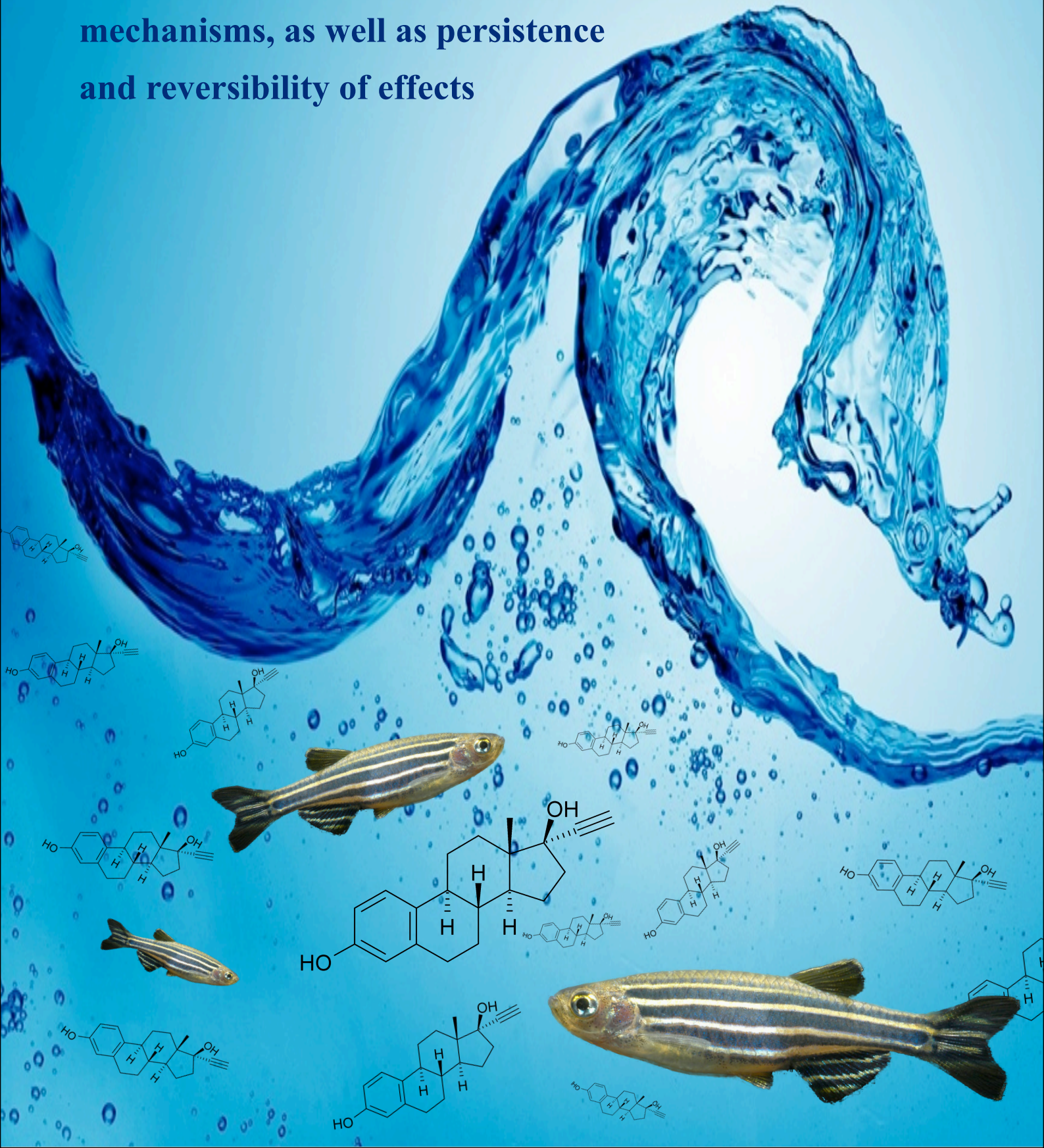
Lisa Baumann - Dissertation 2012

Zebrafish (*Danio rerio*) as a model to study developmental effects of endocrine disruption: molecular mechanisms, as well as persistence and reversibility of effects



Dissertation

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Zebrafish (*Danio rerio*) as a model to study developmental effects of endocrine disruption: molecular mechanisms, as well as persistence and reversibility of effects

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Prof. Dr. Helmut Segner, University of Berne

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Summary

The present study aimed to investigate the reversibility of endocrine disruption in zebrafish (*Danio rerio*) at different effect levels. For this purpose, three different endocrine disrupting chemicals (EDCs) were chosen according to their different modes of action. 17α-Ethinylestradiol (EE2), a semi-synthetic estrogen is the most popular substance in oral contraceptives and is regularly measured in surface waters. 17ß-trenbolone is an anabolic steroid, which binds with high affinity to the androgen receptor. The substance is used in industrial animal farming and sports as muscle growth promoter. Prochloraz is a popular fungicide with multiple modes of action. Its main effect on the endocrine system is that it inhibits the enzyme aromatase, which is essential for the conversion of androgens into estrogens. The effects of those three EDCs were assessed by performance of exposure experiments with developing zebrafish. After exposure of 60 days (from fertilized egg to sexual differentiation), half of the fish were continuously exposed until 100 days post hatch and the other half was held in clean water. In addition, this project focuses on assessing the correlation between different levels of biological organization. For this purpose, five effect levels with different ecological relevance were investigated: (1) population level: sex ratio; (2) individual level: growth; (3) organ/cell level: histology of gonads; (4) protein level: vitellogenin (VTG) induction and (5) mRNA level: aromatase (cyp19b) expression in brain.

The three different EDCs investigated in this study showed strong impact on the sexual development of zebrafish at all effect levels at environmentally relevant concentrations. For trenbolone and prochloraz we could only find tendencies of reversibility, most effects remained unchanged after 40 days of depuration in clean water. Despite different underlying mechanisms, these substances produce an irreversible and considerable drift of the sex ratio towards males, as well as permanent effects on growth, VTG and aromatase levels. A clear reversibility of those effects could only be shown for EE2. Even at population level the impact of the semi-synthetic estrogen was reversible.

These results show that the sexual development of zebrafish is a fragile process that can easily be disrupted permanently by substances that are found in the environment. Moreover, the results indicate that even a periodic exposure to those EDCs can cause severe impairment for wildlife and humans.

Zusammenfassung

In der vorliegenden Arbeit sollten die Effekte von endokrinen Disruptoren (EDs) auf die sexuelle Entwicklung des Zebrabärblings (*Danio rerio*) untersucht werden. Es wurden 3 verschiedene Substanzen ausgewählt die regelmäßig in der Umwelt gefunden werden. Das starke Östrogen Ethinylestradiol, welches einer der wichtigsten Bestandteile in hormonellen Verhütungsmitteln ist; Trenbolon, ein anaboles Steroid, das in der Viehmast und im Sport-Doping als Wachstumshormon eingesetzt wird; und Prochloraz, ein weit verbreitetes Fungizid, welches die Steroidsynthese durch Hemmung des Enzyms Aromatase stört. Die Wirkung der 3 verschieden Chemikalien auf den Zebrabärbling sollten auf verschiedenen Effektebenen mit unterschiedlicher ökologischer Relevanz untersucht werden. (1) Populations-Ebene: Geschlechterverhältnis; (2) Individual-Ebene: Wachstum; (3) Organ-/Zell-Ebene: Histologie der Gonaden; (4) Protein-Ebene: Vitellogenin (VTG) Induktion und (5) mRNA-Ebene: Aromatase (cyp19b) Expression im Gehirn. Zusätzlich wurde untersucht ob die verursachten Effekte reversibel sind. Zu diesem Zweck wurde ein 100-tägiger Belastungsversuch mit Regenerationsphase konzipiert: frisch befruchtete Eier wurden zunächst in verschiedenen Konzentrationen der 3 unterschiedliche EDs aufgezogen. Diese Belastung dauerte bis 60 Tage nach Schlupf. Danach wurde bei der Hälfte der Fische die Belastung beendet und sie hatten die Möglichkeit 40 Tage in sauberem Wasser zu regenerieren. Die andere Hälfte der Fische wurde während dieser 40 Tage weiter exponiert. Die Ergebnisse zeigen, dass alle 3 Substanzen die Sexualentwicklung des Zebrabärblings massiv gestört haben. Sowohl Prochloraz, als auch Trenbolon hatten stark vermännlichenden Einfluss auf die Fische. In hohen Konzentrationen entwickelten sich signifikant weniger weibliche Fische, bei beiden Geschlechtern war die VTG Produktion gehemmt und auch das Wachstum war stark beeinflusst. Trenbolon wirkte wachstumssteigernd, wohingegen Prochloraz sich hier hemmend auswirkte. Ethinylestradiol hatte stark verweiblichende und verzögernde Wirkung auf die sexuelle Entwicklung der Fische: es wurden signifikant mehr Weibchen und unreife Fische gefunden, die VTG Produktion war massiv erhöht und die Aromatase Genexpression reduziert. Für die Reversibilität der Effekte konnte gezeigt werden, dass nach EE2 Belastung sämtliche Parameter nach 40 Tagen Regeneration wieder auf Kontroll-Niveau zurückkamen. Sogar das nach 60 Tagen stark veränderte Geschlechterverhältnis normalisierte sich wieder. Im Gegensatz dazu waren die Effekte von Prochloraz und Trenbolon nicht reversibel. Die starke Vermännlichung war auch nach der Regenerationsphase noch auf allen Organisationsebene zu beobachten. Diese Beobachtungen

zeigen, dass verweiblichende Effekte beim Zebrabärbling offenbar reversibel sind, wohingegen eine Vermännlichung persistent zu sein scheint. Selbst eine periodische Belastung mit EDs kann also zu massiven populationsrelevanten Effekten führen.

1 Introduction

1.1 EDCs: History and current concern

Endocrine disrupting chemicals (EDCs) are natural or synthetic substances that mimic or block hormonal functions in organisms. Research on the environmental impact of these substances is comparably recent, even though the fact that exogenous chemicals can interact with the hormonal system has been well-known since the 1940s (Schueler 1946). Already in those times, steroidal chemicals were used in livestock farming for the control of reproductive cycles and body mass increase. In the 1960s, oral contraceptives, mostly based on the synthetic estrogen mestranol and its metabolite ethinylestradiol, became popular for human birth control, resulting in continuous input of estrogens to the environment, which is still a current topic (Owen & Jobling 2012). The most significant arising contributions to pollution of the environment with EDCs were of industrial or agricultural origin, e.g. the use of plastic materials with integrated softeners and the application of pesticides (Wise et al. 2011). Fig. 1.1 gives an overview on the sources of EDCs that constantly reach surface and drinking water. The consequences of the increasing use of all these substances, with or without intended effect on the hormonal system of animals or humans, became obvious in the early 1990s, when feminization of wildlife populations was observed (Colborn et al. 1993, Sumpter 1995). Those findings arose high interest in the public and regulatory organizations, such as the World Health Organization (WHO). Consequently, much effort has been made in the last years to investigate the dimension of the problem of EDCs in the environment (reviewed by Knacker et al. 2010).

The number of field studies concerning effects of EDCs on wildlife fish populations has increased remarkably over the last 20 years. One of the most complex and popular studies was performed by Jobling et al. (1998 & 2006) in the United Kingdom. The roach (*Rutilus rutilus*), a cyprinid freshwater fish, was found to be significantly feminized, probably due to exposure to estrogens in surface waters. High numbers of male fish with elevated vitellogenin (VTG) levels and intersex gonads were found close to effluents from sewage treatment works. These effects could be correlated to high levels of different estrogenic compounds, of both natural and synthetic origin. Feminized fish have also been found in other European countries such as Denmark, France, Italy, Germany and the Netherlands (e.g. Bjerregaard et al. 2006, Vethaak et al. 2005, Hinfray et al. 2010, Viganò et al. 2001, Solé et al. 2003). Skewed sex ratios or intersex gonads are the most striking effects of endocrine disruption in wildlife fish populations, as they clearly show that EDCs can disrupt sexual development. Unfortunately,

gonad maturation of fish has only rarely been investigated in research on endocrine disrupting effects. Part of the present study has therefore been developed to fill this gap (chapter 2). In general, the main focus of the present study will be on effects of EDCs on the sexual development of zebrafish (*Danio rerio*). Nevertheless, reproductive problems of wildlife populations are likely to be due to very complex mechanisms, not only restricted to effects on the gonads. Therefore, some current topics in EDC-research are described in the following, even though they were not specifically evaluated in the present study.

There is increasing evidence that reproductive problems of wildlife populations are not only due to physiological or morphological alterations, but probably also due to behavioral changes (reviewed by Söffker & Tyler 2012). Sexual behavior, being one of the key elements for the reproductive fitness of a population, can be seriously affected by several EDCs and therefore impair the reproductive success of individuals. Even though it is well known that behavior is generally influenced by hormones, only few studies concerning the effect of EDCs on behavior exist. Effects of EDCs on sexual behavior (e.g. Salierno & Kane, 2009), sexual selection (Saaristo et al., 2009), social dominance hierarchies (Coe et al., 2009) and avoidance of predators (Mc Gee et al., 2009) have recently been reported, and these findings clearly indicate that altered behavior due to EDC exposure may seriously affect wildlife fish populations.

Besides effects on reproduction and behavior, there is increasing evidence that EDCs are also interfering with the immune system of fish. This topic has not been evaluated in the present study, but it has to be considered, e.g. when it comes to increased mortality of animals in exposure experiments with EDCs. As the endocrine and immune systems are closely related to each other (Yada & Nakanishi 2002), there is consequently a connection between exposure to EDCs and diseases in wildlife populations. Casanova-Nakayama et al. (2011) reported increased susceptibility of rainbow trout (*Oncorhynchus mykiss*) to pathogens under exposure to E2. Additionally, they could show that estrogen receptors are not only present in reproductive organs, but also in immune organs like the spleen or the head kidney. Comparable exposure experiments with rainbow trout and estrogens were also performed by Wenger et al. (2011). They could show that E2-treated rainbow trout had a lower capacity to activate their immune system for defense against an infection. Several components of the innate and acquired immunity, e.g. macrophages, immune proteins or lymphocytes, can be stimulated or repressed by exposure to EDCs (reviewed by Milla et al. 2011).

Experimental studies on endocrine disruption in fish have been improved in the last years and are multifaceted, but the major weakness of laboratory studies is that they only roughly represent realistic exposure scenarios in the environment. The main difference to laboratory conditions is that wildlife animals are mostly exposed to a complex mixture of different chemicals with variable bioavailability and that the exposure is not constant. Recent studies have tried to develop more realistic exposure scenarios in order to be able to predict effects in wildlife; e.g., Thorpe et al. (2003) or Brian et al. (2005) could show that mixtures of estrogenic steroids can act additively, based on their estrogenic potencies. Given the facts that there are between 30 000 and 50 000 chemicals on the EU market, and that probably more than 50 000 chemicals are present in surface waters (Matthiessen & Johnson, 2006), the hazard potential for mixture effects has to be considered. This topic has intensively been reviewed by Kortenkamp (2007), who comes to the conclusion that there is a serious lack of knowledge concerning mixture effects and that more realistic exposure scenarios should be created.

The second weakness of laboratory studies on EDCs is that exposure is not necessarily continuous, but rather periodic or peak-like. This fact necessarily leads to the question whether adverse effects caused by EDCs are reversible. This topic is slowly, but steadily moving into the focus of recent investigations on EDCs. Several studies have investigated the reversibility of estrogenic effects, mainly caused by ethinylestradiol (Hill & Janz 2003, Nash et al. 2004, Schäfers et al. 2007, Larsen et al. 2009). Data concerning androgens or aromatase inhibitors are more scarce (Morthorst et al. 20010, Larsen & Baatrup 2010, Fenske & Segner 2004). Consequently, one of the aims of the present study is to investigate the persistence of androgenic and aromatase-inhibiting effects (chapter 3&5) and to compare the results to those of estrogenic effects (chapter 4).

The research field of EDCs is probably not coming to an end in foreseeable time, as only part of the adverse effects are investigated in detail and new chemicals are constantly produced that reach the environment. Relatively new and important topics of EDC research are effects on the immune system (Jin et al. 2010, Milla et al. 2010), effects on the thyroid system of fish (Schmidt & Braunbeck 2011, Thienpont et al. 2011), low-dose effects (Vandenberg et al. 2012) and the use of biomarkers for the reduction of animal testing (Hutchinson et al. 2006).

Ideally, scientific publications and research can serve to help politicians and regulators to develop regulations and guidelines for the protection of humans and the environment against pollution with EDCs. Unfortunately, there is still much room for improvement, especially

concerning the education and information of the public. If people are not aware of the impact of EDCs on their lives and the environment, the acceptance, e.g., of increased costs for water purification will be low (Owen & Jobling 2012). Furthermore, it is essential that reasonable test systems are developed and validated to guarantee for the reliable detection of EDCs. Some ambitions of the OECD (Organization for Economic Co-operation and Development) are discussed in the following.

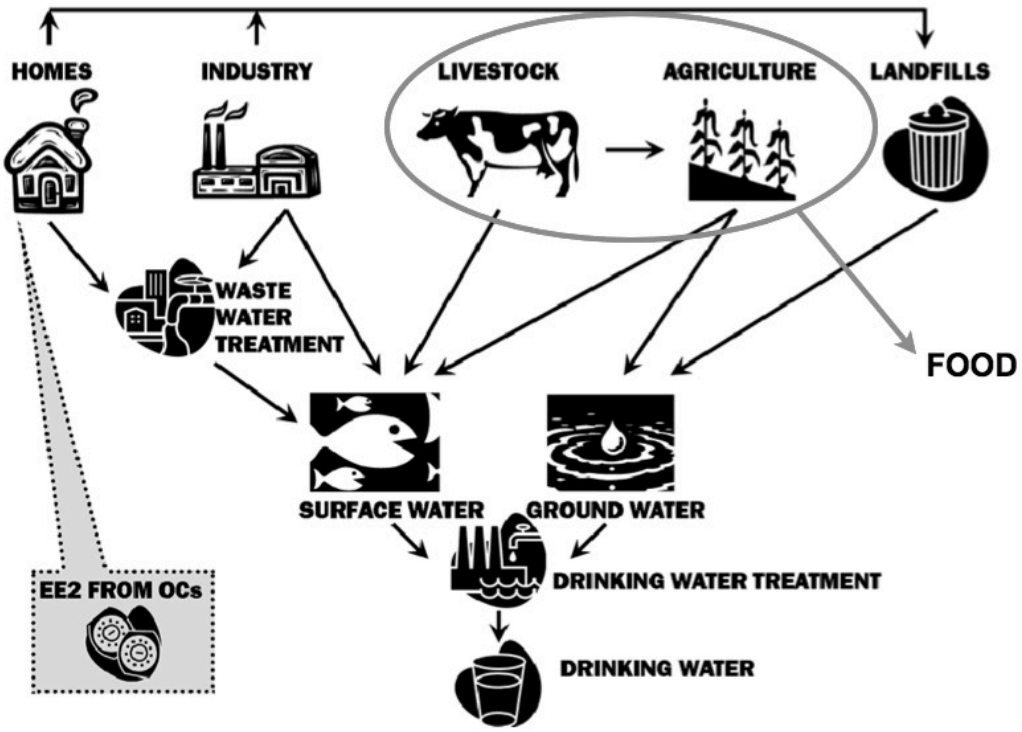


Fig. 1.1: Sources of EDC input into the environment and drinking water
(EE2 = ethinylestradiol, OC = oral contraceptive; adapted from Wise et al. 2011)

1.1.1 Regulatory implications

As EDCs represent a serious hazard to humans, animals and the environment, regulators have initiated several laws, regulations and guidelines for the risk assessment of suspected chemicals. One of the most discussed issues with the most consequences on current risk assessment of chemicals is the EU Regulation REACH (**R**egistration, **E**valuation, **A**uthorization and **R**estriction of **C**hemicals, EU 2006). It is probably the strictest law worldwide dealing with regulation of chemical substances. It enforces the (re-) registration of several thousands of new and existing chemicals and consequently resulted in massive activities of the chemical industry. Not only chemicals in general, but also special products for plant protection or cosmetics have been regulated by the European Commission (EU

2009). Facing the dimension of chemical regulation, adequate test-systems are required for the effective and precise testing of substances. Concerning the evaluation of EDCs, the OECD has developed the following conceptual framework (OECD 2010):

- Level 1: Sorting and prioritization based on existing information

- Level 2: *In vitro* assays providing mechanistic data

- Level 3: *In vivo* assays providing data about single endocrine mechanisms and effects

- Level 4: *In vivo* assays providing data about multiple endocrine mechanisms and effects

- Level 5: *In vivo* assays providing data on effects from endocrine and other mechanisms

Regarding the scope of the present thesis, only *in vivo* test systems for the evaluation of EDCs with fish as test-organisms are described and discussed in the following. Currently, the OECD has 3 established fish test systems that can be performed with zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) or fathead minnow (*Pimephales promelas*):

1. Guideline No. 229: Fish Short Term Reproduction Assay (OECD 2009)

Young adult fish, at least 3 months old, are exposed to an EDC in 3 - 5 different concentrations for a period of 21 days. Most important endpoints for evaluation are: growth, vitellogenin, secondary sex characteristics, and gonadal histopathology. Fecundity and fertility are checked before the exposure by counting the number of eggs produced by a group of fish. This approach is highly questionable, since egg production of fish is very variable and depends on diverse parameters like temperature, population density, food or light (Baroiller et al. 2009). Furthermore, this approach requires a high number of animals, which is not in accordance with animal welfare. Moreover, to check this endpoint after exposure would make much more sense than before, but should also take into account the high variability. It is also doubtful whether effects on growth are to be expected in young adult fish that are almost completely developed. Therefore, effects on secondary sex characteristics are also unlikely to occur within such a short exposure period. The most useful endpoint in this test system is probably gonadal histopathology. E.g. effects on gonad maturation or egg/sperm-quality are likely to be clearly identified. Those endpoints have high predictive value concerning the reproductive fitness of an individual. The OECD has recently published a guidance document for fish gonad histopathology (OECD 2010), which describes the variety of histological techniques and effects on gonads in detail. Another strong endpoint of this test system is VTG induction. The egg yolk precursor protein has successfully served as a biomarker for the

detection of endocrine effects for several years (Holbech et al. 2012). The reaction of the organisms on the exposure to an EDC can be quantified very precisely and the mode of action can also be identified, at least to a certain extent. Estrogenic or anti-androgenic effects are mostly represented by elevated VTG values, whereas androgenic, anti-estrogenic and aromatase-inhibiting effects are represented by lowered values (Holbech et al. 2006).

In summary, the Fish Short Term Reproduction Assay represents a solid, but improvable tool for the detection of endocrine effects in fish.

2. Guideline No. 230: 21-day Fish Assay (OECD 2009)

This assay is performed analogously to TG No. 229, as described before. The difference is that less endpoints are analyzed after the 21 days of exposure. Only VTG and secondary sex characteristics are investigated. As VTG is a highly acknowledged biomarker for the detection of EDCs, this endpoint is probably sufficient to provide a preliminary assessment of an unknown substance and its endocrine activity. On the other hand, detailed analyses or underlying mechanisms cannot be made with this test system. The questionable informative value of the investigation of secondary sex characteristics in adult fish was discussed above.

3. Guideline No. 234: Fish Sexual Development Test (OECD 2011)

The Fish Sexual Development Test (FSDT) has potential as a promising compromise for the gold standards, the full life-cycle or the multi-generation tests, which are not yet established as guidelines. The FSDT has been designed for a safe evaluation of potential EDCs within an extended exposure period of 60 days, which is still much shorter than 6 – 7 months required for a full life-cycle or even a multi-generation test with zebrafish (*Danio rerio*). Nevertheless, it has to be considered that effects on reproduction can only be predicted and are not included themselves in the test design of the FSDT. However, multi-generation tests have not been validated as official guidelines and still only exist as drafts. In contrast to these long-term tests, the FSDT covers only the sensitive period of sexual differentiation, which is known to be very susceptible to EDCs (Maack & Segner 2004). This offers the opportunity to use the sex ratio as meaningful endpoint for the evaluation of a suspected EDC. Several examples show that all-female or all-male populations can be produced by the application of an EDC (reviewed by Scholz & Klüver, 2009). Consequently, in this case of complete sex-reversal, checking for fertility is unnecessary and adverse effects on population fitness can clearly be

predicted anyway. Besides the sex ratio, other very meaningful endpoints of the FSDT are VTG, growth, and histopathology. As growth is under endocrine control, the evaluation of this endpoint is much more appropriate to be examined after the developmental period of young growing fish, as in adults. VTG induction represents a reliable biomarker for the detection of endocrine effects and helps interpreting underlying mechanisms. Histopathology is to some extent controversial in the FSDT: gonad histology has to be performed for definite identification of the gender of each fish and the detection of pathological lesions, but gonad maturity staging is only optional. This can be regarded as a weakness of the FSDT, and, thus, this topic will be discussed in detail in chapter 2.

From the three established test systems for the investigation of endocrine effects in fish, only the FSDT represents a solid and valuable test for the evaluation of population-relevant effects. The Fish Full Life-Cycle Test is still in the validation period, but after that will serve as an important test for the evaluation of long-term effects. Even though this test is very time- and cost-intensive, it is the only possibility to include reproductive endpoints in the evaluation of an EDC. Regulators and industrial representatives constantly highlight the importance of a test system being able to simulate effects for wildlife populations, but this requirement is obviously difficult to fulfill. Exposure of wildlife populations to EDCs and other pollutants is much more complex than under laboratory conditions. Chemical substances reach the environment in complex mixtures and discontinuously, leading to either peak or low-dose exposures. The bioavailability of those compounds can also differ. The ecological context of a species has to be considered as well as seasonal changes or life history traits (Segner 2011). To include all these parameters in an artificial exposure scenario is almost impossible, except if the experiment takes place in a natural ecosystem. This ambitious and controversial approach was performed in Canada from 1999 to 2006, where a complete lake of 34 ha was contaminated with EE2 (Kidd et al., 2007). Fathead minnow populations were massively feminized and almost collapsed by the application of the strong estrogen in environmental relevant, low concentrations. Such intricate exposure scenarios are very interesting from a scientific point of view, but completely unrealistic for routine risk assessment in industries. Nevertheless, there should be more initiatives to try to develop test systems that involve for example peak exposure scenarios, mixture effects, recovery periods, low-dose effects and others. The present study focuses on the consequences of recovery periods after exposure to different EDCs (chapters 3 - 5).

1.2 Test chemicals

1.2.1 17β-Trenbolone

Product details:

IUPAC name: 17β-Hydroxyestra-4,9,11-trien-3-one

Molecular formula: $C_{18}H_{22}O_2$

Molecular weight: 270.37 g/mol

CAS-No.: 10161-33-8

Supplier: Sigma-Aldrich, Deisenhofen, Germany

Product number: 3925

Purity: > 93 %

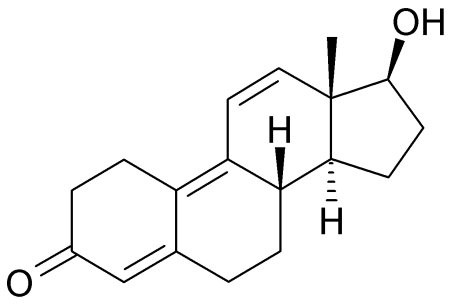


Fig. 1.2: Molecular structure of 17β-trenbolone

The anabolic steroid trenbolone acetate is widely used as a growth promoter in cattle farming in the USA (Bartelt-Hunt et al. 2012) and is thus constantly reaching surface waters (Khan & Lee 2012). Durhan et al. (2006) measured concentrations of trenbolone acetate metabolites up to 20 ng/L in beef cattle feedlot discharge and up to 7 ng/L in associated surface waters. The synthetic androgen is administered to animals *via* subcutaneous implants as a growth promoter. Its stable metabolites 17α-trenbolone and 17β-trenbolone are excreted by the animals and can thus reach the environment (Khan & Lee 2012). These metabolites are stable with a half-life of up to 260 days (Schiffer et al. 2001). 17β-Trenbolone binds as an agonist with very high affinity to the androgen receptor (Durhan et al. 2006), resulting in masculinization and increased growth in many animal species and humans (Ankley et al. 2003). Therefore, 17β-trenbolone is also used illegally as a doping substance in athletic sports and bodybuilding (Bowers et al. 2009, Thevis et al. 2009), but this specific contribution to environmental pollution with steroids is difficult to assess. Nevertheless, studies with questionnaires among gymnasium users indicate that almost 4 % of amateur athletes use anabolic steroids (Leifman et al. 2011). The World Anti-Doping Agency Laboratory Statistics Report of 2010 shows that 60.8% of prohibited substances found in samples of professional athletes in Olympic and non-Olympic sports belong to the class of anabolic steroids, of which trenbolone is on rank no. 9 (WADA 2010). The strong and fast body mass increase after the use of trenbolone is enforced by stimulated appetite and its anti-catabolic properties caused by the activity as an anti-glucocorticoid (Meyer et al. 2001). In contrast to most endogenous and

also exogenous androgens, 17β-trenbolone cannot be metabolized by the enzyme aromatase to estrogens.

Several ecotoxicological studies have used trenbolone as model substance for androgenic effects on fish and other vertebrates. E.g., Wilson et al. (2002) reported strong androgenic effects of 17β-trenbolone *in vitro* and *in vivo* in the Hershberger assay with rats. Ankley et al. (2003) found reduced fecundity of male and female fathead minnow (*Pimephales promelas*) after exposure to 17β-trenbolone for 21 days. The androgenic effect was also obvious by the production of dorsal (nuptial) tubercles in female fish, structures normally only present on the heads of mature males. Plasma steroid levels and vitellogenin were also significantly affected by the exposure to 17β-trenbolone. Effects on VTG, sex ratio and gonad morphology were also published on other fish species such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). Örn et al. (2006) exposed these 2 species to 10 and 50 ng/L 17β-trenbolone from 0 - 60 dph and found significant decrease in VTG compared to the controls at the highest concentration of 17β-trenbolone. Gonad maturity of these individuals was also influenced, showing much higher percentages of mature spermatozoa. Seki et al. (2006) also reported elevated gonadosomatic indices (GSI) in zebrafish after exposure to 17β-trenbolone. The same stimulating effects on testis maturation were observed by Morthorst et al. (2010), who exposed zebrafish to 17β-trenbolone for 60 days, followed by a recovery period of 170 days. Skewed sex ratios towards males were also reported in this study, as well as by Holbech et al. (2006), Larsen & Baatrup (2010) and Örn et al. (2005). All these studies found strong masculinizing effects of 17β-trenbolone at very low environmental relevant concentrations. The most striking finding was that these effects seemed to be irreversible, which makes the ecological relevance for wildlife fish populations exposed to anabolic steroids even higher.

1.2.2 Prochloraz

Product details:

IUPAC name: N-propyl-N-[2-(2,4,6-trichlorphenoxy)ethyl] imidazole-1-carboxamide

Molecular formula: $C_{15}H_{16}C_{l3}N_3O_2$

Molecular weight: 376.67 g/mol

CAS-No.: 67747-09-5

Supplier: Sigma Aldrich, Deisenhofen, Germany

Product number: 45631

Purity: 99.9 %

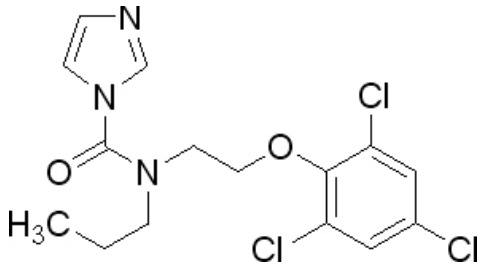


Fig. 1.3: Molecular structure of prochloraz

Prochloraz is a popular fungicide belonging to the group of imidazoles. It inhibits the biosynthesis of ergosterol, which is part of the cell membrane in many fungi (Johnston et al. 1996). Imidazole derivatives are widely used as antifungal agents in agriculture, but also in textile- and paint-producing industries. Through these applications, they can reach the environment and accumulate in aquatic organisms and their predators (reviewed by Mnif et al. 2011). The agricultural use of prochloraz (and fungicides in general) does not only affect wildlife, but also makes them a potential risk for humans, especially for farmers or gardeners. Recent epidemiological studies have indicated a causal relation between human exposure to pesticides and poor sperm quality (Swan et al. 2003) or increased incidence of cryptorchidism in sons of female gardeners (Weidner et al. 1998). Several studies report significant effects of prochloraz on fish and other vertebrates, but the underlying mechanisms are diverse and not yet fully understood. Prochloraz antagonizes the androgen and the estrogen receptors, agonizes the Ah receptor and inhibits aromatase activity (reviewed by Vingaard et al. 2006). Ankley et al. (2009) investigated the effects of prochloraz on the HPG axis in adult fathead minnow (*Pimephales promelas*) in an 8-day exposure experiment. Multiple alterations such as inhibition of estradiol and testosterone production were found. These effects were compensated during a short recovery period of 8 days, indicating that the multiple modes of action of prochloraz on the hormonal system are not persistent in adults. In contrast, exposure during sexual differentiation seems to have irreversible effects on sexual development of zebrafish (chapter 5). Kinnberg et al. (2007) and Holbech et al. (2012) investigated the effects of prochloraz on zebrafish in the FSDT and found altered gonad development with increased

percentages of males and intersex individuals accompanied by decreased VTG levels. Thorpe et al. 2011 found similar effects in zebrafish and fathead minnow.

There are also indications that prochloraz has adverse effects on the thyroid system of vertebrates (Liu et al. 2011, Brande-Lavridsen et al. 2010). All these findings suggest that exposure of humans and wildlife to prochloraz can have severe consequences, and, thus, the use of the fungicide should be regulated.

1.2.3 17α-Ethinylestradiol

Product details:

IUPAC name: 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol

Molecular formula: $C_{20}H_{24}O_2$

Molecular weight: 296.40 g/mol

CAS-No.: 57-63-6

Supplier: Sigma Aldrich, Deisenhofen, Germany

Product number: E4876

Purity: ≥ 98 %

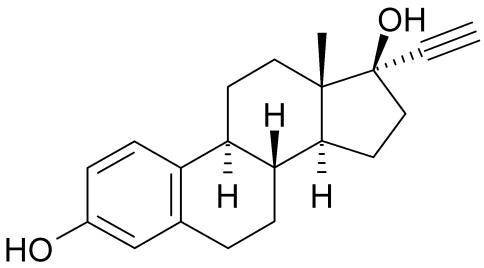


Fig. 1.4: Molecular structure of 17α-ethinylestradiol

The most popular and best-investigated substance in the field of endocrine disruption is probably 17α-ethinylestradiol (EE2). It is one of the most potent and significant xenoestrogens and has been measured in sewage effluents at concentrations from 0.1 – 5.1 ng/L. Even drinking water, especially in Germany, is contaminated with EE2 in ranges around 0.15 – 0.5 ng/L EE2 (see Wise et al. 2010 for review). It is the most important substance in combined oral contraceptive pills, which are consumed by over 100 million women worldwide (UN 2009). Its high prevalence in the environment is due to its release through urine and feces of women who take it as medication. EE2 is hormonally effective by activating the estrogen receptor with even higher affinity than natural estradiol, and thus acts as a strong estrogen. Several years of research have proven that EE2 and other estrogens cause serious adverse effects in the aquatic environment by disrupting endocrine systems in wildlife animals (Owen & Jobling 2012). The massive impact on fish populations has been underlined by Kidd et al. (2007), who contaminated a whole lake in Canada with environmentally relevant concentrations of EE2. This exposure experiment resulted in

massive feminization and an almost total collapse of the fish population. Multiple other studies gave the same results on population fitness in laboratory experiments with different fish species (see Scholz & Klüver 2009 for review). The feminizing effect of EE2 is obvious in many different outcomes, all depending on the hormonal system, such as reproductive behavior, fecundity, fertility, egg quality or sexual differentiation. Since wildlife animals and humans are constantly exposed to EE2, the question about reversibility of effects seems to be obsolete. Nevertheless, several studies report reversibility of effects by EE2 on fish sexual development (Hill & Janz 2003, Nash et al. 2004, Schäfers 2007, Larsen et al. 2009) after depuration in clean water. However, this process is highly dependent on the mode of sexual development and the timing of exposure. Gonochorists such as fathead minnow (*Pimephales promelas*), are not able to recover from strong estrogenic exposure during sexual development (Länge et al. 2001), whereas protogynic fish like zebrafish are (Fenske et al. 2005).

Research on xenoestrogens has a long history in the evaluation of EDCs, and recent publications focus on concentration- and time-dependent effects on reproductive capabilities of zebrafish (Schäfers 2007). Several long-term exposure experiments with EE2 have been carried out to simulate the effects of chronic exposure in the environment (Nash et al., 2004; Xu et al., 2008; Zha et al., 2008). All these studies could show that male fish, when continuously exposed to low concentrations of EE2, have impaired sexual maturation, resulting in reproductive failure.

Recently, several studies reported adverse effects of EE2 and other estrogens on the immune system (reviewed by Milla et al. 2011). Fish exposed to environmentally relevant concentrations of estradiol were much more susceptible to pathogens than controls (Wenger et al. 2011). Histopathological lesions in the gonads of fish exposed to estrogens could also be correlated to the estrogenic exposure by measurement of immunmarkers in the gonads (Cabas et al. 2011).

Taken these experimental results and documentations of wildlife effects together, it can be concluded that, if the continuous EE2 exposure is not restricted by regulation, e.g. by optimizing water purification with activated charcoal filtration, massive effects on populations are likely to increase during the next years (see Owen & Jobling 2012).

1.3 Zebrafish (*Danio rerio*)

Fig. 1.5: Zebrafish (*Danio rerio*)

1.3.1 History and Biology

The zebrafish (*Danio rerio*) is a small freshwater fish belonging to the teleost family of the Cyprinidae. The name "Danio" comes from the Bengali word "dhani", which means "from the rice field". Its natural habitat is the Indian subcontinent, where it lives in rivers, but also in rice fields. Zebrafish are omnivorous predators of small fish, invertebrates and their eggs, but also ingest algae, spores and detritus. The maximum size of their laterally compressed body is 4 cm. The trivial name "zebra-"fish is based on the flamboyant blue and yellow stripes that reach from behind the gills to the tail fin. Male and female zebrafish look very similar, but differ slightly in the shape of their bodies. Adult females have a more round body shape due to the high number of big eggs in their ovaries. The sexual development of zebrafish will be described in chapter 1.3.2.

Zebrafish is a very popular vertebrate model in genetics, developmental biology, neurophysiology, biomedicine and ecotoxicology. Over 750 research laboratories and 140 companies worldwide use zebrafish for their research (www.zfin.org). Some striking features make it an ideal laboratory organism: it is comparably small and can be kept in high numbers under easy and cheap conditions. The reproductive cycle does not depend on seasonal changes, and, thus, egg production is possible during the whole year. Females are able to spawn 1-2 times per week and to produce up to several hundred eggs per spawn. The eggs are comparably large (0.7 mm in diameter) and are optically transparent. This is one of the most striking features, which made zebrafish such a popular model in developmental biology and ecotoxicology. The development of the embryo and its organs can easily be observed and manipulated under a normal microscope. Generation time is only 3 - 4 months, providing ideal conditions for genetic or reproduction experiments. Consequently, zebrafish represents a very important model for research on EDCs (see Spence et al. 2007 for review).

1.3.2 Sexual development

Zebrafish have a so-called protogynic sexual development, which means, that all individuals first develop ovaries, from which around 50% will be transformed to testes later (Takahashi 1977, Maack & Segner 2004). This process is accomplished within the first 60 days post-hatch, which was consequently chosen as the exposure period for the FSDT. The final maturation of the gonads is normally accomplished within 3 - 4 months. This transformation from ovary to testis is a very fragile process, which is under the control of steroids. High estrogen levels induce the differentiation of ovaries, whereas androgens lead to the differentiation of testis (Orban et al. 2009, Clelland & Peng 2009). Definite sex chromosomes or sex-related genes have not yet been identified in zebrafish (Anderson et al. 2012, Jorgensen et al. 2008, Orban et al. 2009). However, several genes are known to be involved in gonadal differentiation of zebrafish such as sox9a and sox9b (Chiang et al., 2001) or anti-Müllerian hormone (von Hofsten & Olsen 2005; Rodriguez-Mari et al., 2005). The responsible hormonal system for reproduction and sexual development is the hypothalamus-pituitary-gonad (HPG) axis, which is a very complex system of different organs and hormones working together. Fig. 1.7 gives an overview on the hormonal system of zebrafish. In short, the hypothalamus secretes gonadotropin releasing hormones (GnRH3) and regulates the release of gonadotropins (GtH) from the pituitary. In fish, these are GtH I and GtH II, which are analogous to mammalian follicle-stimulating hormone (FSH) and mammalian luteinizing hormone (LH), respectively (Swanson et al. 1991). Both hormones stimulate maturation and steroid hormone production of the gonads. Steroid hormones like estradiol or testosterone, in turn regulate hypothalamic and pituitary components of the HPG axis *via* feedback systems. Estrogen/androgen levels are regulated by the enzyme aromatase, which exists in 2 isoforms in zebrafish, either expressed in the brain (Cyp19b) or in the gonads (Cyp19a). The enzyme represents a key element in the hormonal regulation, since it converts testosterone to estradiol. Aromatase activity can directly or indirectly be influenced by EDCs. Several chemicals are aromatase inhibitors per definition and thus inhibit the production of estradiol, resulting in masculinization (Fenske & Segner 2004). Aromatase expression is also known to be regulated through estrogen-responsive elements and can thus be up- or down-regulated by estrogenic EDCs (Brion et al. 2012). Depending on the timing and intensity of exposure, the application of exogenous steroids or hormone-like substances can easily disrupt this process and lead to development of gonads different from the genetic predisposition. This has been documented in several studies concerning the investigation of EDCs, as well for estrogenic, androgenic or aromatase-inhibiting substances (reviewed by Scholz & Klüver 2009). Not only the sexual

differentiation itself, but also the grade of maturation of the gonads can be affected by EDCs (chapter 2). Substances that inhibit gonad maturation can have the same adverse effects on the reproductive capacities of a population as a skewed sex ratio, since immature gametes cannot be fertilized. Intersex or testis-ova is one of the most striking observations made in zebrafish after exposure to EDCs. To which extent intersex gonads cannot be used for reproduction is not clearly predictable, it probably depends on the grade of severity. This topic has been discussed controversially, as it depends on the mode of action of a substance, if the intersex gonad is only a consequence of delayed sexual differentiation or a real malformation. As the normal gonad development of male zebrafish includes a state of juvenile hermaphroditism, intersex gonads can often be interpreted as an effect of inhibited development caused by an EDC (Fenske et al. 2005). Immature small eggs, partially in a degenerated state, surrounded by immature testis-tissue characterize such gonads. Individuals displaying those intersex gonads are probably "arrested" males (Maack & Segner 2004). "Real" intersex or hermaphroditic gonads are more likely to contain either mature ovaries or testis tissue, indicating that sexual differentiation was not delayed, but seriously disrupted by an EDC. Histological investigations provide the only safe method to assess these direct effects on gonad development. Fig. 1.6 gives an overview on different types of gonads that can be found in zebrafish. Nevertheless, these findings can additionally be supported by the use of biomarkers like VTG-induction or aromatase-expression to understand the underlying modes of action.

As mentioned before, aromatase is the key enzyme in the steroid synthesis and can therefore be used as a tool for the detection of endocrine effects (Kallivretaki et al. 2006). This approach is especially useful for preliminary tests in embryos, where gonad development is not yet accomplished (Brion et al. 2012). VTG can also be used in comparably early live stages, but also in adults that were only exposed to EDCs for a short period (e.g. OECD 21-day assay). VTG is an estrogen-dependent precursor of yolk proteins in oviparous vertebrates. It is produced under the control of estradiol in the liver, from where it is transported to the oocytes and taken up by the control of GtH1 for the production of yolk. Yolk basically consists of lipovittelin and phosvitin, which result from cleavage of VTG. Due to its function, VTG is normally found in females, but can also be synthesized by males. The basic level of VTG in males is much lower than in females but can reach the same level under exposure to estrogens (chapter 2). *Vice versa*, the exposure to androgens causes decreased levels of VTG in both genders (chapter 3).

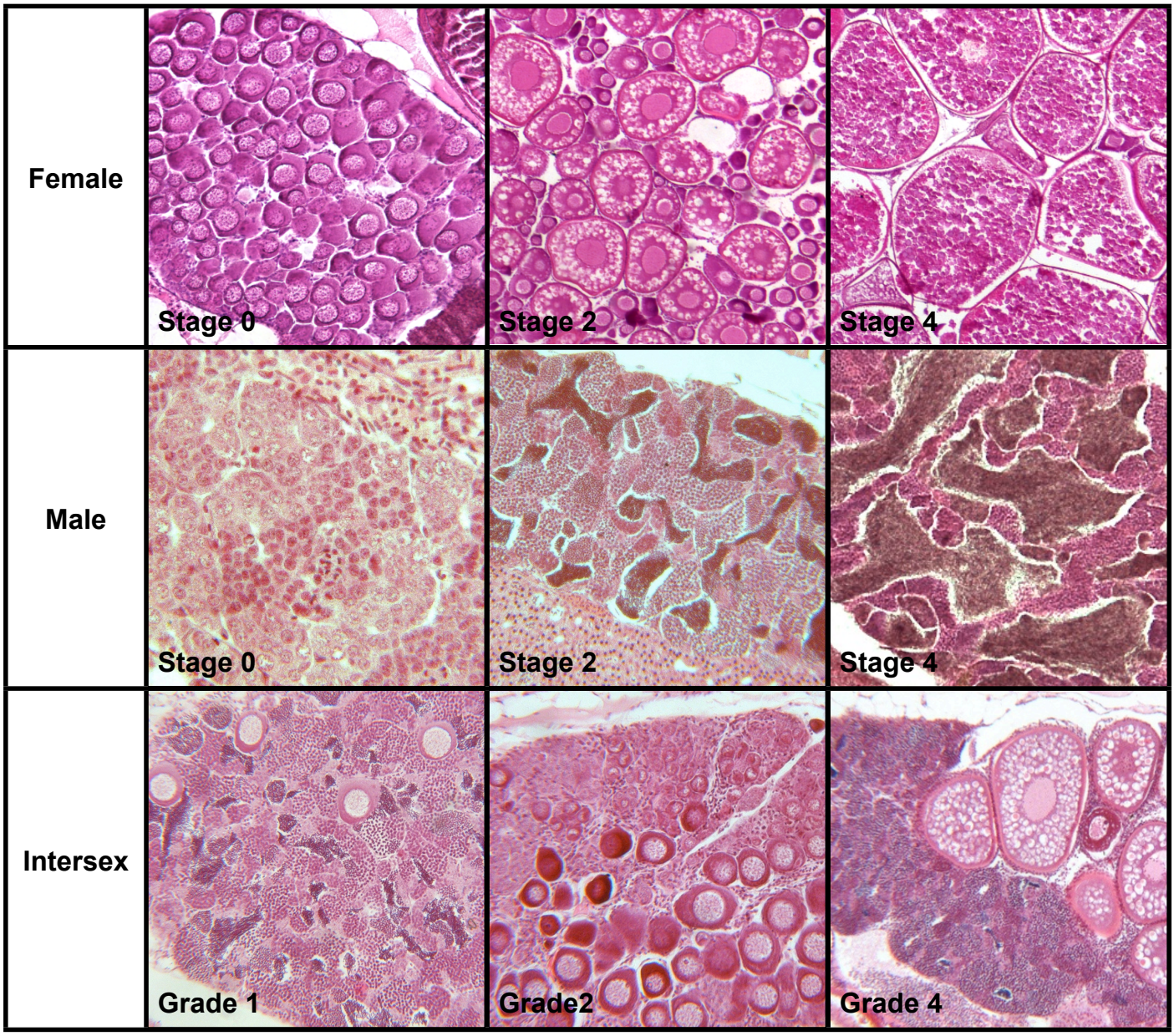


Fig. 1.6: Gonad histology of zebrafish (*Danio rerio*): examples of different gonad maturity stages of female and male zebrafish, and examples of severity grades of intersex gonads

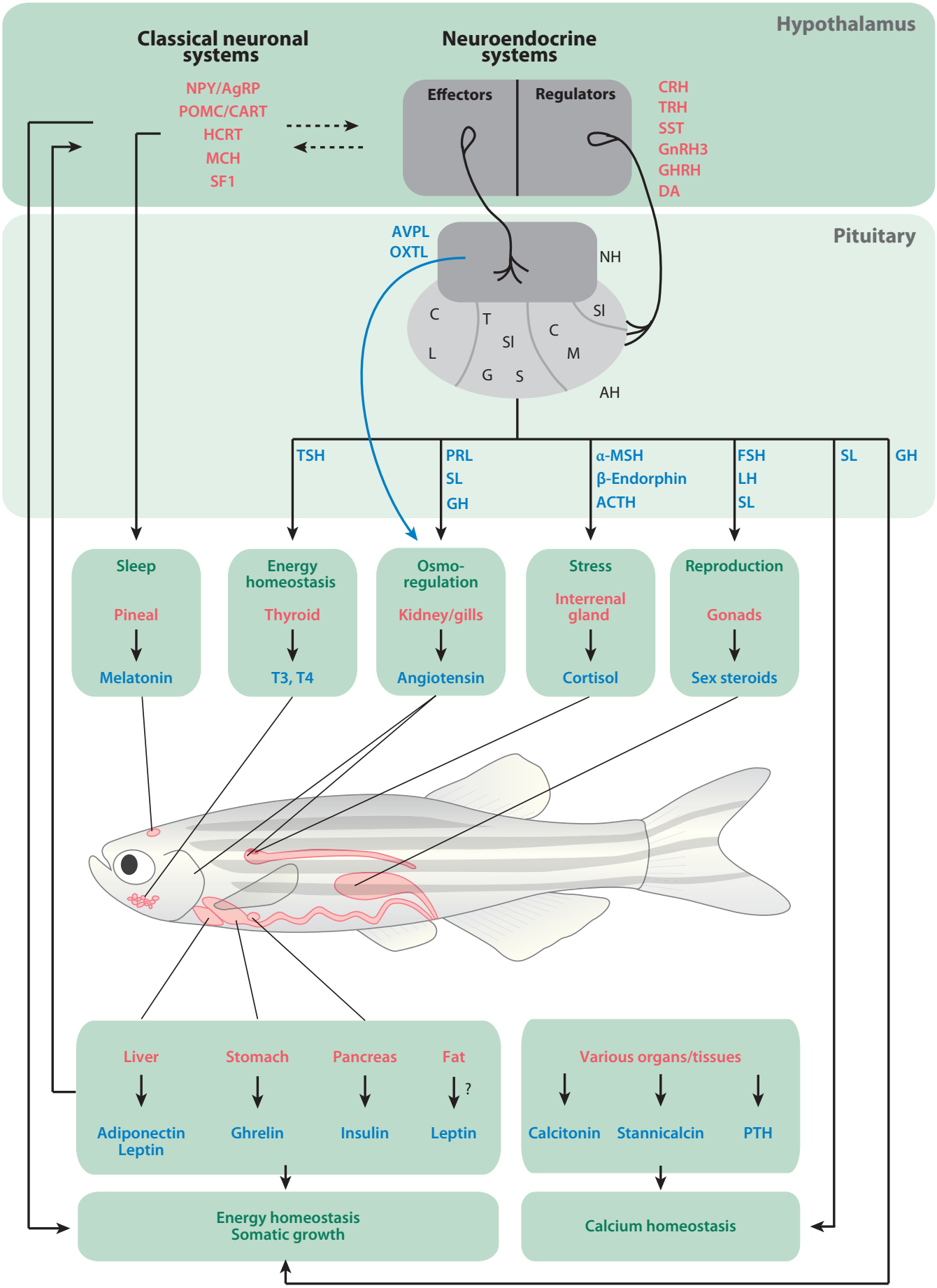

Hypothalamus
Classical neuronal systems
Neuroendocrine systems
NPY/AgRP
POMC/CART
HCRT
MCH
SF1
Effectors
Regulators
CRH
TRH
SST
GnRH3
GHRH
DA
Pituitary
AVPL
OXTL
NH
C
T
SI
C
SI
L
M
G
S
AH
TSH
PRL
SL
GH
α-MSH
β-Endorphin
ACTH
FSH
LH
SL
SL
GH
Sleep
Pineal
Melatonin
Energy homeostasis
Thyroid
T3, T4
Osmo-regulation
Kidney/gills
Angiotensin
Stress
Interrenal gland
Cortisol
Reproduction
Gonads
Sex steroids
Liver
Stomach
Pancreas
Fat
?
Adiponectin
Leptin
Ghrelin
Insulin
Leptin
Various organs/tissues
Calcitonin
Stannicalcin
PTH
Energy homeostasis
Somatic growth
Calcium homeostasis

Fig. 1.7: The endocrine system of zebrafish (Löhr & Hammerschmidt 2011). Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; AH, adenohypophysis; AVPL, arginine vasopressin like; C, corticotropes; CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; DA, dopamine; FSH, follicle-stimulating hormone; G, gonadotropes; GH, growth hormone; GHRH, growth hormone–releasing hormone; GnRH, gonadotropin- releasing hormone; HRCT, hypocretin; L, lactotropes; LH, luteinizing hormone; M, melanotropes; MCH, melanin-concentrating hormone; MSH, melanocyte-stimulating hormone; NH, neurohypophysis; NPY, neuropeptide Y; OXTL, oxytocin like; POMC, proopiomelanocortin; PRL, prolactin; PTH, parathyroid hormone; S, somatotropes; SF1, steroidogenic factor; Sl, somatolactotropes; SL, somatolactin; SST, somatostatin; T, thyrotropes; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

1.4 Design and aims of the present study

EDCs can cause effects at very low concentrations and result in serious problems for the hormonal homeostasis of various organisms. Exposure of wildlife to EDCs is not necessarily continuous, but may often occur intermittently. Consequently, the evaluation of the long-term effects on populations is dependent on their reversibility. The aim of the present study was to elucidate the persistence of effects of three different EDCs on the hormonal system of zebrafish (*Danio rerio*). An exposure scenario including a recovery phase was chosen to assess the potential of reversibility of these effects. Three different substances, selected according to different modes of action, were tested for their long-term impact on zebrafish: the androgen 17β-trenbolone, the estrogen 17α-ethinylestradiol and the aromatase inhibitor prochloraz. All three compounds can be found in the environment and have previously been shown to cause striking effects in zebrafish, but recovery has only partly been studied. Environmentally relevant, or even lower, concentrations were chosen for the exposure experiments with zebrafish. In addition, this project focused on assessing the correlation between different effect levels of endocrine disruption. For this purpose, five effect levels with different ecological relevance were investigated: (1) population level: sex ratio; (2) individual level: growth; (3) organ/cell level: histology/maturity of gonads; (4) protein level: vitellogenin (VTG) induction and (5) mRNA level: aromatase (Cyp19b) expression in the brain. By assessing both, morphological effects and molecular biomarkers, a more complex insight into the mechanisms and effects caused by EDCs in zebrafish should become possible.

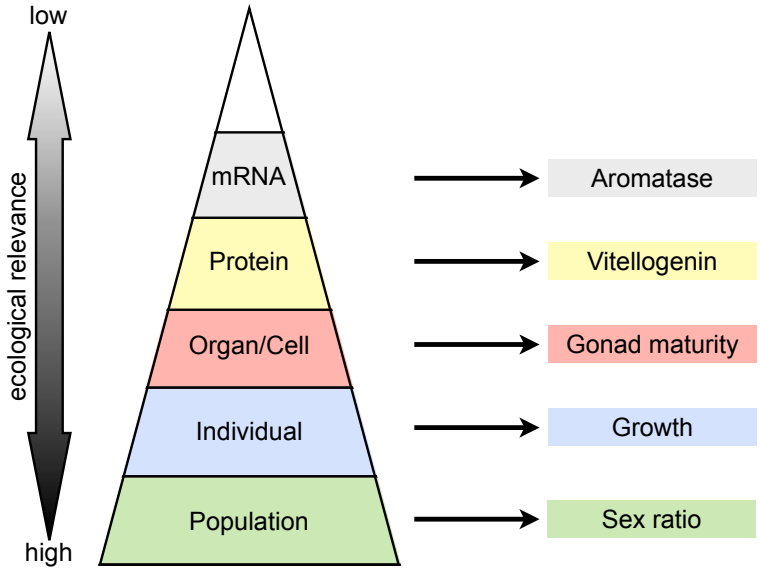


Fig. 1.8: Ecological relevance of effect levels and endpoints investigated within the present study

2 The maturity index as a tool to facilitate the interpretation of changes in vitellogenin production and sex ratio in the Fish Sexual Development Test

2.1 Abstract

In July 2011, the Fish Sexual Development Test (FSDT) has officially been adopted as OECD test guideline 234 for the detection of endocrine disrupting chemicals (EDCs). Sex ratio and vitellogenin (VTG) induction are the mandatory endocrine endpoints within this test, whereas gonad staging is only included as an option. In the present study, five FSDTs with zebrafish (*Danio rerio*) were conducted with EDCs with different modes of action (17α-ethinylestradiol, dihydrotestosterone, 17β-trenbolone, prochloraz and 4-*tert*-pentylphenol). Results document that not only sex ratio and VTG production of the exposed fish were massively affected, but also gonad maturation. As a novel approach for the quantification of gonad maturation in zebrafish, the maturity index was developed to allow not only an improved assessment of dose-dependent EDC-related effects on gonad maturation, but also statistical analysis of histological data. VTG induction and maturity index showed an excellent correlation for all five EDCs tested. Most importantly, the maturity index often helped to find appropriate interpretations for results that seemed contradictory at first sight. Results show that histological analyses and their predictive power for population fitness are currently underestimated and should become a standard component in the evaluation of potential EDCs.

2.2 Introduction

Endocrine disrupting chemicals (EDCs) have become a major research field in ecotoxicology over the last 20 years. Especially aquatic vertebrates are continuously affected by EDCs and have, therefore, frequently been used for EDC research. In the environment, numerous chemicals have been identified as EDCs showing effects like shift of sex ratio or occurrence of intersex gonads, on wildlife populations (Guillette et al. 1996, Jobling & Tyler 2003, Lye 2000, Sumpter 1998) In the future, even more compounds might be identified to show endocrine activity, especially under the EU regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, EU 2006), which enforces the (re-) registration of thousands of new and existing chemicals. As a consequence, considerable efforts have been made to reduce the impact of EDCs on nature and humans, and a suite of standardized test systems were developed at the OECD level (Organization for Economic Co-operation and Development, OECD 2009a, b, c, 2011).

The most critical issue of an *in vivo* test system is its capability to predict endocrine effects in wildlife populations. Full life-cycle or even multi-generation tests are usually regarded as the method of choice for the detection of population-relevant endpoints such as reproductive failure, but they are very time- and cost-intensive (Nash 2004). In July 2011, the Fish Sexual Development Test (FSDT) has officially been adopted as OECD test guideline no. 234 for the detection of EDCs within the OECD conceptual framework at level 4 (OECD 2011). Although reproduction is not included as an endpoint, the FSDT has potential as a promising compromise for the gold standards, the full life-cycle or the multi-generation test, since it has been designed for a safe evaluation of potential EDCs within an extended exposure period of 60 days, which is still much shorter than $\geq 6 - 7$ months for a full life-cycle or even a multi-generation test with, e.g., zebrafish (*Danio rerio*).

As a protogynic fish species, zebrafish represents an interesting test organism for the investigation of effects on reproduction and the hormonal system. Its gonad development covers an all-female state, which means that all individuals first develop ovaries, from which around 50% will be transformed to testis later (Takahashi 1977). This developmental process is under hormonal control and makes zebrafish particularly sensitive to EDCs (Andersen et al. 2000, Maack & Segner 2004). All experiments conducted so far during the validation of the FSDT and several studies before (for reviews, see Holbech et al. 2012 as well as Scholz & Klüver 2009) demonstrated that exposure of zebrafish to EDCs in this sensitive phase of gonadal transformation resulted in a dose-dependent shift of sex ratio and an up- or down-regulation of vitellogenin (VTG). Histopathological lesions in the gonads have been given less attention, even though a guidance document on the diagnosis of endocrine-related histopathology in fish gonads has been made available (OECD 2010) and although several chemicals are known to inhibit sexual differentiation and gonad maturation (for review, see Danzo 1998). Moreover, there are numerous reports showing significant effects in the gonads of fish exposed to EDCs (Dang et al. 2011, Keiter et al. 2012, van der Ven et al. 2003, Weber et al. 2003).

VTG induction is usually regarded as an essential biomarker for the evaluation of EDCs affecting the sexual hormone system (Holbech et al. 2001, Matozzo et al. 2008, Örn et al. 2003, Sumpter & Jobling 1996). However, an appropriate interpretation of VTG data is frequently only possible with information about the individual gender (Holbech et al. 2006). Since the genetic sex of zebrafish can neither be detected genetically (Tong et al. 2010), nor be unequivocally assessed by phenotype at 60 days post-hatch (dph), especially after exposure to EDCs, gonad histology as the only method for safe and definite sex determination is an

indispensable component in the FSDT with zebrafish. However, while sex ratio and VTG induction are mandatory endocrine endpoints in the FSDT, gonad staging is only included as an option in OECD TG 234 (OECD 2011), thus giving rise to potential misinterpretations, since differentiation to the female or male gender does not necessarily coincide with reproductive capability. Since EDCs may not only affect differentiation itself, but also the time-scale of gonadal maturation, staging of gonad maturity seems most helpful for the interpretation of EDC-related effects, a fact that should be considered when it comes to the evaluation of an unknown substance, e.g. with respect to decisions at the regulatory level.

In the present study, data from five FSDTs with different EDCs covering diverse modes of action are compared with particular focus on the correlation between the mandatory endocrine endpoints of the FSDT (VTG induction and sex ratio) and the stage of gonadal maturity. The maturity index was developed as a novel approach for the quantification of gonad maturation in young zebrafish, which should allow not only improved visualization of dose-dependent EDC-related effects on gonad maturation, but also the statistical analysis of histological data.

2.3 Material and Methods

Tab. 2.1: Chemicals and test concentrations

Test substance	CAS-No.	Replicates	Test concentrations
4-*Tert*-Pentylphenol	80-46-6	4	32, 100 and 320 µg/L
17α-Ethinylestradiol	57-63-6	2	0.1, 1, 3 and 10 ng/L
17β-Trenbolone	10161-33-8	2	1, 3, 10 and 30 ng/L
Dihydrotestosterone	512-18-6	4	100, 320 and 1000 ng/L
Prochloraz	67747-09-5	2	10, 30, 100 and 300 µg/L

Test Substances

4-*Tert*-pentylphenol, prochloraz, 17α-ethinylestradiol, dihydrotestosterone and 17β-trenbolone and all other substances, unless stated otherwise, were obtained from Sigma-Aldrich (Deisenhofen, Germany).

Table 2.1 gives an overview of the test substances and concentrations used for zebrafish exposure in the FSDTs. The steroids 17α-ethinylestradiol, dihydrotestosterone and 17β-tren-

bolone were dissolved in dimethylsulfoxide (DMSO) at maximum final solvent concentrations of 0.01 %. All treatments were run in at least two replicates. A water- and a solvent-control were run in duplicates.

Exposure

The Fish Sexual Development Test (FSDT) has been performed as described in the OECD Test Guideline no. 234 (OECD 2011). Exposure of zebrafish (*Danio rerio*) to the test chemicals started at latest 1 hour post-fertilization (hpf) and ended at 60 days post-hatch (dph). At minimum, 40 eggs were used for each replicate. Fish were held in aerated 8 - 12 L flow-through glass tanks at 26 - 27 °C and a dark:light cycle of 10:14 hours. The exposure water was mixed from tap water and demineralized water to reduce the total hardness. Constant aeration in the reservoirs ensured high oxygen saturation. The effluent was purified by passing over a charcoal filter, before it was released to the municipal sewage treatment plant. Water temperature and flow-through rates (complete water exchange every 8 hours) were controlled twice daily. Water hardness (200 - 280 mg/L), conductivity (600 - 750 µS), pH (8.0 - 8.2) and oxygen saturation (90 – 95 %) were checked once weekly. Feces and food remains in the tanks were removed daily. From day 4 to 14, larvae were fed with powdered dry food (Sera Micron, Heinsberg, Germany or Tetra AZ 100™ starter food (Tetra-Werke, Melle, Germany), followed by feeding with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

Sampling

Fish were euthanized in a saturated solution of benzocaine (ethyl-4-aminobenzoate) or buffered MS-222 (100 mg/L). Length and wet weight of each individual were documented. Head and tail were cut off with a razor blade behind the operculum and behind the anal fin, weighed together and frozen immediately in liquid nitrogen for subsequent quantification of VTG *via* enzyme-linked immunosorbent assay (ELISA). Remaining trunks were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidsons's fixative (Romeis & Böck 2001) for subsequent histological analyses.

ELISA

The measurement of the VTG concentration in head and tail homogenate of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1.5 ml centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris-HCl, pH 7.4: 1 % protease inhibitor cocktail (P

8340; (Sigma-Aldrich)). The homogenate was centrifuged for 30 min at minimum 25,000 × g at 4°C where after the supernatant was collected and stored at -80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).

Histology

Samples were incubated in modified Davidsons's fixative or Bouin´s fixative at 4 °C for at least 24 h before embedding into paraffin by a tissue processor. Embedding was performed with a heated paraffin embedding module with trunks orientated ventrally to the cutting surface. Sections of the gonads with a thickness of 4 - 5 µm were cut with a microtome, mounted on glass slides and then stained with hematoxylin-eosin (Romeis & Böck 2001) the next day.

Light microscope evaluation of the tissue sections was performed according to the OECD Histopathology Guidance Document (OECD 2010). Each fish was identified either as female, male or intersex, or recorded as undifferentiated. Additionally, the maturation stages of the gonads were categorized as relative proportions of various gametogenic cell types into a numerical staging system (ovary: stages 0 - 5, testis: stages 0 - 4) according to the OECD Histopathology Guidance Document (OECD 2010).

Maturity index

As an enhancement to the regular staging system in the OECD Histopathology Guidance Document (OECD 2010), each stage of maturity was given a fixed maturity value, increasing with the maturity of the fish (Stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The sum of these values from all individuals of each replicate was divided by the number of fish. The sex-specific mean values for each treatment were calculated from the replicates and termed the maturity indices for females or males. Completely undifferentiated or intersex individuals were not included into this assessment, since they could not be classified as female or male.

Chemical analyses

Water samples for chemical analyses were taken weekly and frozen at -80 °C for subsequent use. Except for prochloraz, actual concentration data were determined by means of HPLC. Analyzes for prochloraz were performed with LC–MS as described by Kinnberg et al. (2007). For 17β-trenbolone, no chemical analyzes were conducted, since previous experiments with

the same substance in the same facility had documented high stability and only minor deviations from nominal concentrations (Böttcher 2011).

Statistics

For statistical evaluation, data were tested for normality and homogeneity of variance and analyzed by one-way ANOVA - either parametric Bonferroni-Holm adjusted or non-parametric Kruskal-Wallis followed by Dunn´s or Dunnett´s Test using SigmaStat 12.0 (Statsoft-Jandel Scientific, Erkrath, Germany).

2.4 Results

Chemical analyses

Measured concentrations of prochloraz and 17α-ethinylestradiol did not vary from nominal concentrations by more than 20 % over the entire test period (details not shown) and did, thus, meet requirements of the OECD guideline (OECD 2011). As mentioned above, for 17β-trenbolone, no chemical analyzes were conducted. Due to inadequate storage conditions, chemical analyses of 4-*tert*-pentylphenol could not be performed (for details, see OECD validation report phase 1, 2011). Chemical analyses of dihydrotestosterone revealed around 50 % lower measured than nominal concentrations in all treatments (for details, see OECD validation report phase 2, 2011).

Mortality

No significant effects on hatchability or mortality were observed in any of the experiments with prochloraz, 17α-ethinylestradiol and 17β-trenbolone (details not shown). Increased mortality was observed in the experiments with dihydrotestosterone and 4-*tert*-pentylphenol (for details, see OECD validation reports phase 1 & 2, 2011).

Body size

No significant effects on body size were observed, except for prochloraz, where zebrafish in the highest concentrations were smaller than in the controls (details not shown).

Sex ratio, VTG induction and maturity index

17α-Ethinylestradiol and 4-*tert*-pentylphenol caused feminization and retardation of sexual maturation; dihydrotestosterone and 17β-trenbolone caused masculinization. For prochloraz, masculinization and intersex development were observed (Fig. 2.1, Tab. 2.2). Figs. 2.2 – 2.6 illustrate changes in vitellogenin induction and maturity index for the five EDCs.

Tab. 2.2: Semiquantitive summary of effects found in the 5 different FSDTs. ⬇ – decrease, ⬆ – increase; the number of arrows represents intensity of effects.

	Females		**Males**		**Sex ratio**	
	VTG	Maturity Index	VTG	Maturity Index	Females	Males
4-*Tert*-Pentylphenol	⬇	⬇⬇	⬆	⬇⬇	⬆	⬇
17α-Ethinylestradiol	⬆⬆⬆	⬇	⬆⬆	⬇⬇	⬆	⬇
17β-Trenbolone	⬇	⬇	⬇	⬆⬆⬆	⬇	⬆
Dihydrotestosterone	⬇	⬇⬇	⬇	⬆⬆	⬇	⬆
Prochloraz	⬇	⬇⬆	⬇	⬇⬆	⬇	⬆

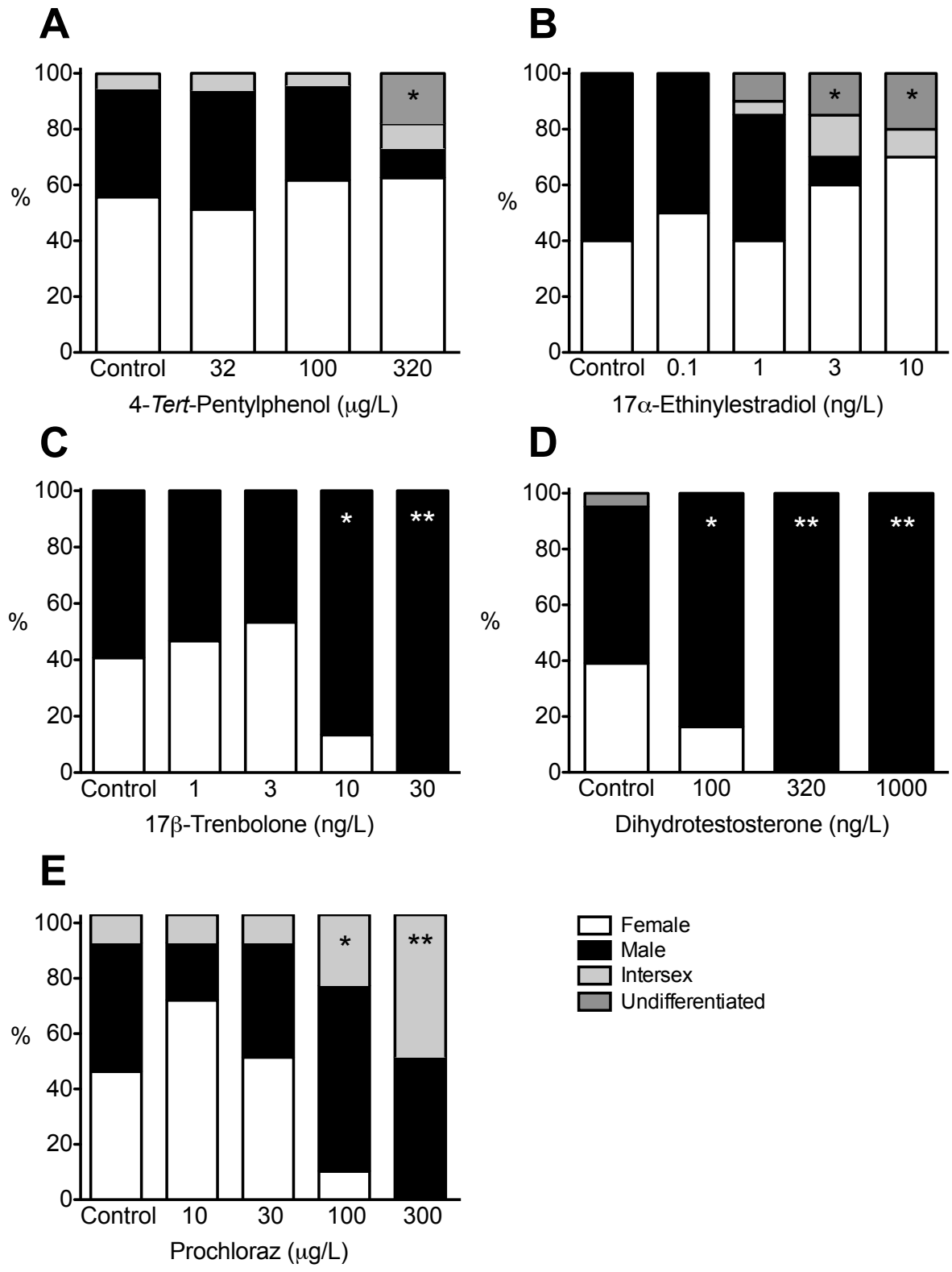


Fig. 2.1: Sex ratio of zebrafish (*Danio rerio*) at 60 dph exposed to (A) 4-*tert*-pentylphenol, (B) 17α-ethinylestradiol, (C) 17β-trenbolone, (D) dihydrotestosterone and (E) prochloraz. The data were analyzed by one-way ANOVA, non-parametric Kruskal-Wallis followed by Dunn´s Test. (*$p < 0.05$, **$p < 0.01$).

4-*Tert*-pentylphenol: Male zebrafish exposed to 320 µg/L 4-*tert*-pentylphenol showed significantly increased VTG concentrations and decreased testicular maturity (Fig. 2.2). The dose-dependent decrease of VTG concentrations in female zebrafish coincided with a marked decline of gonad maturity. Both effects were significant at 100 and 320 µg/L. Exposure to 4-*tert*-pentylphenol resulted in a change of the sex ratio towards females and undifferentiated fish at concentrations of 100 and 320 µg/L (Fig. 2.1). These findings document a strong retardation of gonad maturation caused by 4-*tert*-pentylphenol.

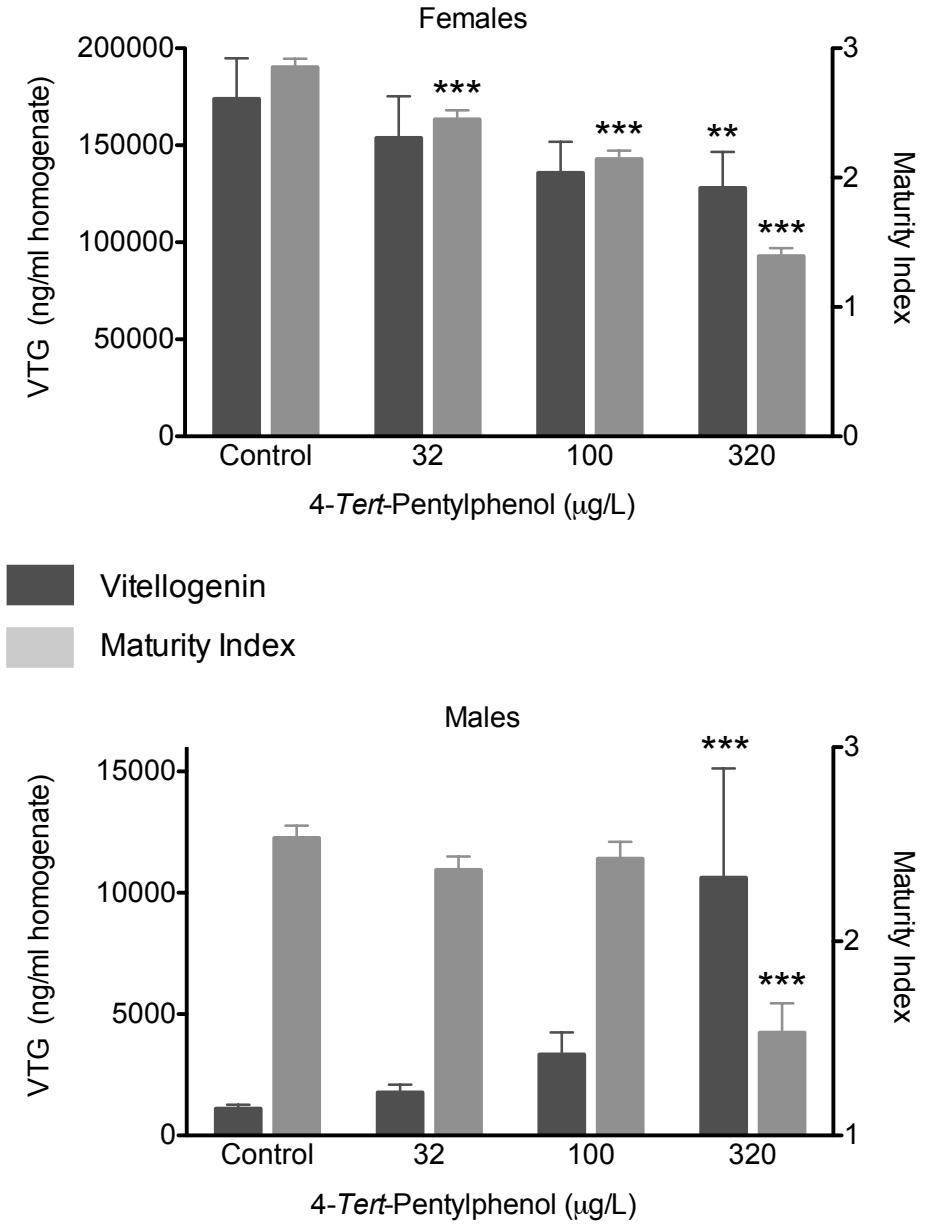


Fig. 2.2: Induction of vitellogenin and maturity index in female and male zebrafish (*Danio rerio*) after exposure to 4-*tert*-pentylphenol at 60 dph (**$p < 0.05$, ***$p < 0.01$; Dunnett´s Test).

17α-Ethinylestradiol: Exposure to 17α-ethinylestradiol caused very strong VTG induction in both female and male zebrafish from 3 and 10 ng/L, respectively. Maturity indices of both genders were already significantly decreased from concentrations > 0.1 ng/L (Fig. 2.3). A strong dose-dependent feminization was evident in the sex ratio from 3 and 10 ng/L. Additionally, the percentage of completely undifferentiated and immature zebrafish was more than 30 % in the highest concentration of 17α-ethinylestradiol (Fig. 2.1). In summary, the results show that 17α-ethinylestradiol inhibits gonad maturation and stimulates VTG production in both sexes.

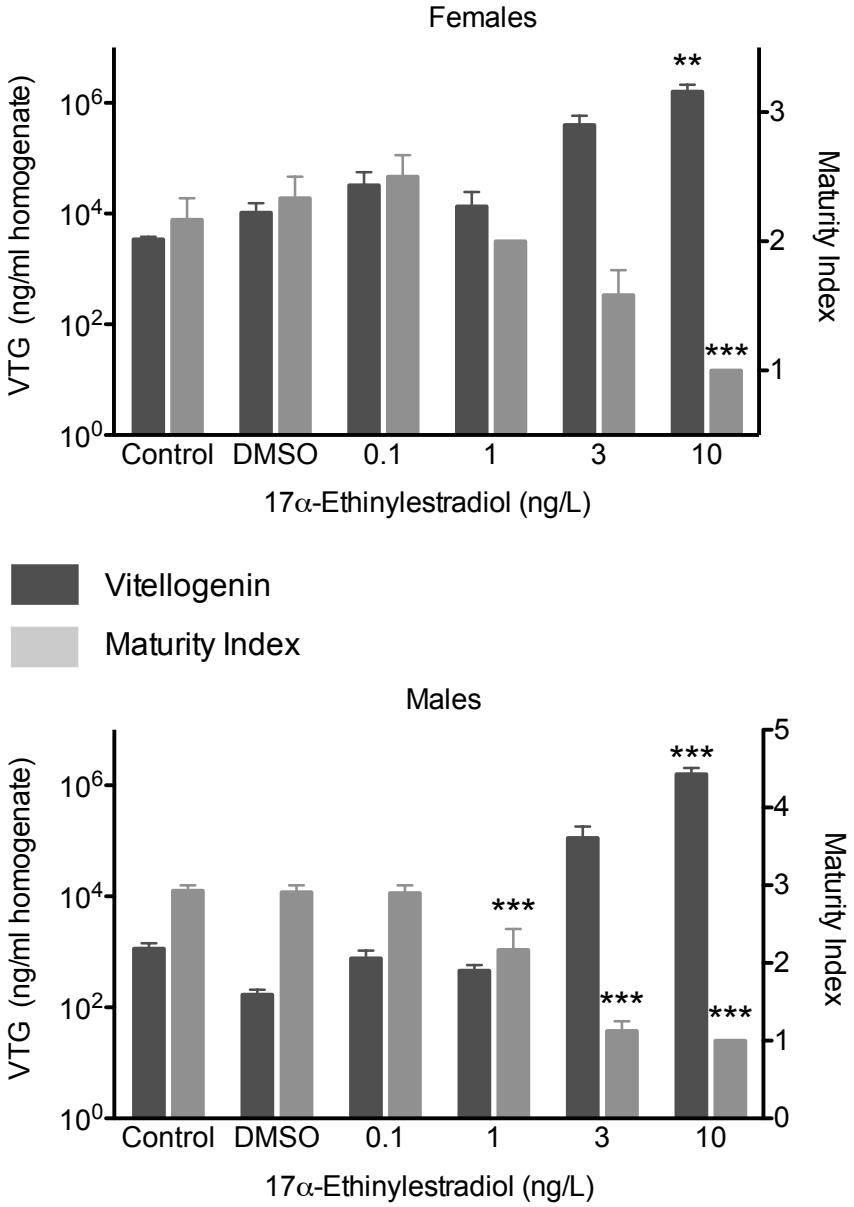


Fig. 2.3: Induction of vitellogenin and maturity index in female and male zebrafish (*Danio rerio*) after exposure to 17α-ethinylestradiol at 60 dph (**$p < 0.05$, ***$p < 0.01$; Dunnett´s Test).

17β-Trenbolone: Zebrafish exposed to the synthetic androgen 17β-trenbolone showed reduced levels of VTG, when compared to controls (Fig. 2.4). A significant decrease in males was found at 10 and 30 ng/L, whereas a tendency for decreased VTG concentrations in females was found already at the lowest concentration of 1 ng/L 17β-trenbolone. At the highest concentration of 30 ng/L, no fish developed female gonads (Fig. 2.1) and, therefore, no VTG concentrations were measured for females at 30 ng/L. At 10 ng/L 17β-trenbolone, only 10 % of the individuals were identified as females, and only these individuals displayed slightly increased VTG values and gonad maturity compared to controls. In contrast, elevated maturity indices were prominent in male zebrafish. Their gonads were significantly advanced in maturity at 10 and 30 ng/L 17β-trenbolone, which correlated to decreased VTG values.

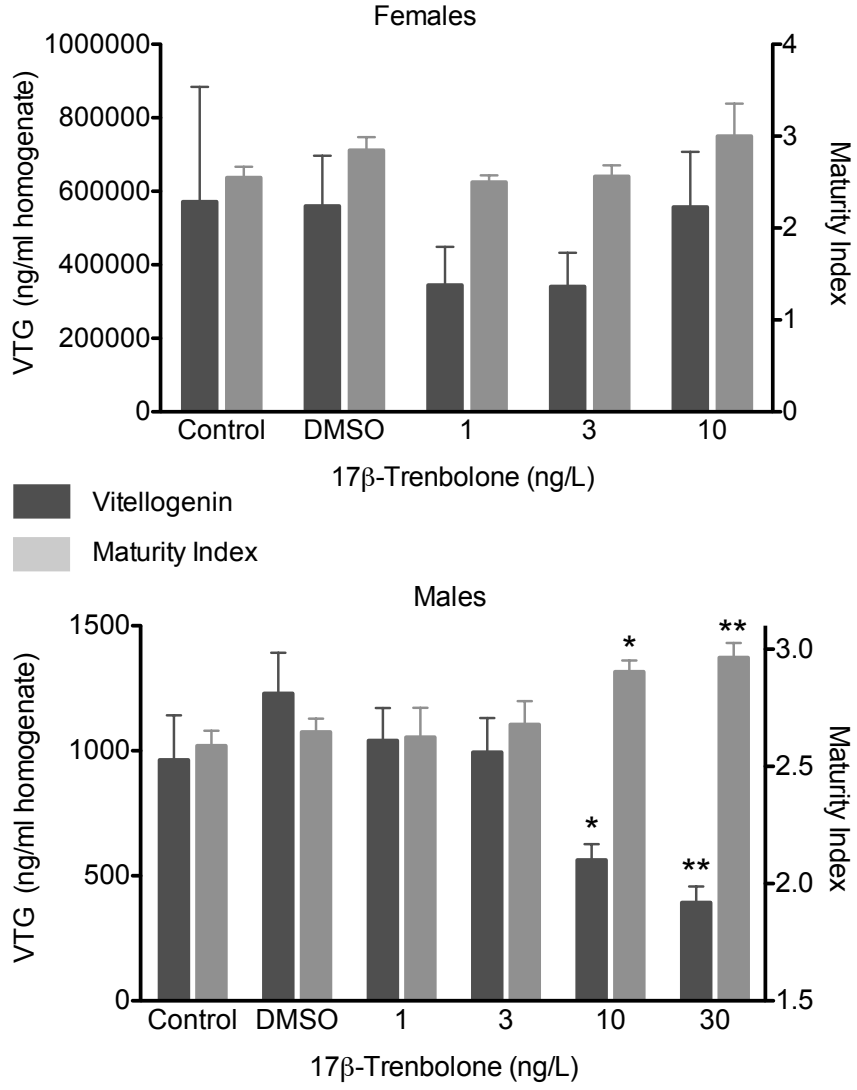


Fig. 2.4: Induction of vitellogenin and maturity index in female and male zebrafish (*Danio rerio*) after exposure to 17β-trenbolone at 60 dph (*$p < 0.05$, **$p < 0.01$; Dunnett´s Test). No female individuals developed in treatment groups > 10 ng/L.

Dihydrotestosterone: Dihydrotestosterone had a very strong impact on the sex ratio of zebrafish. At ≥ 300 ng/L, no females could be identified (Fig. 2.1). At 100 ng/L, 16 % of the individuals were female and displayed a significant decrease of VTG induction and maturity indices (Fig. 2.5). In contrast, the maturity of males was increased in a dose-dependent manner, accompanied by very low VTG levels at ≥ 100 ng/L dihydrotestosterone.

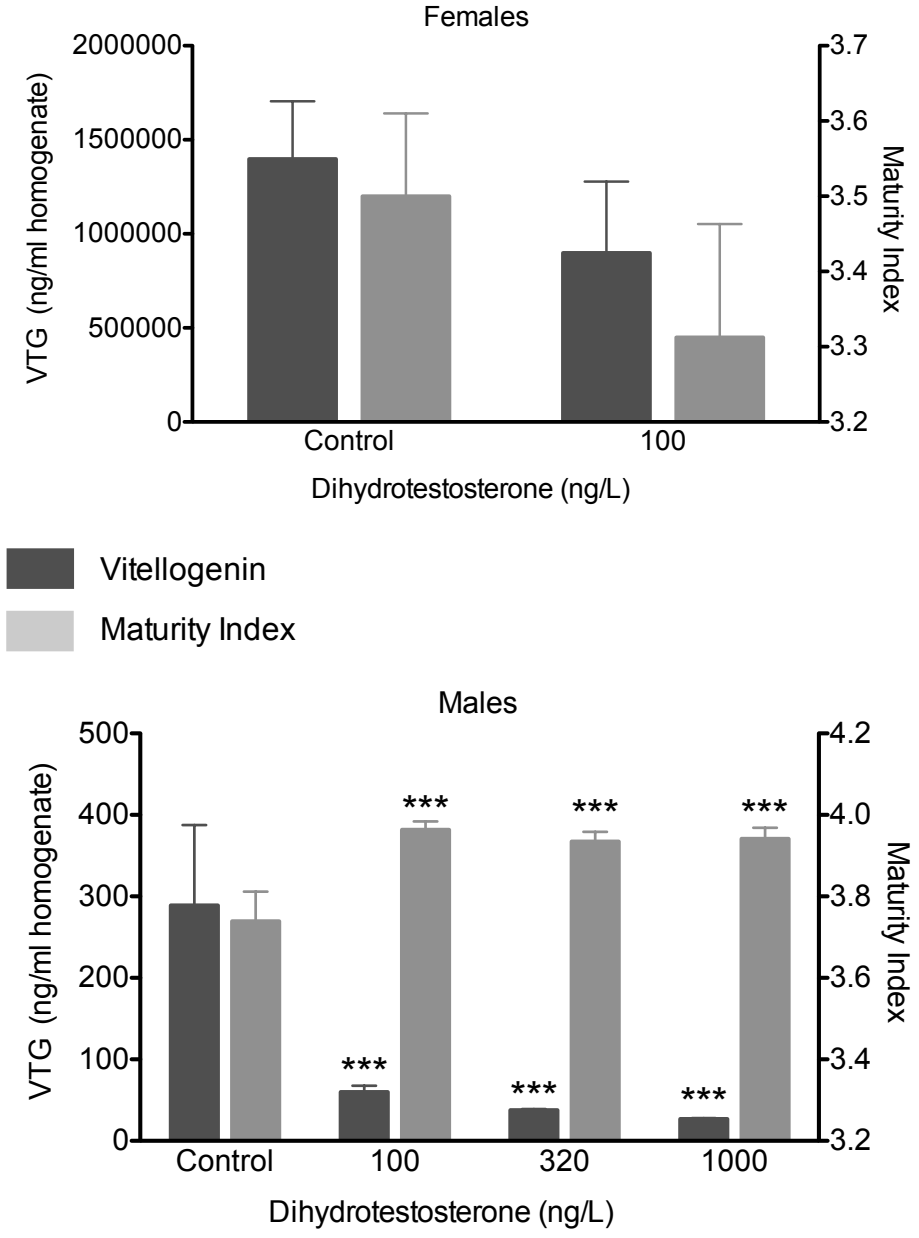


Fig. 2.5: Induction of vitellogenin and maturity index in female and male zebrafish (*Danio rerio*) after exposure to dihydrotestosterone at 60 dph (***$p < 0.01$; Dunnett´s Test). No female individuals developed in treatment groups > 100 ng/L.

Prochloraz: Exposure to the fungicide prochloraz did not produce a linear dose-response relationship; however, VTG concentrations and maturity indices again correlated in both female and male zebrafish (Fig. 2.6). A peak of VTG concentrations and maturity indices was found at 30 µg/L for both genders. However, from 100 µg/L prochloraz, males showed a decrease of VTG levels, but elevated maturity indices, if compared to controls. Following exposure to ≥ 100 µg/L prochloraz, ≤ 10 % of the individuals were females. In contrast, at 320 µg/L, 40 % of the fish were categorized as intersex (Fig. 2.1), indicating that prochloraz has a strong impact on sexual development of zebrafish.

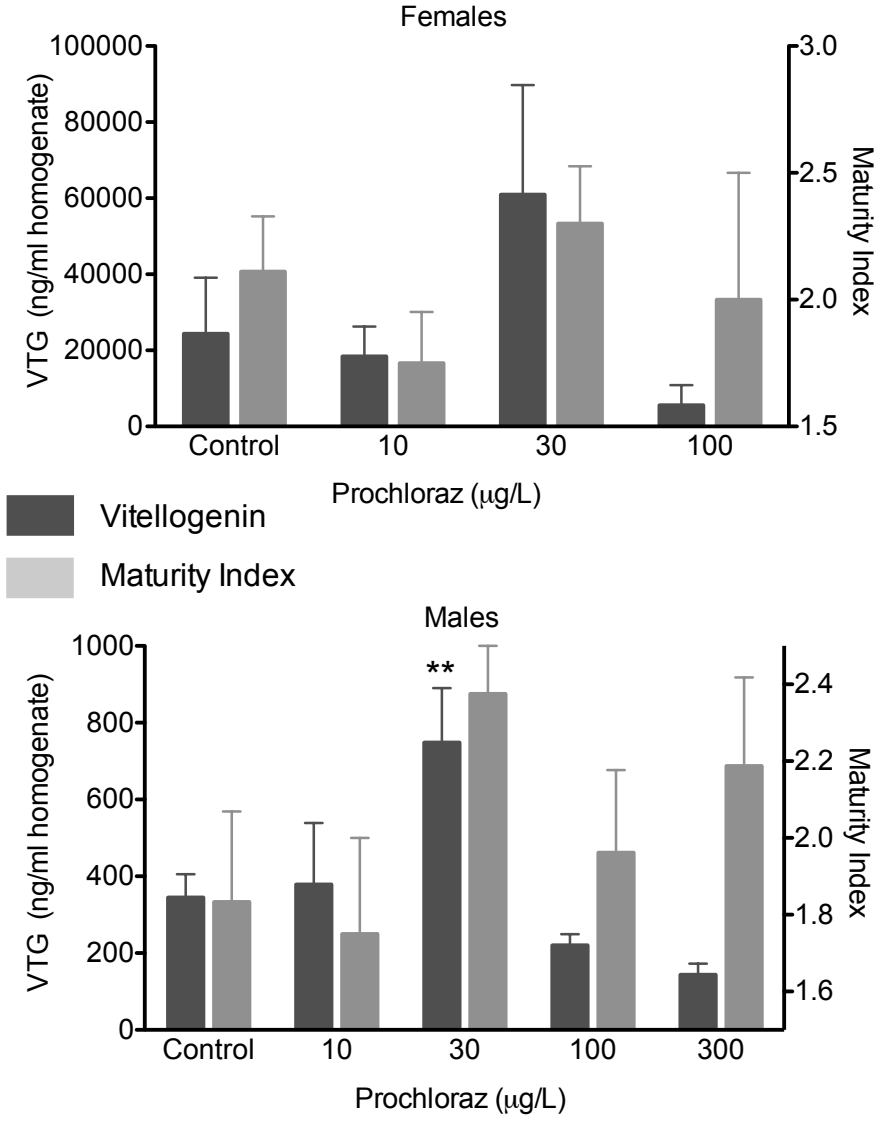


Fig. 2.6: Induction of vitellogenin and maturity index in female and male zebrafish (*Danio rerio*) after exposure to prochloraz at 60 dph (**$p < 0.05$; Dunnett´s Test). No female individuals developed in treatment groups > 100 µg/L.

2.5 Discussion

There is increasing evidence that disruption of the sex ratio and gonad maturity by EDCs may lead to reproductive failure of fish at the population level (Fenske et al. 2005, Kidd et al. 2007, Van den Belt et al. 2003, Weber et al. 2003). As a consequence, morphological changes and delayed maturity of the gonads are indicative of impaired reproduction, and consequently, reduced fitness of the population. The EDCs examined in this study are well-known to have impact on gonad development in fish (Davis et al. 1992, Gimeno et al. 1998, Holbech et al. 2006, Le Gac et al. 2001, Örn 2003), but their effects on gonad maturity and its correlation to VTG induction have never been compared systematically. Even though gonad staging is an accepted method with clear references in the OECD Histopathology Guidance Document (OECD 2010), data on gonad staging are scant in literature. As a potential reason for this (beside the work load), the quantification of histological results has been discussed controversially, and the presentation of dose-dependent morphological effects is difficult. Several studies have used the gonado-somatic index (GSI) as an indirect parameter for reproductive capacity (Brion et al. 2004, Noaksson et al. 2005, Panter et al. 2006). However, spawning cycles may limit the reliability of the GSI and may give rise to false negative results. In the FSDT, however, gonad histology has to be performed for the identification of each individual's gender, and, consequently, gonad staging makes more sense than the GSI. The maturity index was developed as a parameter to describe the state of maturity of a specific exposure group, which is easy to record and to quantify. All endocrine disruptors used in the present study had a remarkable impact on gonad maturity and VTG induction of the exposed fish.

Prochloraz, e.g., is a popular fungicide, well known as an aromatase inhibitor and androgen antagonist and, therefore, commonly used in endocrine disruptor research (Hinfray et al. 2006, Trant et al. 2001). Its multiple modes of action have influence on several hormonal axes of vertebrates (Laier et al. 2006, Liu et al. 2011). In our experiment, prochloraz caused a significant shift of the sex ratio towards males and intersex and, thus, confirmed previous studies (Holbech et al. 2012, Kinnberg et al. 2007, Thorpe et al. 2011). However, not all of these studies reported the same effects on gonad maturity and VTG induction: Thorpe at al. (2011), e.g., did not detect any changes in gonad maturity of zebrafish in an FSDT. In the present study, very low concentrations of prochloraz were used, if compared to other studies, in order to analyze if the dose-response relationship would be different. In fact, results indicate that low concentrations of prochloraz may cause unexpected hormesis-like effects. After 60 days of exposure, both genders reached a maximum average maturity at low

concentrations (30 µg/L). Most likely, at higher concentrations, aromatase-inhibition and toxic effects of prochloraz prevail and cause under-development and masculinization of females as well as a remarkable dose-dependent decrease in the maturity index of males. Kinnberg et al. (2007) also found a significant decrease of VTG concentrations and alterations of gonad maturity in female fish exposed to 200 µg/L prochloraz in an FSDT. In FSDTs with prochloraz in four different laboratories, Holbech et al. (2012) showed decreased VTG levels in females and sex ratios skewed towards males at different concentrations. Gonad staging was performed in both studies, but unfortunately data were not presented in detail because the OECD VMG-Eco decided to not include histopathology as a mandatory endpoint in the FSDT, since they were of the opinion that histology implies a heavy workload and is difficult to interpret. The maturity index though provides an opportunity to add a third conclusive endpoint in the evaluation of the results, which allows a better understanding, visualization and interpretation of VTG data and the sex ratio.

A masculinizing effect on fish was also found for dihydrotestosterone (OECD phase 2 validation report, 2011) and 17β-trenbolone (Örn et al. 2006, Panter et al. 2004, Seki et al. 2006). The strong affinity of these substances to the androgen receptor leads to increased spermatogenesis and accelerated male gonad maturation, which is reflected in the high maturity indices of exposed males in our study. The high maturity indices correlated highly with VTG levels in males, which declined dose-dependently after exposure to dihydrotestosterone and 17β-trenbolone. Looking at effects in females exposed to these androgens, the significant decrease in the number of females with increasing exposure concentrations makes statistical approaches very difficult, and thus can only be interpreted as a tendency. Nevertheless, females seem to be able to compensate for the massive androgenic effects of 17β-trenbolone and tend to develop strong feminine attributes once they have succeeded in developing according to their genetic sex. Aromatase activity, e.g., is known to be up-regulated by a high surplus of androgens (Brion et al. 2012), resulting in elevated estrogen levels. Similar observations concerning VTG induction and sex ratio of zebrafish have been made in other studies and were reported to be irreversible after depuration (Larsen et al. 2010, Morthorst et al. 2010, Örn et al. 2006). Effects on gonad maturity have been presented in different manners in these studies, i.e. by showing the proportions of the stages, by counting the number of sperm or by measuring the size of testis area. This diversity of presentation modes shows that there is a need for a consistent and simple method to quantify effects on gonad maturity.

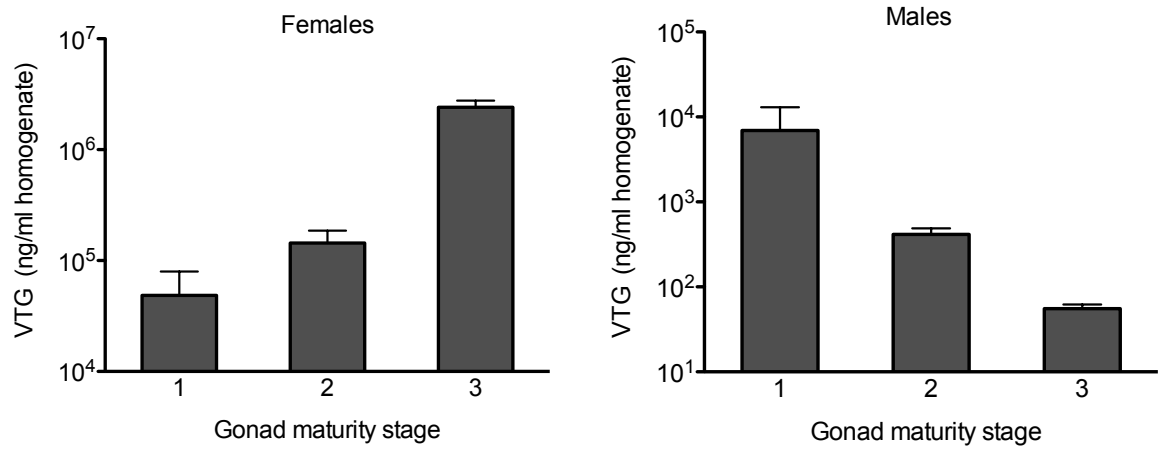


Fig. 2.7: Correlation between grade of gonad maturity and VTG concentration in untreated female and male zebrafish at 60 dph (note different scales on the y-axis).

Moreover, the absence of baseline data in literature for the correlation between VTG production and gonad maturation in zebrafish reveals that there is a gap in our knowledge, which could build the basis of interpretation of EDC-related effects in zebrafish. The correlation of VTG concentration and gonad maturity in untreated female and male zebrafish from all five FSDTs investigated in the present study is presented in Fig. 2.7. Plotting the maturity indices against corresponding VTG values of the individual clearly shows that these two parameters are closely connected. In females, the increasing concentration of VTG with increasing gonad maturity is obvious. An inverse correlation can be found in males: The more mature male zebrafish are, the less VTG can be measured. Mean values of the basic VTG concentrations are also sex-specific. At the age of 60 dph, female zebrafish have average VTG concentrations of 50.000 – 2.500.000 ng/ml homogenate, whereas males only have 50 – 7.000 ng/ml. As shown in Fig. 2.7, increasing gonad maturity of unexposed females always correlates to higher VTG values, which results from the need for yolk production in the ovaries. The reason why this correlation cannot be observed after exposure to strong estrogens might be that some of the morphological females are genetic males with immature ovaries or individuals that are arrested in the all-female stage of sexual development in zebrafish. Sexual arrest of males exposed to estrogenic substances during sensitive developmental periods has been shown (Fenske et al. 2005), though genetic determination of the gender of zebrafish would be needed to confirm this. To our knowledge, this is not yet possible (in contrast to other fish species like the medaka (*Oryzias latipes*)), even though several genes have been correlated to sexual differentiation of zebrafish (Jørgensen et al. 2008, Orban et al. 2009). For a better understanding of the underlying mechanisms, the possibility to distinguish between phenotypic and genetic sex would help to elucidate whether feminization of male zebrafish is

a real sex reversal or only arrested sexual development. This question remains unanswered, even though estrogens are much better investigated than androgens (Scholz & Klüver 2009).

The standard positive substance in research on estrogenic EDCs is 17α-ethinylestradiol. It is commonly used in oral contraceptives, and thus constantly found in surface waters of the environment (Barel-Cohen et al. 2006, Belfroid et al. 1999, Campell et al. 2006). Its effective contribution to feminization of populations is discussed controversially, because a high quantity of industrial chemicals also significantly accounts for estrogenic effects in wildlife (Wise et al. 2011). Other recent publications suggest that the impact of 17α-ethinylestradiol on human health is highly underestimated and that the level of exposure should be drastically lowered (Owen & Jobling 2012). In contrast to most other estrogenic compounds found in the environment, 17α-ethinylestradiol has an even stronger effect than natural 17β-estradiol, due to its strong binding to the estrogen receptor. Other synthetic endocrine disruptors like alkylphenols are much weaker in their affinity to the estrogen receptor than 17α-ethinylestradiol and 17β-estradiol, and have multiple other effects, probably due to their different structures (Routledge & Sumpter 1997). The semi-synthetic properties of 17α-ethinylestradiol may, to some extent, also explain why many organisms are able to recover from 17α-ethinylestradiol exposure (Van den Belt et al. 2003, Hill & Janz 2003, Kidd et al. 2007). This difference between the two endocrine disruptors 4-*tert*-pentylphenol and 17α-ethinylestradiol is clearly evident from our results: 17α-ethinylestradiol is a classic substance for estrogenic VTG induction: Female and male zebrafish exposed to high concentrations of the semi-synthetic estrogen have up to 100.000-fold higher plasma VTG concentrations than in the controls. The sex ratio was significantly skewed towards females, intersex and undifferentiated individuals at 3 and 10 ng/L and accompanied by massive underdevelopment of the gonads in both genders. These findings give another hint to the assumption that feminization of male zebrafish is probably rather an arrest of sexual development than a real sex reversal (Maack & Segner 2004).

4-*Tert*-Pentylphenol, like other alkylphenols, is also known to be estrogenic (Routledge & Sumpter 1997), but its effect on VTG induction is not a "typical" estrogenic response. VTG levels of males exposed to high concentrations of 4-*tert*-pentylphenol were significantly higher than in controls, whereas those of females were significantly lower. However, female medaka exposed to concentrations of up to 220 µg/L showed no elevated VTG levels, whereas males exposed to high concentrations showed significantly increased VTG levels (Seki et al. 2003). Likewise, adult zebrafish exposed for 3 weeks to 4-*tert*-octylphenol (12.5-100 µg/L), an alkylphenol with comparably high estrogenic potency, showed no effect in

VTG levels, but both females and males had decreased GSIs (Van den Belt et al. 2001). Similar results were reported by Weber et al. (2003), who found dose-dependent suppression of gametogenesis in zebrafish after exposure to 17α-ethinylestradiol and nonylphenol. While these findings may appear contradictory to the estrogenic properties of the alkylphenols, a closer look at the maturity index makes them more understandable: The maturity indices found in our experiment clearly prove 4-*tert*-pentylphenol to cause strong maturity retardation in females and males (Fig. 2.3). Additionally, 20 % of the individuals in the highest concentration of our experiment were identified as undifferentiated, which is another indicator for delayed sexual development after exposure to 4-*tert*-pentylphenol. Normally, the sexual differentiation of zebrafish should be accomplished after 60 days (Takahashi 1977), but our data clearly show 4-*tert*-pentylphenol to produce a massive retardation of gonadal development, which could not have been identified without gonad staging and the calculation of the maturity index. The VTG results of the females alone might even have indicated that 4-*tert*-pentylphenol is not estrogenic.

Fig. 2.7 illustrates the correlation between gonad maturity and VTG concentration in control fish. From these findings, it can be concluded that the weak estrogenic properties of 4-*tert*-pentylphenol were not sufficient to induce high VTG expression in immature ovaries, whereas the semi-synthetic and very potent 17α-ethinylestradiol was powerful enough to induce massive VTG production in immature ovaries. 17α-Ethinylestradiol has a twofold higher affinity to the estrogen receptor (Blair et al. 2000) than the natural steroid 17β-estradiol, which also induces very high levels of VTG (Esterhuyse et al. 2009). Moreover, toxic properties of 4-*tert*-pentylphenol might also influence the sexual development of exposed zebrafish, delaying or even inhibiting gonad maturation and VTG induction. This example proves that the maturity index has additional informative value helping to explain the effects of a substance. Lacking a strong affect on the sex ratio of a population, an endocrine disruptor might be categorized as harmless by evaluation of only the basic mandatory endpoints required in the OECD guideline for the FSDT (OECD 2011). Effects on the maturity of the individuals, however, may be a limiting aspect for a population, especially for seasonal spawners. Sex specific behavior, which is essential for the timing of gamete release, is closely linked to sexual maturity (Munakata & Kobayashi, 2010). Consequently, exposure to environmental pollutants that accelerate or decelerate gonad maturation can lead altered behavior resulting in impaired individual reproductive success and, thus, to adverse effects at the population level (Söffker & Tyler 2012). Therefore, additional information about the reproductive state of the exposed fish is needed to be able to predict the fitness of populations.

Our results show that the implementation of the maturity index into the evaluation of endocrine disruptors helps to interpret the underlying mechanisms of the test substances, providing a much more complete argumentative basis for regulatory authorities to predict adverse effects on populations. Cost-benefit analyses clearly indicate that the maturity index should be a mandatory component of OECD TG 234 (OECD 2011), since histological techniques will have to be applied for gender identification anyway. As gonad staging can also be performed with medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*), there is no reason to raise doubts as to whether the maturity index can be applied to these species as well. Especially under the new EU chemicals policy, REACH, which enforces the (re-) registration of several thousands of chemicals, a quick and effective evaluation of relevant endpoints is of prime interest. The presence of mature, healthy gonads is a major precondition for successful reproduction in wildlife populations and is, therefore, as necessary for the prediction of population fitness as the sex ratio. Furthermore, the statistical approach of the maturity index seems to be much more appropriate to visualize dose-depended effects than only showing proportions of different maturity stages. Gonad development and VTG induction go hand-in-hand and must be investigated as a complex, especially under the influence of endocrine disrupting chemicals. Since, for OECD TG 234, histological analyses have to be carried out anyway to determine the sex of each individual, it seems well worth having a second look. In our view, a detailed histological analysis of gonad histology in combination with staging is indispensable for an appropriate interpretation of VTG induction and sex ratio in the FSDT and should be obligatory in OECD TG 234.

3 Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after periodic exposure to 17β-trenbolone

3.1 Abstract

The aim of the present study was to investigate the effects of the androgenic endocrine disruptor 17β-trenbolone on the sexual development of zebrafish (*Danio rerio*) with special emphasis on the persistence of adverse outcomes. An exposure experiment with recovery phase was chosen to assess the potential of reversibility of androgenic effects. Zebrafish were exposed to environmental relevant concentrations of 17β-trenbolone (1 - 30 ng/L) from fertilization until completion of gonad sexual differentiation (60 days post-hatch). Afterwards exposure was followed by 40 days of recovery in clean water or continuous until 100 dph. Fish exposed for 100 days to 10 or 30 ng/L 17β-trenbolone were masculinized at different effect levels, as evidenced from a dose-dependent shift of sex ratio towards males, and significantly increased gonad maturity of males. At protein level, vitellogenin concentrations in head/tail homogenates of both genders were decreased. Brain aromatase (cyp19b) mRNA expression was up-regulated at low exposure concentrations (1 and 3 ng/L), but no effect was found at 10 and 30 ng/L. All those effects were persistent after depuration of 40 days in clean water, suggesting that zebrafish are not able to recover from androgenic effects on their sexual development, already at very low levels of biological organization.

3.2 Introduction

Exposure of wildlife to endocrine disrupting chemicals (EDCs) is not necessarily continuous, but may often occur intermittently, resulting from seasonal changes, variable industrial and agricultural activities. For example, industrial livestock farming is responsible for the discharge of multiple chemicals and pharmaceuticals to surface waters and the environment (Kümmerer 2009). This input is dependent on the number of animals, and the time point of application and, therefore not continuously. Whereas antibiotics are predominately found near pig and poultry farms (Wei et al. 2011), steroids are mainly found near beef feedlots (Bartelt-Hunt et al. 2012, Soto et al. 2004); Durhan et al. (2005), e.g., measured concentrations of trenbolone acetate metabolites up to 20 ng/L in beef cattle feedlot discharge and 7 ng/L in associated surface waters. The synthetic androgen is administered to animals *via* subcutaneous implants as a growth promoter for more effective meat production. Its stable metabolites 17α-trenbolone and 17β-trenbolone are excreted by the animals and can thus reach the environment (Khan & Lee 2012). These metabolites are stable with a half-life of up

to 260 days (Schiffer et al. 2001). 17β-Trenbolone binds with very high affinity as an agonist to the androgen receptor (Durhan et al. 2005), resulting in masculinization and increased growth in many animals and humans (Ankley et al. 2003). Therefore, 17β-trenbolone is also used illegally as a doping substance in athletic sports and bodybuilding (Bowers et al. 2009), but this contribution to environmental pollution with steroids is difficult to assess. Nevertheless, recent data reveal that the use of anabolic steroids in amateur and professional sports is common practice (Leifman et al. 2011, WADA 2010). The strong and fast body mass increase is even reinforced by stimulated appetite and its anti-catabolic properties caused by the activity as an anti-glucocorticoid (Meyer 2001). In contrast to most endogenous and also exogenous androgens, 17β-trenbolone cannot be metabolized by the enzyme aromatase to estrogens. However, recent studies have shown elevated cyp19b mRNA levels in zebrafish embryos after exposure to 17β-trenbolone (Brion et al. 2012), which is normally triggered by estrogens. Given the fact that there are probably no androgen responsive elements in the cyp19b promoter in zebrafish, this influence must occur *via* other, indirect mechanisms that involve estrogen receptors (Mouriec et al. 2009).

Other studies have focused on the question whether these androgenic effects in fish are reversible and have developed more realistic exposure scenarios with depuration periods; for instance, Morthorst et al. (2010) as well as Larsen & Baatrup (2010) could show an irreversible masculinization of zebrafish after exposure to environmentally relevant concentrations of 17β-trenbolone. Based on these findings, the present study was designed to elucidate if the persistence of these androgenic effects can be found at different effect levels, from mRNA to population-relevant endpoints. For this purpose, zebrafish were exposed to environmental relevant concentrations of 17β-trenbolone until 100 days post-hatch (dph), either under continuous exposure or with a recovery period of 40 days. Gonad differentiation of zebrafish takes place within the first 60 days post fertilization, therefore this exposure period was chosen to cover the sexual development. Five effect levels with different ecological relevance were investigated: (1) population level: sex ratio; (2) individual level: growth; (3) organ/cell level: histology of gonads; (4) protein level: vitellogenin (VTG) production and (5) mRNA level: aromatase (cyp19b) expression in the brain. By assessing both morphological effects and molecular biomarkers, a more complex insight into the mechanisms and effects caused by androgenic EDCs in zebrafish becomes possible. Moreover, taking into account the potential persistence of such effects, the ecological consequences for wildlife after periodic exposure to anabolic steroids is more likely to be evaluated in a realistic context.

3.3 Material and Methods

Test substance

17β-Trenbolone (CAS-No.: 10161-33-8) was obtained from Sigma-Aldrich (Deisenhofen, Germany). The following test concentrations were used for the exposure of zebrafish in the 100 days assay: 0, 1, 3, 10 and 30 ng/L, dissolved in dimethylsulfoxide (DMSO; maximum concentration in the final test solutions ≤ 0.01 %). Fresh stock solutions were produced every second day in light-isolated glass reservoirs, from which they were added to the exposure tanks *via* peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany).

Exposure and sampling

The exposure of zebrafish (*Danio rerio*, Westaquarium strain) to the different chemicals started at latest 1 hour post-fertilization and ended at 100 days post-hatch (dph). Fish were held in aerated 12 L flow-through glass tanks at 26 - 27 °C and a dark-light cycle of 10/14 hours. Water temperature and flow-through rate (complete water exchange every 8 hours) were controlled twice daily. Hardness (200 - 280 mg/L), conductivity (600 - 750 µS), pH (8.0 - 8.2) and oxygen saturation (90 - 95 %) were tested at least once weekly. Feces and food leftovers were removed daily. From days 4 to 14, larvae were fed with powdered dry food (Staubfutter, Sera Micron, Heinsberg, Germany); from day 14 larvae were fed with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

The exposure to 17β-trenbolone was carried out until 60 dph, followed by a recovery-period in clear water of 40 days. Additionally, control groups with continuous and without any exposure over the whole time of the experiment (100 days) were analyzed. Each treatment was run in two replicates. 100 Eggs per replicate were used at the beginning. After 30 and 60 days, 30 individuals from each tank were randomly removed. In case of slight differences in the amount of individuals per tank, more or less fish were removed to avoid density-dependent effects. Fish were euthanized with a saturated solution of benzocaine (ethyl-*p*-aminobenzoate, Sigma-Aldrich). Length and wet weight of each individual were documented. Head and tail (for ELISA) or only the head (for qPCR) were cut off with a razor blade immediately behind the operculum and behind the anal fin, weighed and frozen in liquid nitrogen for subsequent quantification of VTG or cyp19b mRNA. Half of the sampled fish were used for VTG analyzes, the other half for cyp19b. Trunks of all 30 fish were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidson´s fixative (Romeis & Böck 2001) for subsequent histological analyses.

Histology

Samples were incubated in modified Davidsons's fixative (Romeis & Böck 2001) at 4°C for at least 24 h before embedding into paraffin by a tissue processor (TP 1020, Leica Microsystems, Nussloch, Germany). Embedding into blocks was performed with a heated paraffin embedding module (EG 1140 H, Leica Microsystems, Nussloch, Germany) with trunks orientated ventrally to the cutting surface. Sections of the gonads with a thickness of 4 - 5 µm were cut with a microtome (HN 40, Reichert-Jung, Heidelberg, Germany), mounted on glass slides (Langenbrinck, Langenseibold, Germany) and then stained with hematoxylin-eosin (Romeis & Böck 2001) the next day. Light microscopical evaluation of the tissue sections was performed according to the OECD Histopathology Guidance Document (OECD 2010). Each fish was identified either as female, male or intersex individual or recorded as undifferentiated. Additionally, the maturation stages of the gonads were categorized as relative proportions of various gametogenic cell types into a numerical staging system (ovary: stages 0 - 5, testis: stages 0 - 4) according to the OECD Histopathology Guidance Document (OECD 2010).

Maturity index

As an enhancement to the regular staging system in the OECD Histopathology Guidance Document (OECD 2010), each stage of maturity was given a fixed maturity value, increasing with the maturity of the fish (Stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The sum of those values from all individuals of each replicate was divided by the number of fish. The sex-specific mean values for each treatment were calculated from the replicates and termed the maturity indices for females or males (Baumann et al. 2012). Completely undifferentiated or intersex individuals were not included into this assessment, since they could not be classified as female or male.

ELISA

The measurement of the VTG concentration in head and tail homogenates of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1.5 ml centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris-HCl, pH 7.4: 1 % protease inhibitor cocktail, Sigma-Aldrich). The homogenate was centrifuged for 30 min at 25,000 × g at 4°C (Multifuge 1 S-R, Heraeus, Hanau, Germany), the supernatant was collected and stored at -80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).

qPCR

Total RNA isolation of zebrafish heads was performed with TriReagent (guanidine thiocyanate and phenol, Sigma-Aldrich) according to the manufacturer's instructions. The tissue was homogenized with the aid of Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at a frequency of 18 beats per second. RNA concentration and purity was measured with the NanoVueTM Plus Spectrophotometer (General Electric, Fairfield, USA). Subsequent cDNA synthesis was performed with the following reagents per reaction with 1 µg RNA: 1 µg hexanucleotide random-primer-mix (Roth, Karlsruhe, Germany), 2.5 µl M-MLV RT reaction buffer (Sigma-Aldrich), 1.25 µl deoxynucleotide (dNTP) Mix (Sigma Aldrich), 1 µl RiboLock RNase inhibitor (40 U; Fisher Scientific, Schwerte, Germany), 1 µl M-MLV reverse transcriptase (Sigma-Aldrich), 4.25 µl RNase-free water. The qPCR reaction with the cDNA was performed using the StepOne^{TM} real-time PCR System (Applied Biosystems, Foster City, USA) and the Fast SYBR Green master mix (Applied Biosystems, Foster City, USA). Expressions of 18S-rRNA (endogenous control) and cyp19b mRNA were measured using following primers (5`-3`):

18S Forward-Primer: CACTTGTCCCTCTAAGAAGTTGCA

18S Reverse-Primer: GGTTGATTCCGATAACGAACGA

Cyp19b Forward-Primer: GCTCCAGACACGCTCTCCAT

Cyp19b Reverse-Primer: CATCCTCCAGAGACTGCCTCA

A cDNA pool of 5 female or male unexposed fish was used as negative control for the measurement of female and male fish, respectively. In each qPCR run, cDNA of the pooled control fish (negative control) was measured in duplicates with 18S and cyp19b as targets. For all other samples, measurement of 18S cDNA was performed in duplicate, and measurement of cyp19b cDNA was performed in triplicate. In addition, there were duplicates of water controls for each master mix that did not contain any template cDNA.

qPCR data were analyzed using the comparative quantification method (ΔΔCT) in the integrated software (Applied Biosystems, StepOne).

Statistics

For statistical evaluation, data were analyzed by one-way ANOVA and Dunnett´s Test using SigmaStat 12.0 (Statsoft-Jandel Scientific, Erkrath, Germany).

Chemical analyses

No chemical analyzes were conducted, as previous experiments with the same substance in the same facility had revealed high stability and only minor deviations from nominal concentrations after chemical analyses (Böttcher 2011).

3.4 Results

Mortality

No significant mortality due to 17β-trenbolone exposure could be observed. All exposure groups showed survival of > 95%.

Cyp19b (brain aromatase) mRNA expression

There was a high inter-individual variability in cyp19b mRNA expression not only in the negative control groups, but also in exposed zebrafish. At 100 dph, the relative amount of cyp19b mRNA (RQ-value) in male negative control zebrafish was ca. 0.5 relative to the pool of unexposed adults (RQ = 1). In contrast, in the 1 ng/L recovery and both groups of 3 ng/L 17β-trenbolone, transcript abundance of cyp19b was significantly increased over negative controls with RQ-values of ca. 2 (Fig. 3.1 a). After continuous exposure to 1 ng/L 17β-trenbolone, there was also a tendency towards increased, yet non-significant expression. Comparison of transcript abundance between continuous and 60 d exposure plus recovery to 1 and 3 ng/L 17β-trenbolone did not reveal any significant differences. Likewise, cyp19b mRNA expression in groups exposed to 10 and 30 ng/L 17β-trenbolone were not significantly different from negative controls independent of recovery. Furthermore, there was also no difference between recovery and continuous exposure in both concentrations.

Exposure to 17β-trenbolone caused a significant decrease in the number of female individuals and an increase of males at high concentrations, which made the analysis of cyp19b expressions in females difficult. Again, the inter-individual variance was high in all groups, except for the group exposed to 1 ng/L 17β-trenbolone over 100 days. The RQ-value of the female negative control group was ca. 0.9, and in none of the female exposure groups, the RQ of cyp19b mRNA was significantly different from the negative control (Fig 3.1 b). However, there was a tendency to increased expression in the 1 ng/L recovery group, as well as after continuous exposure to 3 ng/L 17β-trenbolone. Comparison of recovery and continuous exposure to 1 and 3 ng/L 17β-trenbolone also gave a significant difference.

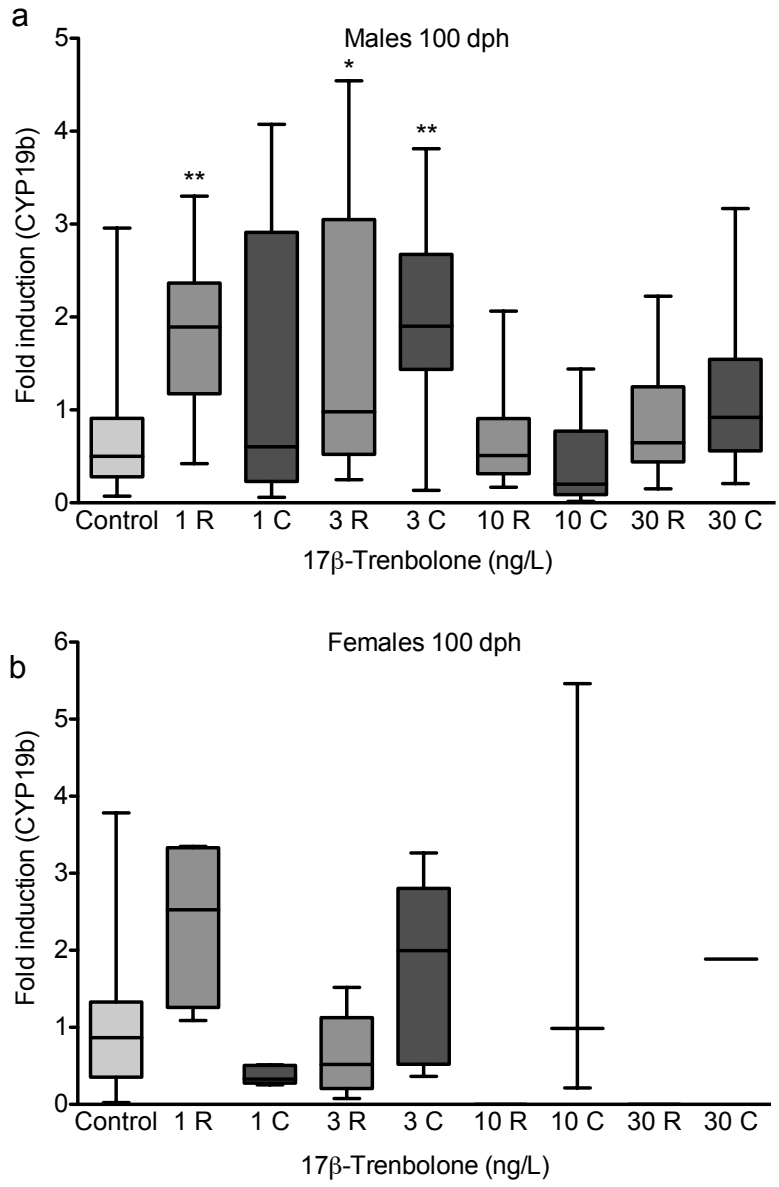
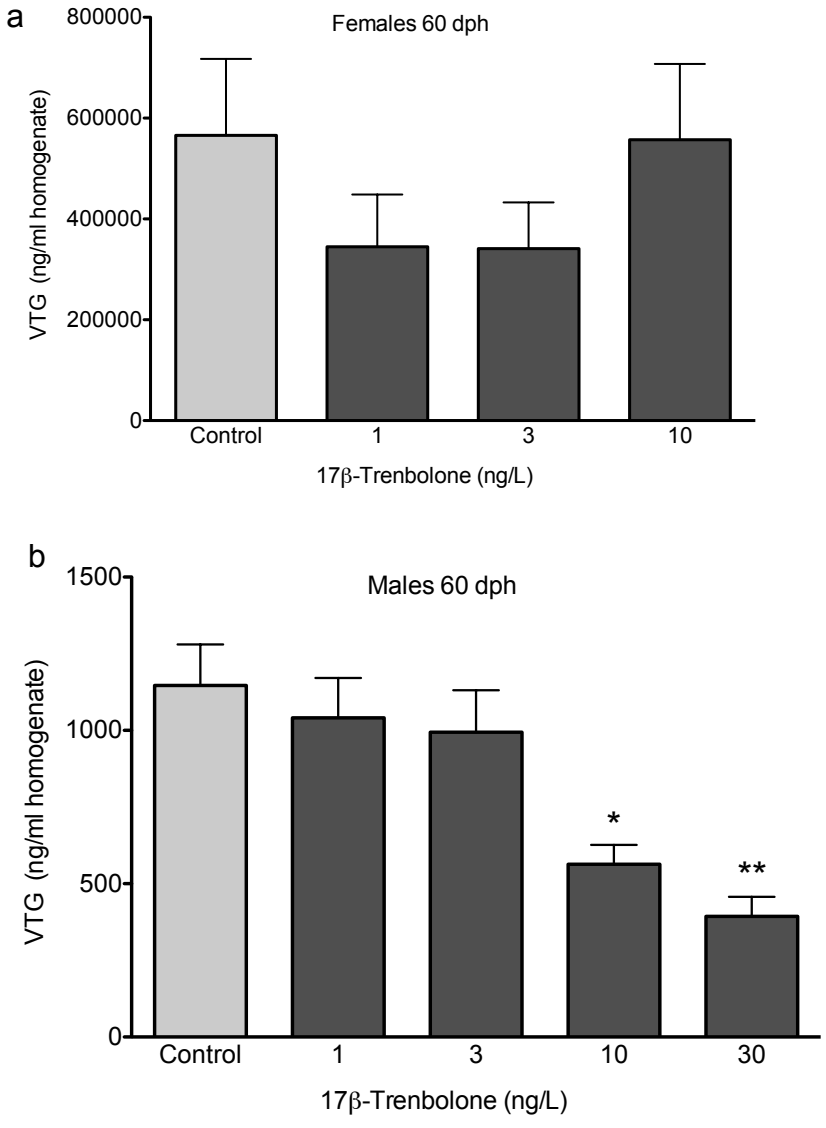


Fig. 3.1: Cyp19b (brain aromatase) mRNA expression of zebrafish (*Danio rerio*) exposed to 17β-trenbolone at 100 days post-hatch (dph), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett`s test compared to controls.

Vitellogenin (VTG)

At 60 dph, VTG concentrations in males exposed to 17β-trenbolone showed a clear dose-response decrease, which was significant for 10 and 30 ng/L exposure (Fig. 3.2). VTG levels in females also tended to decrease at low concentrations of 17β-trenbolone, but returned to control levels at 10 ng/L. Nevertheless, it has to be considered that only 3 female individuals were left for analyses in this exposure group and that no female was found in the highest concentration.

At 100 dph, female zebrafish exposed to 17β-trenbolone over 100 days with or without recovery phase showed no significant effect concerning VTG concentrations (Fig. 3.2). A slight tendency to decrease was found for 30 ng/L 17β-trenbolone with continuous exposure; however, due to a low number of individuals, this was not significant. For male zebrafish, a significant decrease of VTG levels could be detected in the recovery groups of the highest concentrations (10 and 30 ng/L) and for the continuous exposure to 30 ng/L 17β-trenbolone. VTG production of males was not affected by low concentrations of 17β-trenbolone.



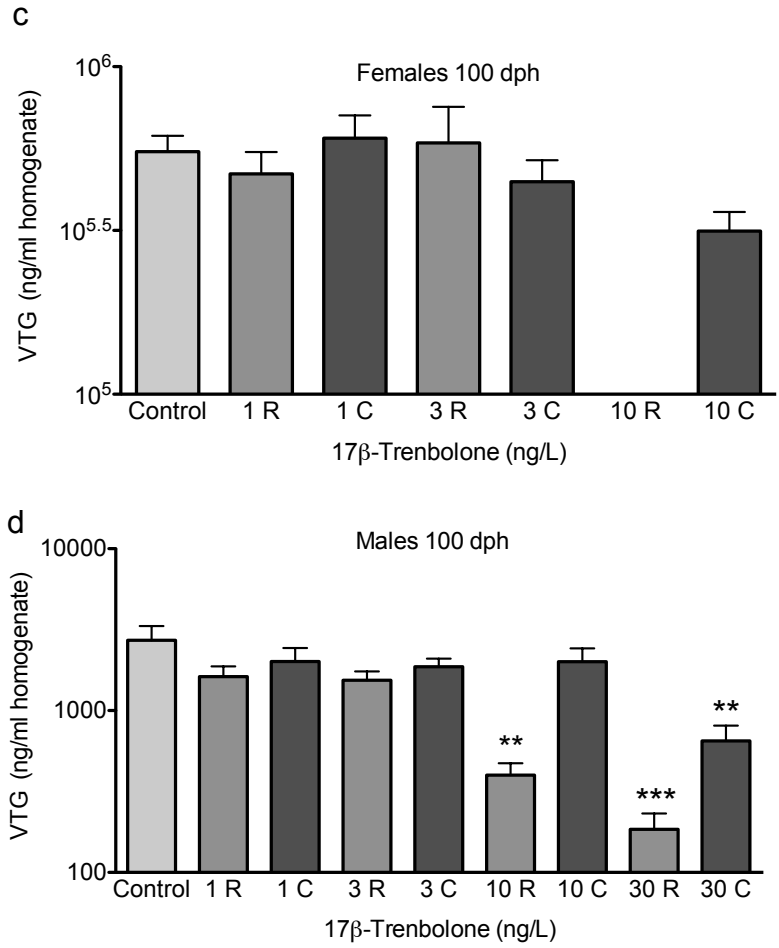


Fig. 3.2: Vitellogenin levels of zebrafish (*Danio rerio*) exposed to 17β-trenbolone at 60 dph (a, b) and 100 dph (c, d), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett`s test compared to controls.

Maturity index

Gonad staging at 60 dph revealed increased maturity for males after exposure to 17β-trenbolone (Fig. 3.3). Significantly increased maturity indices were found for males exposed to high concentrations of 17β-trenbolone (10 and 30 ng/L); these fish had considerably more spermatozoa in their testicular tissues than control fish. Female gonads at 10 ng/L were also more mature than in the controls; however, due to a low number of individuals, statistical significance could not be shown. A slight decrease of maturity could be assessed at 1 and 3 ng/L. At 30 ng/L 17β-trenbolone, no more female individuals were found.

At 100 dph, all female zebrafish were staged as maturity grade 3 independent of exposure, resulting in a maturity index of 4; thus, an influence from the exposure to 17β-trenbolone could not be found. In contrast, in all groups with continuous exposure, males showed elevated maturity indices, if compared to controls (Fig. 3.3). In addition, an increase in the

occurrence of histolytic cells in such mature testes areas could be correlated to 17β-trenbolone concentrations (Fig. 3.4). No other pathological changes in the gonads could be observed. The recovery groups formerly exposed to concentrations of 3 and 10 ng/L 17β-trenbolone were at control levels, whereas males formerly exposed to 30 ng/L 17β-trenbolone had more mature gonads.

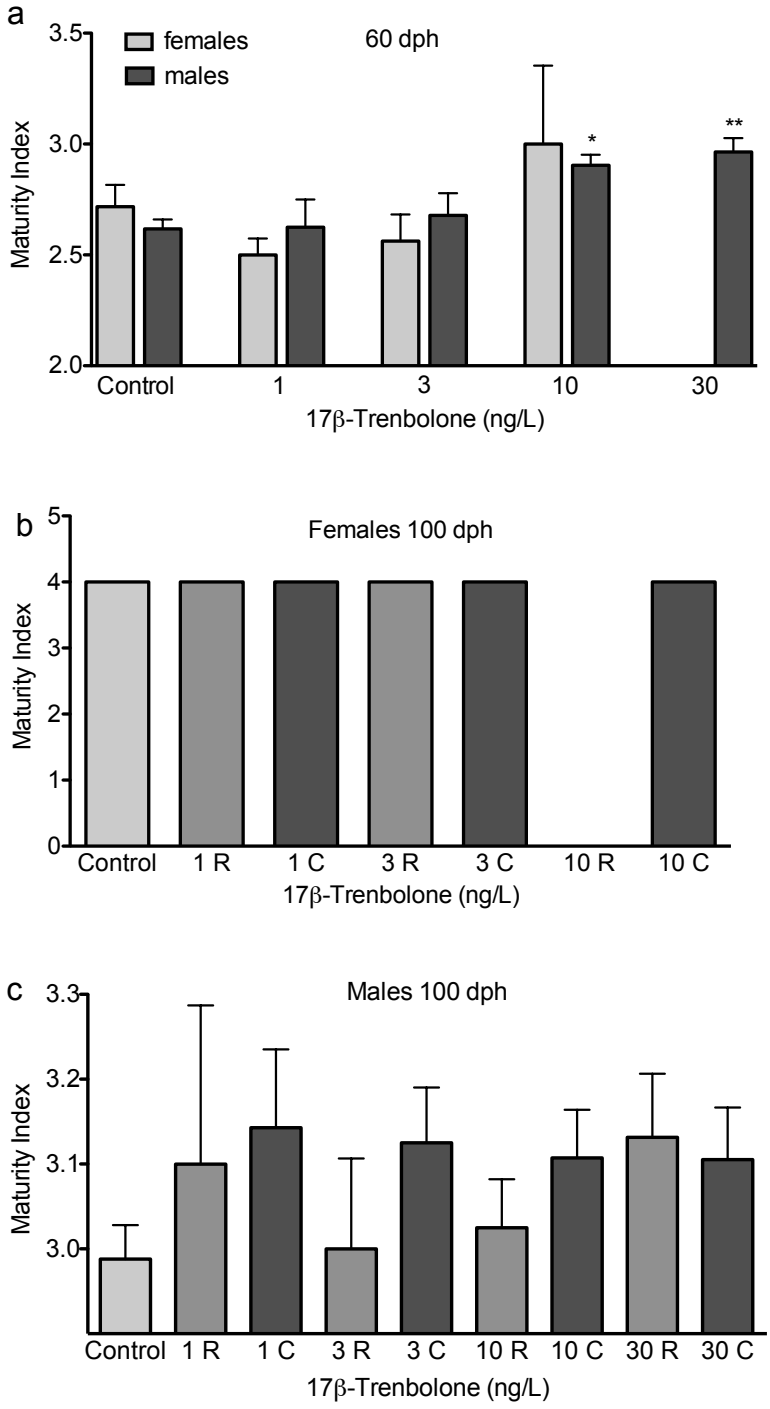


Fig. 3.3: Maturity index of zebrafish (*Danio rerio*) exposed to 17β-trenbolone at 60 dph (a) and 100 dph (b, c), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, Dunnett`s test compared to controls.

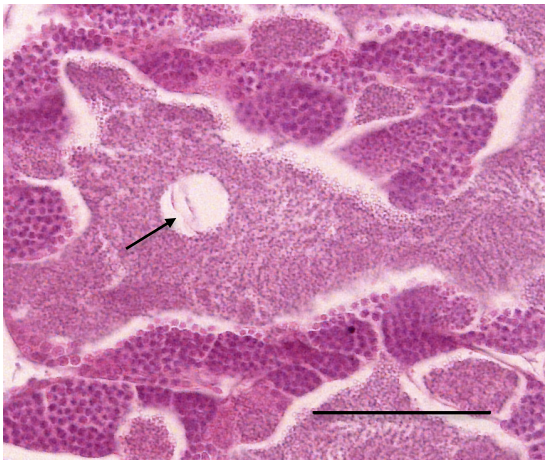
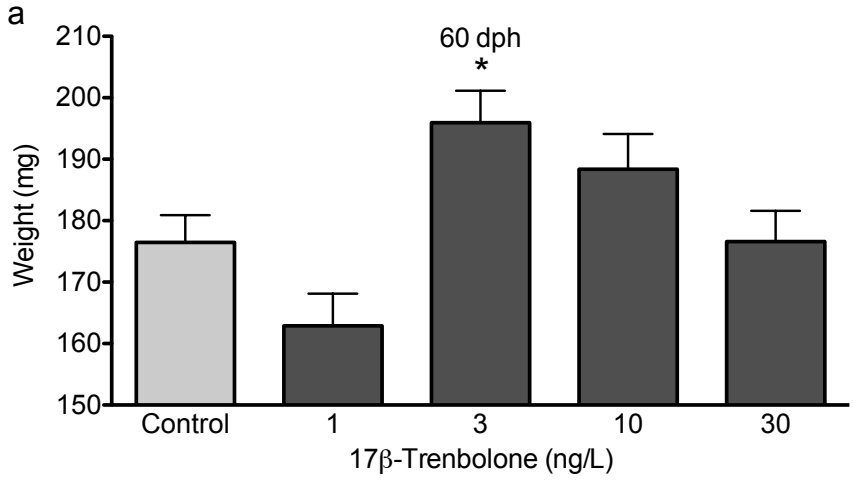


Fig. 3.4: Histolytic cell in the center of a round lumen within a spermatocyst containing spermatozoa. Mature testis tissue of a male zebrafish (*Danio rerio*) exposed to 30 ng/L 17β-trenbolone for 100 days, bar: 2 µm

Body size

At 60 dph, the average length and weight of control zebrafish was 26.5 mm and 175 mg, respectively. Length and weight were decreased in fish exposed to 1 ng/L, but elevated for 3 and 10 ng/L 17β-trenbolone (Fig. 3.5). At 30 ng/L 17β-trenbolone, no effects on body length and weight were seen.

After 100 days of exposure to 17β-trenbolone, clear differences in body length between constant and periodic exposure were evident (Fig. 3.5): All individuals from the recovery groups were smaller than the control fish, whereas all fish that had constantly been exposed were bigger. This effect was not as pronounced for the weight, where only the recovery groups formerly exposed to 10 and 30 ng/L 17β-trenbolone and the continuous exposure to 30 ng/L were significantly smaller.



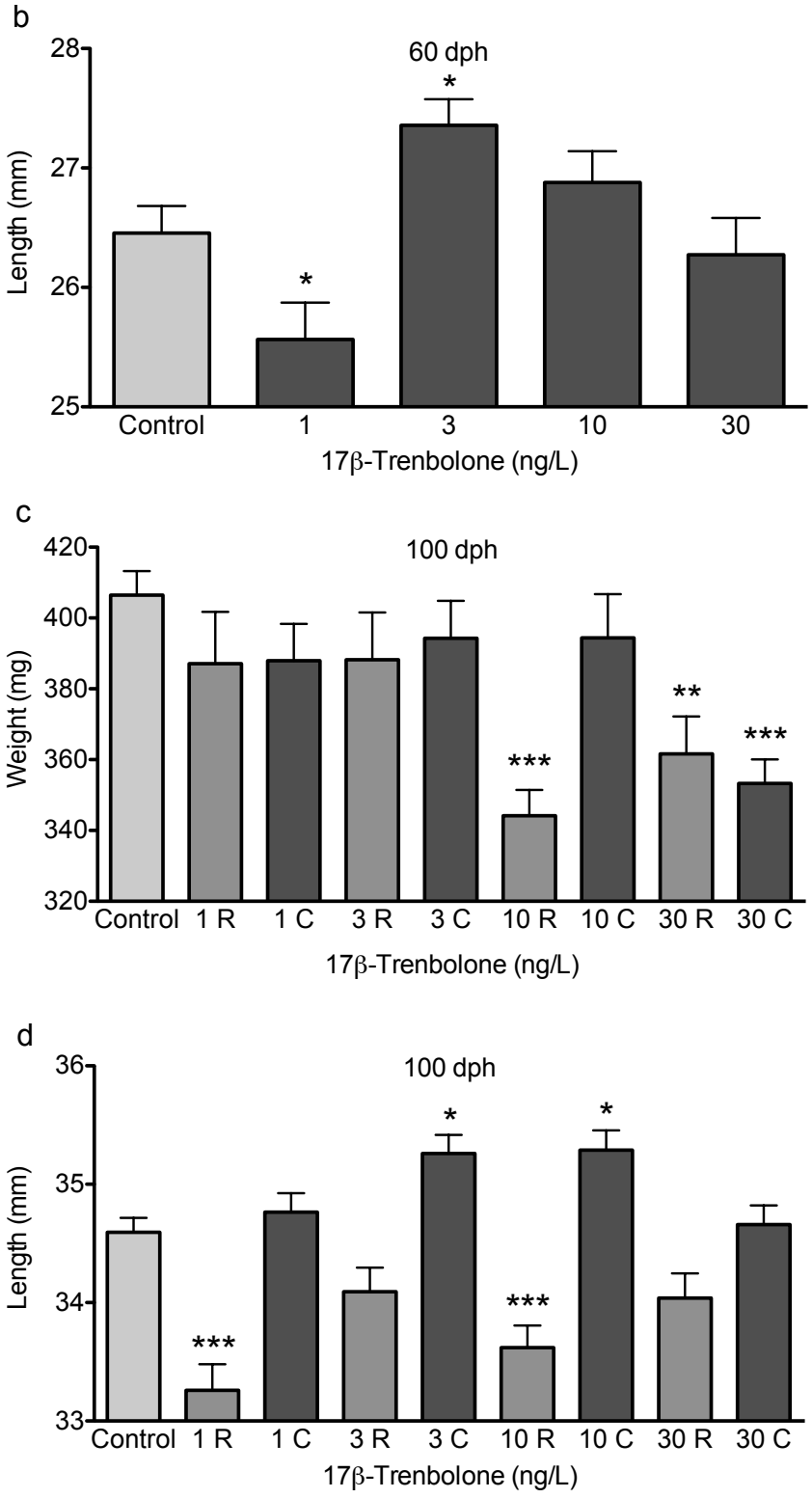


Fig. 3.5: Length and weight of zebrafish (*Danio rerio*) exposed to 17β-trenbolone at 60 and 100 days post-hatch (dph), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p <$ 0.05, **$p < 0.01$, ***$p < 0.001$, Dunnett`s test compared to controls.

Sex ratio

At 60 dph, 30 individuals were randomly picked from each exposure group of 70 zebrafish. The sex ratio (females:males) in the control groups was 41:59 (Tab. 3.1). Comparable values were found at 1 and 3 ng/L, but from 10 ng/L a significant increase of males was found. Not one female was picked at 30 ng/L and only 13 % at 10 ng/L. No intersex or undifferentiated fish were found during the whole experiment.

At 100 dph, the remaining 40 fish were sampled from the tanks. In the negative control, the ratio of female to male individuals was 45:55 (Tab. 3.1). The sex ratios at 1 and 3 ng/L 17β-trenbolone were approximately similar to controls. Exposure to 10 and 30 ng/L 17β-trenbolone resulted in a dose-dependent increase in the percentage of males in the groups that were continuously exposed (10 ng/L: 76 %, 30 ng/L: 97 %) and in all-male populations in the recovery groups.

Tab. 3.1: Sex ratio (%) of zebrafish (*Danio rerio*) exposed to 17β-trenbolone at 60 and 100 days post-hatch (dph), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett`s test compared to controls

	60 dph		**100 dph (C)**		**100 dph (R)**	
	Females	Males	Females	Males	Females	Males
Control	41	59	45	55	45	55
1 ng/L	47	53	50	50	53	47
3 ng/L	53	47	50	50	36 *	64 *
10 ng/L	13 **	87 **	24 *	76 *	0 ***	100 ***
30 ng/L	0 ***	100 ***	3 ***	97 ***	0 ***	100 ***

3.5 Discussion

17β-trenbolone exposure during the first 100 days of development of zebrafish had strong influence on their sexual development at different effect levels.

Cyp19b (aromatase) expression

The enzyme aromatase (cyp19b) is a key element in the synthesis of steroids, since it catalyzes the conversion of androgens (C19-steroids) into estrogens (C18-steroids). Therefore, adequate aromatase activities are essential for all body functions dependent on steroids, including growth, neural and sexual development and behavior (Diotel et al. 2010). Several studies could show an increased cyp19b expression or activity in zebrafish and other fish species after exposure to estrogens and xenoestrogens (for review, see Cheshenko et al. 2008 as well as Diotel et al. 2011). This made cyp19b a useful biomarker, at least for (pseudo-) estrogenic compounds. Nevertheless, an influence of androgens on the cyp19b expression has also been shown in different studies (Brion et al. 2012, Fenske & Segner 2004, Lassiter & Linney 2007, Mouriec et al. 2009, Zhang et al. 2012).

In the present study, measurement of cyp19b mRNA expression in 100 days old zebrafish revealed great individual variations in the expression within both sexes, but no significant differences between male and female controls. Goto-Kazeto et al. (2004) found similar inter-individual differences in transcript abundances of cyp19b mRNA in zebrafish, but also differences between males and females, with higher amounts of cyp19b mRNA in males. Tong et al. (2009) determined cyp19b expression in adult zebrafish *via* GFP (green fluorescent protein) expression driven by the cyp19b promoter; they could not reveal differential cyp19b expressions between sexes. As these few examples show, the constitutive expression of cyp19b in adult zebrafish is apparently not yet fully understood. Regulation of cyp19b is known to depend on numerous factors including age, reproductive cycle or social status (Cheshenko et al. 2008, Kazeto et al. 2003, Piferrer & Blazquez 2005). Filby et al. (2012) revealed significant differences of cyp19b expression in dominant and subordinate male zebrafish. Mong et al. 2011 showed variations due to diurnal changes in cyp19b expression. Since it takes several hours to sample all individuals from all exposure tanks, the changes seen in the present study might as well be due to such diurnal changes and be reflected in the measured cyp19b mRNA levels.

Exposure to 1 and 3 ng/L 17β-trenbolone resulted in an up-regulation of cyp19b mRNA expression in male zebrafish at 100 dph. In contrast, higher concentrations of 17β-trenbolone (10 and 30 ng/L) did not cause significant changes of cyp19b transcript abundance compared

to the negative control. Both effects were independent of the 40 d recovery period. There are no comparable studies that investigated the effect of 17β-trenbolone on cyp19b expression in adult zebrafish. However, exposure to the androgenic compound methyltestosterone did not affect cyp19b activity in the brain of adult male zebrafish (Andersen et al. 2006). The androgens testosterone and dihydrotestosterone, on the other hand, have been shown to increase cyp19b mRNA expression in zebrafish embryos (Mouriec et al. 2009). In general, the presence of an estrogen-responsive element in the promoter region of the cyp19b gene correlates cyp19b expression to sex steroid levels in the brain (Diotel et al. 2010, Kazeto et al. 2004). There are several potential explanations for the differential responses to low and high concentrations of 17β-trenbolone: For example, this might be due to sex-specific mechanisms, i.e. exposure to 10 and 30 ng/L 17β-trenbolone might cause an up-regulation of cyp19b expression in genetic males and an inverse response in genetic females. The high number of phenotypically masculinized genetic females in the 10 and 30 ng/L groups could thus be one reason why the overall response to 17β-trenbolone exposure in these groups varied from the response of groups with genetic males solely (1 and 3 ng/L). Gender-specific responses to 17β-trenbolone were, e.g., shown in fathead minnow (Ankley et al. 2003). Another possibility for such concentration-dependent differences might be the high number of phenotypic male individuals in the groups of 10 and 30 ng/L that might have influenced the social behavior of the fish (Söffken & Tyler 2012). Since the social status is most likely related to cyp19b expression, altered transcription levels might follow (Cheshenko et al. 2008, Filby et al. 2012, Larsen & Baatrup 2010). These are only assumptions, since as long as it is not possible to genetically distinguish male and female zebrafish, gender-specific responses to EDCs cannot be unequivocally determined.

For the analysis of the effects of 17β-trenbolone on cyp19b expression in female zebrafish, the groups exposed to 30 ng/L could not be used, as they had developed into all-male populations. The same applies to the recovery group of 10 ng/L. Considering the remaining exposure groups, cyp19b mRNA levels in female zebrafish were not significantly different from the negative control. There were only tendencies towards an increased expression after exposure to 1 ng/L (recovery group) and 3 ng/L (continuous exposure). In fact, increased cyp19b expression in female zebrafish has been shown after exposure to the androgen methyltestosterone (Fenske & Segner 2004). On the other hand, Dorts et al. (2009) found decreased cyp19b mRNA levels in female fathead minnow after exposure to 17β- trenbolone, albeit at higher concentrations (0.1 and 1 µg/L) than used in the present study. Again, regulation of cyp19b mRNA expression due to steroid exposure might occur – directly or

indirectly – *via* estrogen-responsive elements. A 17β-trenbolone-induced up-regulation of cyp19b expression in female zebrafish would make sense, since the increased androgen levels might be compensated by an increased production of estrogens. This would be in line with the fact that there was no phenotypic masculinization in the groups with a potential up-regulation. However, the slightly increased levels of cyp19b mRNA in these two groups were not statistically significant, if compared to the negative control and might, thus, be random.

In each exposure concentration of the present study, recovery and continuous exposure did not result in significantly different cyp19b mRNA levels. This illustrates that effects on cyp19b expression provoked by 17β-trenbolone are irreversible, which is in line with the persistent effects of 17β-trenbolone on sex ratio and VTG induction in zebrafish (this study, Larsen & Baatrup 2010, Morthorst et al 2010).

Vitellogenin (VTG)

VTG has been a popular biomarker in research on endocrine disruption of fish for several years (Tyler et al. 1999). The protein is a precursor for egg yolk, which is predominately expressed in females, but also detectable in males. Especially under influence of (xeno-) estrogens, VTG is able to illustrate dose-dependent effects on the hormonal system of fish (Holbech et al. 2001). Male fish have been shown to produce female-typical levels of the protein if exposed to strong estrogens (Rose et al. 2002), and the opposite effect can be observed after exposure to androgens, e.g. 17β-trenbolone (Holbech et al. 2006). The production of VTG is closely correlated to gonad maturation and should thus not be investigated separately (Bauman et al. 2012). In fact, VTG levels in males of the present study declined dose-dependently after exposure to 17β-trenbolone in parallel to an increase in gonad maturity. Both parameters clearly indicate masculinization by 17β-trenbolone. In females, however, the strong decrease in the number of female individuals makes statistical approaches very difficult, and only tendencies can be shown. Nevertheless, a clear decrease of VTG production could be documented in females after exposure to 1 and 3 ng/L 17β-trenbolone, which was accompanied by lowered gonad maturity. Those results are in line with previous findings in zebrafish by Holbech et al. (2006) in an FSDT with 17β-trenbolone and by Örn et al. (2006), who reported decreased VTG concentrations in both genders at 50 ng/L.

Surprisingly, the inhibiting effect of 17β-trenbolone on VTG production in male zebrafish was even stronger after a recovery period of 40 days. The reason may be that gonad maturity is not only linked to VTG production (Baumann et al. 2012), it is also correlated to growth. In fact, small fish are likely to have less mature gonads than bigger fish (Baumann 2008). The

comparison of Figs. 3.1, 3.3 and 3.4 shows that the curves for body size, maturity index and VTG induction follow similar shapes, especially in males. This shows that the masculinizing properties of 17β-trenbolone can be visualized at different effects levels, since these are strongly correlated to each other.

Maturity index

Male gonad maturation was massively affected by exposure to 17β-trenbolone. Both at 60 and 100 dph, male zebrafish showed a dose-dependent increase of gonad maturity, whereas recovery, at least from exposure to low concentrations showed tendencies to lower maturity indices. A dose-depended increase of histolytic cells in the mature testis tissue could be revealed, indicating that the mature spermatozoa could not be released and were thus degraded. This effect is probably due to the strong affinity of 17β-trenbolone to the androgen receptor (Ankley et al. 2003). Androgens like natural testosterone are necessarily involved in sperm maturation (Orban et al. 2009); consequently, substances with even higher affinity to the androgen receptor should therefore be expected to provoke high testicular maturity. These results are in accordance with reports by Morthorst et al. (2010) and Örn et al. (2005). Also Seki et al. (2006) presented elevated gonadosomatic indices (GSI) of male zebrafish, and decreased GSI of females after exposure to 17β-trenbolone at comparable concentration ranges.

Female zebrafish in the present study were not affected in their gonad maturity at 100 dph, whereas at 60 dph a dose-depended increase of maturity could be determined. Although Morthorst et al. (2010) reported similar effects in female zebrafish after exposure to 17β-trenbolone, this finding seems to be inconsistent with the androgenic properties of 17β-trenbolone. Potentially, this effect could either be interpreted as a compensating effect against the masculinization of 17β-trenbolone. Or this is simply randomly as a consequence of a very low number of female individuals found after exposure to high concentrations of 17β-trenbolone, which is statistically not significant.

Sexual maturity is a key factor for the reproductive fitness of animal populations in wildlife, especially for seasonal spawners. Sex-specific behavior, which is essential for the timing of gamete release, is closely linked to sexual maturity (Munakata & Kobayashi, 2010). Consequently, exposure to environmental pollutants that accelerate or decelerate gonad maturation can lead altered behavior resulting in impaired individual reproductive success and, thus, to adverse effects at the population level (Söffker & Tyler 2012).

Body size

Besides effects on sexual development and differentiation, 17β-trenbolone also had strong impact on growth of zebrafish. Androgens like 17β-trenbolone are not only involved in sexual processes, but also provoke the development of secondary male characteristics, such as increased musculature (Bhasin et al. 2001). Hence, anabolic steroids are commonly used by athletes as muscle growth promoters (Bowers et al. 2009, Leifman et al. 2011). Actually, in zebrafish both genders showed dose-related high values in length and weight after exposure to the anabolic steroid 17β-trenbolone. While for length, significantly lower values were found after depuration in all exposure groups, weight was about equal at low concentrations, and lowered values were only found at high exposure concentrations. For mammals, an augmentation in bone growth and skeletal muscle mass accompanied by a reduction of fatty tissue was reported (Yarrow et al. 2010). Thus, it is not surprising that androgen receptors are found in many tissues other than gonads and in both genders (De Waal et al. 2008). Notably low values for weight were measured after 100 dph at the highest concentration of 30 ng/L, especially after constant exposure, which might have been due to a combination of toxic and endocrine effects of 17β-trenbolone. It has to be considered that no female fish developed in high exposure groups, but that females normally weigh much more than males. Additionally, behavioral aspects should also be considered because the all-male populations probably lacked any sexual mating activities. Full maturation in conjunction with the inability to spawn might even cause stress in males. Effects of EDCs on behavior of fish are evident, but are currently heavily underestimated in research (Söffken & Tyler 2012).

Sex ratio

The sexual differentiation of zebrafish is known to be susceptible to EDCs, especially during the sensitive period when the juvenile female gonad develops into either definite ovary or testis (Andersen et al. 2000, Maack & Segner 2003, Örn et al. 2003). This critical phase takes place within the first 60 days of development, which was, therefore, chosen as exposure period in the FSDT to evaluate endocrine effects (OECD 2011). The present study again corroborates that exposure to the androgen 17β-trenbolone during this sensitive developmental period has a strong and irreversible influence on sexual differentiation and thereby on the sex ratio of a zebrafish population. A dose-dependent shift of the sex ratio towards males caused by the exposure to 17β-trenbolone could be documented after both 60 and 100 dph (with and without recovery phase for the last 40 d). In all exposure groups > 10 ng/L, at least 90 % of the fish were found to be males, which correlates well with the

masculinization reported in other studies on 17β-trenbolone (Holbech et al. 2006, Larsen & Baatrup 2010, Morthorst et al. 2010). In line with the study of Morthorst et al. (2010), there was very low intersex prevalence. With respect to the environmental relevance of 17β-trenbolone (Bartelt-Hunt et al. 2012), it can be concluded that even periodic exposure may have massive and irreversible impact on wildlife fish populations by affecting sexual differentiation.

Most other studies about the reversibility of endocrine disruption investigated environmental estrogens such as ethinylestradiol, the feminizing effect of which proved to be reversible (Fenske et al. 2005, Hill & Janz 2003, Maack & Segner 2004). Some studies, however, reported partial reversibility in addition to irreversible effects, depending on time, duration and concentration of exposure (Schäfers et al. 2007, Van den Belt et al. 2002). Further research into the reversibility of EDCs with other modes of action is scarce. Fenske & Segner (2004) treated zebrafish with the aromatase inhibitor fadrozol during sexual differentiation, followed by 3 months of depuration. The gonads of all treated fish still displayed testicular morphology, indicating that the masculinizing effect of EDCs on gonad differentiation may be very persistent, as also shown by Morthorst et al. (2010) and Larsen & Baatrup (2010) for 17β-trenbolone.

Conclusions

At environmentally relevant concentrations, 17β-trenbolone had a strong impact on sexual development of zebrafish (*Danio rerio*), which remained almost completely unchanged after depuration. Reversibility of adverse effects by 17β-trenbolone could, therefore, not be shown; only minor tendencies to less pronounced effects were evident. Given the presence of 17β-trenbolone in the aquatic environment, it can be concluded that wild fish populations may be permanently affected in their development even by periodic exposure to this anabolic steroid or comparable androgenic compounds. Since exposure of animals to EDCs in the environment may mostly not be constant, but intermittent, more research on the persistence of effects seems indispensable.

In contrast to masculinization, feminization of zebrafish is mostly reversible. The difference between these two effects will be discussed in the following chapter.

4 Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after periodic exposure to 17α-ethinylestradiol

4.1 Abstract

Exposure to estrogenic chemicals represents a serious hazard for wildlife populations, especially in the aquatic environment. One of the best-investigated substances is probably 17α-ethinylestradiol (EE2), which continuously reaches surface waters due to its worldwide use in oral contraceptives. The aim of the present study was to investigate the persistence of the feminizing effect of EE2 in zebrafish (*Danio rerio*). An exposure scenario covering the sensitive phase of sexual differentiation as well as final gonad maturation was chosen to examine the effects of EE2 on sexual development of zebrafish. Exposure to environmental relevant concentrations (0.1 – 10 ng/L EE2) was either continuous up to 100 days post hatch (dph) or stopped at 60 dph, followed by 40 days of depuration in clean water. The persistence of effects was investigated at different biological organization levels from mRNA to population-relevant endpoints. As expected, EE2 had a strong feminizing and inhibiting effect on the sexual development of zebrafish. Brain aromatase (cyp19b) mRNA expression was decreased, VTG levels were significantly elevated, gonad maturation and body growth were inhibited in both genders, and sex ratio was skewed towards females and undifferentiated individuals. Interestingly, all these effects were reversible after 40 days of recovery, leading to the conclusion that exposure to EE2 results in very strong, but reversible underdevelopment and feminization of zebrafish. If compared to a previous study on the effects of 17β-trenbolone, the same exposure scenario led to irreversible effects at all organization levels.

4.2 Introduction

17α-Ethinylestradiol (EE2) is one of the most potent and important xenoestrogens that can be found in the aquatic environment. It is regularly measured in surface waters at concentrations in the lower ng/L range, which is mostly due to human excretion (see Wise et al. 2011 for review). It is the most important substance in contraceptive pills, which are consumed by over 100 million women worldwide, leading to environmental problems (Owen & Jobling 2012,). The semi-synthetic estrogen is very potent, with a twofold higher binding affinity to the estrogen receptor than natural 17β-estradiol (Blair et al. 2000). Very low concentrations in the ng/L range are sufficient to disrupt reproductive capacities of fish populations (Länge et al. 2001, Kidd et al. 2007, Nash et al. 2004, Pawlowski et al. 2004), induce sex reversal (Örn et

al. 2003 & 2006) or influence sexual behavior (Coe et al. 2010, Filby et al. 2012, Reyhanian et al. 2011). Consequently, EE2 has become one of the most popular substances in research on estrogenic EDCs in fish (see Scholz & Klüver 2009 for review). The zebrafish (*Danio rerio*) has proven to be especially interesting for such studies, as it has a protogynic sexual development, which is very sensitive to EDC exposure (Maack & Segner 2004). Sex reversal in zebrafish is easily inducible with different EDCs, such as estrogens (Hill & Janz 2003, Van den Belt et al. 2002), androgens (Holbech et al. 2006, Örn et al. 2006) or aromatase inhibitors (Kinnberg et al. 2007, Thorpe et al. 2011). Nevertheless, feminization of zebrafish can easily be misinterpreted as sex reversal, if a substance only inhibits and/or retards sexual differentiation, which results in an arrest of genetic males in the "all-female" stage (Fenske et al. 2005).

Since exposure of wildlife to EDCs is mostly not continuous, but intermittent, realistic exposure scenarios with recovery phase have become more popular over the last years. Some publications already report the ability of zebrafish to recover from estrogen exposure (Hill & Janz 2003, Larsen et al. 2009, Nash et al. 2004, Schäfers 2007), whereas others have shown that exposure to androgens is much more persistent (Larsen & Baatrup 2010, Morthorst et al. 2010). In the previous chapter, results of a long-term exposure to the anabolic steroid 17β-trenbolone with subsequent recovery phase were presented (chapter 3). The masculinizing effects of the androgen were irreversible at all organization levels in zebrafish. Brain aromatase (cyp19b) expression, vitellogenin (VTG) levels, gonad maturation, growth, as well as sex ratio were irreversibly affected after 60 days of exposure followed by 40 days of recovery. In the present chapter, 17β-trenbolone was compared to EE2. Exposure scenarios were identical for both experiments. Again, the focus has been on the evaluation of various endpoints at different levels of biological organization, covering molecular biomarkers like cyp19b expression or VTG induction, as well as population-relevant endpoints, such as gonad maturity or sex ratio. This approach gives the opportunity to determine the complexity of effects leading to an adverse outcome and to compare the difference between estrogenic and androgenic endocrine disruption in zebrafish.

4.3 Material and Methods

Test substance

17α-Ethinylestradiol (EE2; CAS-No.: 57-63-6) was obtained from Sigma-Aldrich (Deisenhofen, Germany). Following test concentrations were used for the exposure of zebrafish in the 100 days assay: 0, 0.1, 1, 3 and 10 ng/L, dissolved in dimethylsulfoxide (DMSO; maximum concentration in the test solutions ≤ 0.01 %). Fresh stock solutions were produced every second day in light-isolated glass reservoirs from which they were added to the exposure tanks *via* peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany).

Exposure and sampling

The exposure of zebrafish (*Danio rerio*, Westaquarium strain) to the different chemicals started at latest 1 hour post-fertilization and ended at 100 days post-hatch (dph). Fish were held in aerated 12 L flow-through glass tanks at 26 - 27 °C and a dark-light cycle of 10/14 hours. Water temperature and flow-through rate (complete water exchange every 8 hours) were controlled twice daily. Hardness (200 - 280 mg/L), conductivity (600 - 750 µS), pH (8.0 - 8.2) and oxygen saturation (90 - 95 %) were tested at least once weekly. Feces and food leftovers were removed daily. From days 4 to 14, larvae were fed with powdered dry food (Staubfutter, Sera Micron, Heinsberg, Germany); from day 14 larvae were fed with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

The exposure to EE2 was carried out until 60 dph, followed by a recovery-period in clear water of 40 days. Additionally, control groups with continuous and without any exposure over the whole time of the experiment (100 days) were analyzed. Each treatment was run in two replicates. 100 Eggs per replicate were used at the beginning. After 30 and 60 days, 30 individuals from each tank were randomly removed. In case of slight differences in the number of fish per tank, more or less individuals were removed to avoid density-dependent effects. Fish were euthanized with a saturated solution of benzocaine (ethyl-*p*-aminobenzoate, Sigma-Aldrich). Length and wet weight of each individual were documented. Head and tail (for ELISA) or only the head (for qPCR) were cut off with a razor blade immediately behind the operculum and behind the anal fin, weighed and frozen in liquid nitrogen for subsequent quantification of VTG or cyp19b mRNA. Half of the sampled fish were used for VTG analyzes, the other half for cyp19b. Trunks were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidson´s fixative (Romeis & Böck 2001) for subsequent histological analyses.

Histology

Samples were incubated in modified Davidsons's fixative (Romeis & Böck 2001) at 4°C for at least 24 h before embedding into paraffin by a tissue processor (TP 1020, Leica Microsystems, Nussloch, Germany). Embedding into blocks was performed with a heated paraffin embedding module (EG 1140 H, Leica Microsystems, Nussloch, Germany) with trunks orientated ventrally to the cutting surface. Sections of the gonads with a thickness of 4 - 5 µm were cut with a microtome (HN 40, Reichert-Jung, Heidelberg, Germany), mounted on glass slides (Langenbrinck, Langenseibold, Germany) and then stained with hematoxylin-eosin (Romeis & Böck 2001) the next day. Light microscopical evaluation of the tissue sections was performed according to the OECD Histopathology Guidance Document (OECD 2010). Each fish was identified either as female, male or intersex or recorded as undifferentiated. Additionally, the maturation stages of the gonads were categorized as relative proportions of various gametogenic cell types into a numerical staging system (ovary: stages 0 - 5, testis: stages 0 - 4) according to the OECD Histopathology Guidance Document (OECD 2010).

Maturity index

As an enhancement to the regular staging system in the OECD Histopathology Guidance Document (OECD 2010), each stage of maturity was given a fixed maturity value, increasing with the maturity of the fish (Stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The sum of those values from all individuals of each replicate was divided by the number of fish. The sex-specific mean values for each treatment were calculated from the replicates and termed the maturity indices for females or males (Baumann et al. 2012). Completely undifferentiated or intersex individuals were not included into this assessment, since they could not be classified as female or male.

ELISA

The measurement of the VTG concentration in head and tail homogenates of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1.5 ml centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris-HCl, pH 7.4: 1 % protease inhibitor cocktail, Sigma-Aldrich). The homogenate was centrifuged for 30 min at 25,000 × g at 4°C (Multifuge 1 S-R, Heraeus, Hanau, Germany), the supernatant was collected and stored at -80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA, based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).

qPCR

Total RNA isolation of zebrafish heads was performed with TriReagent (guanidine thiocyanate and phenol, Sigma-Aldrich) according to the manufacturer's instructions. The tissue was homogenized with the aid of Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at a frequency of 18 beats per second. RNA concentration and purity was measured with the NanoVueTM Plus Spectrophotometer (General Electric, Fairfield, USA). Subsequent cDNA synthesis was performed with following reagents per reaction with 1 µg RNA: 1 µg hexanucleotide random-primer-mix (Roth, Karlsruhe, Germany), 2.5 µl M-MLV RT reaction buffer (Sigma-Aldrich), 1.25 µl deoxynucleotide (dNTP) Mix (Sigma Aldrich), 1 µl RiboLock RNase inhibitor (40 U; Fisher Scientific, Schwerte, Germany), 1 µl M-MLV reverse transcriptase (Sigma-Aldrich), 4.25 µl RNase-free water. The qPCR reaction with the cDNA was performed using the StepOne^{TM} real-time PCR System (Applied Biosystems, Foster City, USA) and the Fast SYBR Green master mix (Applied Biosystems, Foster City, USA). Expression of 18S-rRNA (endogenous control) and cyp19b mRNA were measured using following primers (5`-3`):

18S Forward-Primer: CACTTGTCCCTCTAAGAAGTTGCA
18S Reverse-Primer: GGTTGATTCCGATAACGAACGA

Cyp19b Forward-Primer: GCTCCAGACACGCTCTCCAT
Cyp19b Reverse-Primer: CATCCTCCAGAGACTGCCTCA

A cDNA pool of 5 female or male unexposed fish was used as negative control for the measurement of female and male fish, respectively. In each qPCR run, cDNA of the pooled control fish (negative control) was measured in duplicates with 18S and cyp19b as targets. For all other samples, measurement of 18S cDNA was performed in duplicate, and measurement of cyp19b cDNA was performed in triplicate. In addition, there were duplicates of water controls for each master mix that did not contain any template cDNA. qPCR data were analyzed using the comparative quantification method (ΔΔCT) in the integrated software (Applied Biosystems, StepOne).

Statistics

For statistical evaluation, data were analyzed by one-way ANOVA and Dunnett´s Test using SigmaStat 12.0 (Statsoft-Jandel Scientific, Erkrath, Germany).

Chemical analyses

No chemical analyzes were conducted, as previous experiments with the same substance in the same facility had revealed high stability and only minor deviations from nominal concentrations after chemical analyses (LUBW 2012).

4.4 Results

Mortality

No significant mortality due to EE2 exposure could be observed. All treatment groups showed survival >95%.

Cyp19b (brain aromatase) mRNA expression

Cyp19b mRNA expression revealed high variability between individuals of both genders. No gender-specific differences could be assessed (data not shown), and therefore females and males were analyzed together (Fig. 4.1). Cyp19b abundance tended to decrease after exposure to EE2, but significant differences from controls could only be assessed after continuous exposure to the highest concentration (10 ng/L EE2). The corresponding recovery group did not differ significantly from the control, but was slightly lower.

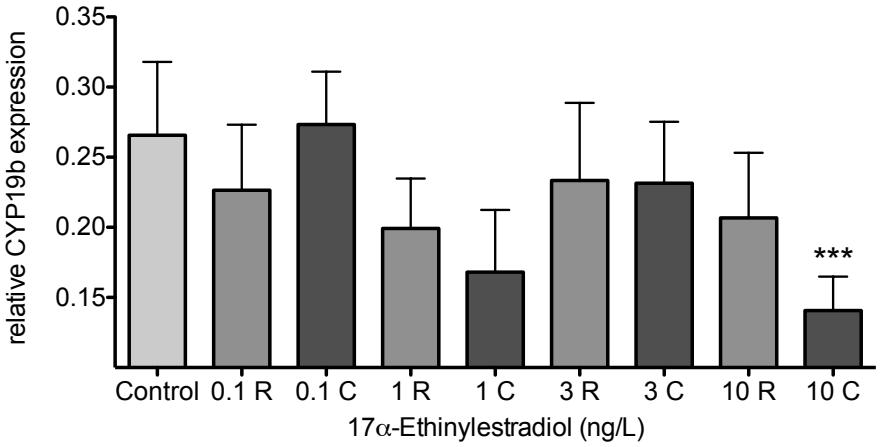


Fig. 4.1: Cyp19b mRNA expression of zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol at 100 dph, C = continuous exposure, R = 60 d exposure + 40 d recovery, ***$p < 0.05$; Dunnett´s Test compared to controls.

Vitellogenin (VTG)

VTG production of zebrafish was massively affected in a dose-dependent manner by exposure to EE2 at 60 dph in both genders (Fig. 4.2). VTG levels in females of the highest exposure group (10 ng/L EE2) were over 200fold higher than in the controls and in males even 2000fold higher.

The same effect could be observed at 100 dph after continuous exposure to EE2. Females as well as males in the highest concentration had significantly increased VTG levels, if compared to controls. However, this was not seen after a recovery period of 40 days. Zebrafish of both genders in the recovery groups had even lower VTG levels, if compared to the controls.

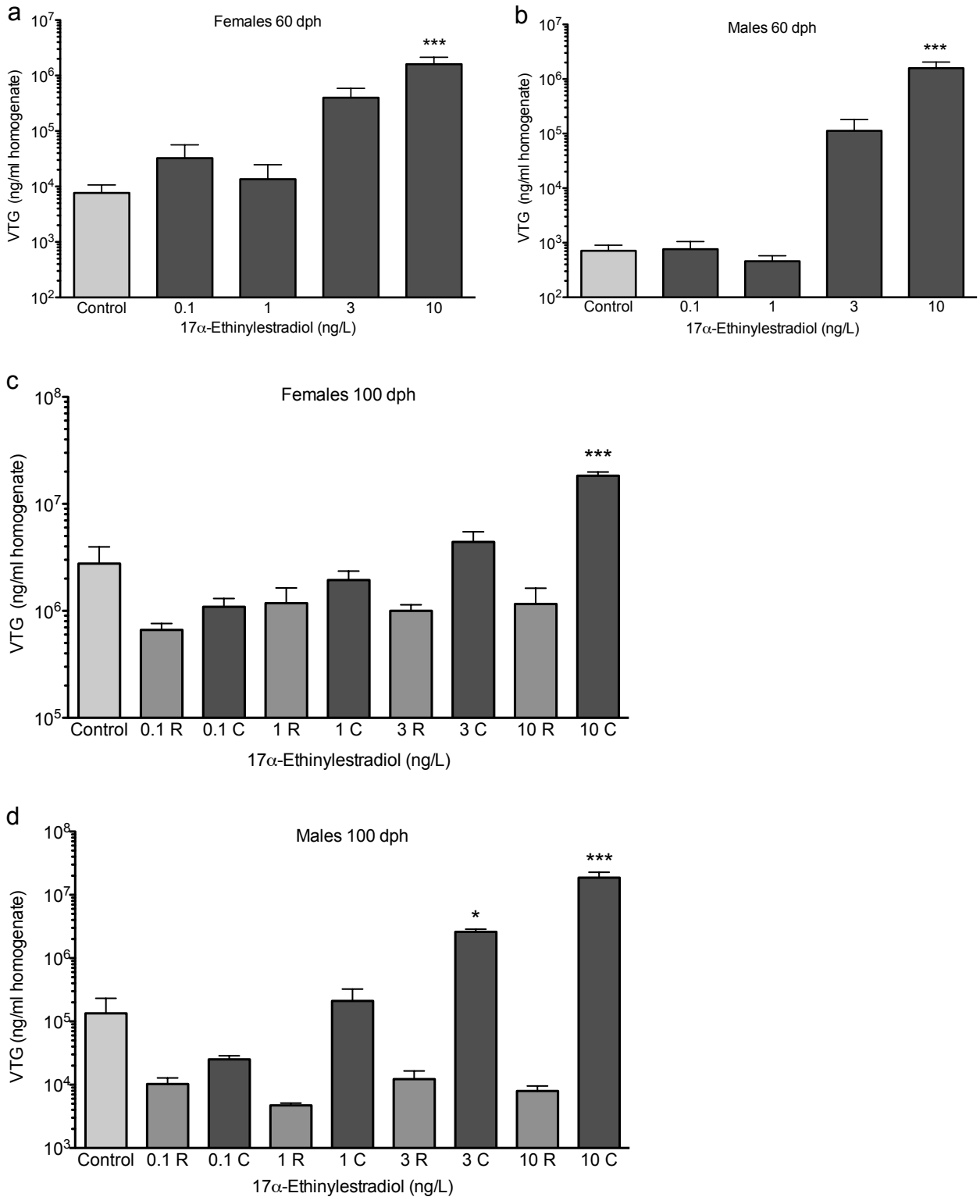
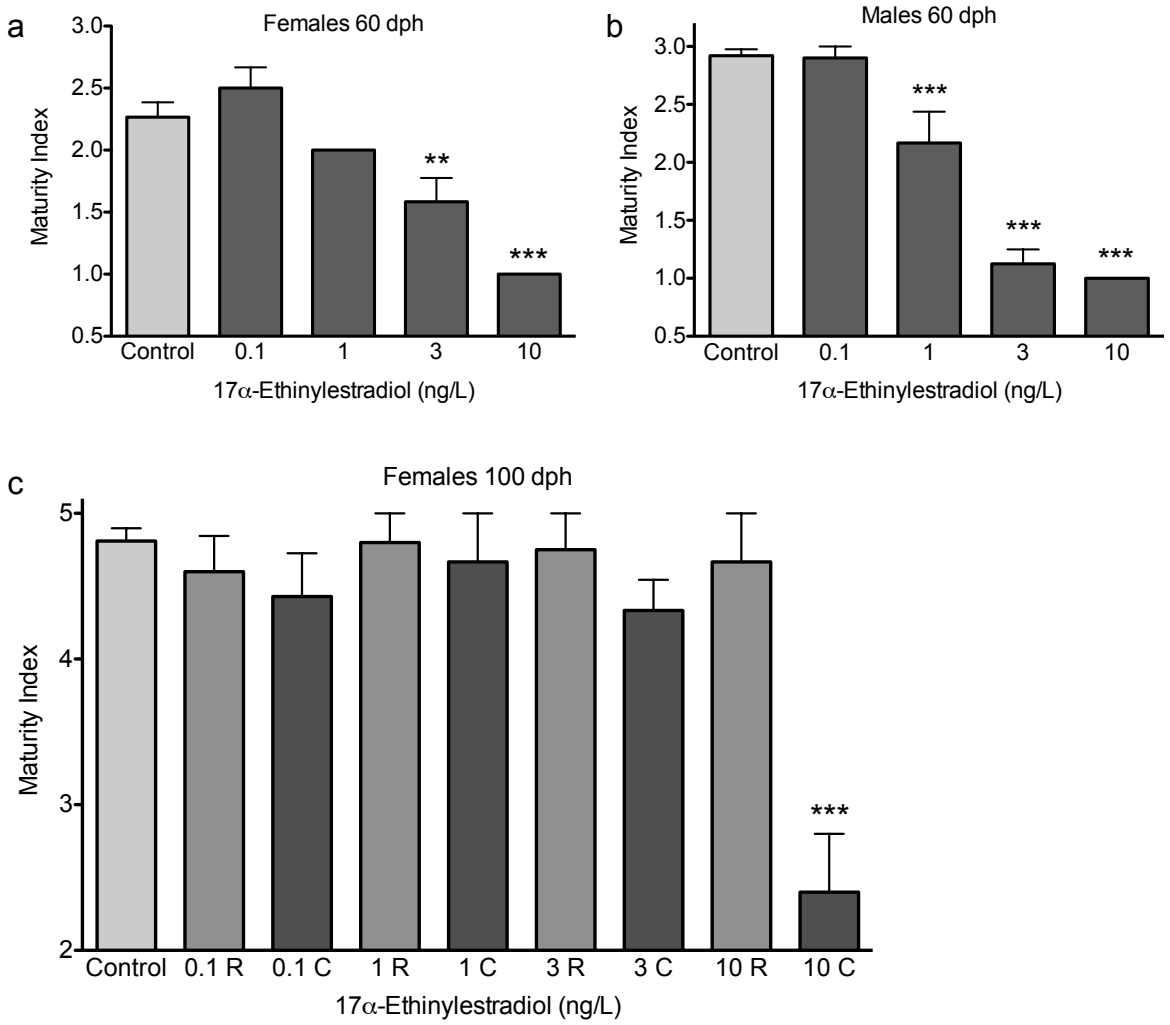


Fig. 4.2: Vitellogenin levels of zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol at 60 dph (a,b) and 100 dph (c,d), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, ***$p < 0.001$; Dunnett´s Test compared to controls.

Maturity index

At 60 dph, a clear dose-dependent decrease of gonad maturity was obvious for female and male zebrafish exposed to EE2 (Fig. 4.3). Statistically significantly lowered maturity indices for females were calculated for 3 and 10 ng/L EE2 and for males in all exposure groups except the lowest concentration of 0.1 ng/L EE2.

At 100 dph, zebrafish of both genders had significantly less mature gonads after continuous exposure to high concentrations of EE2 (Fig. 4.3). The corresponding recovery groups showed no effects versus controls.



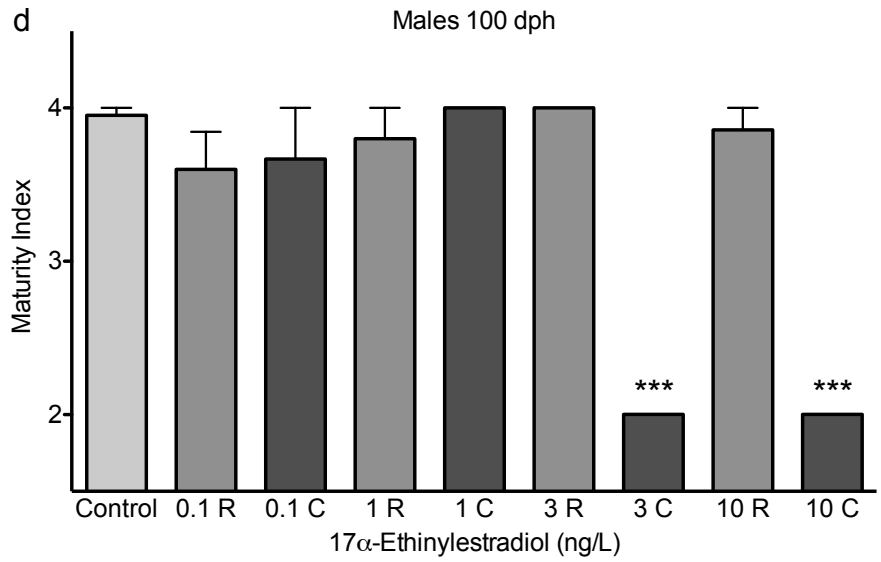


Fig. 4.3: Maturity index of zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol at 60 dph (a, b) and 100 dph (b, c), C = continuous exposure, R = 60 d exposure + 40 d recovery, $^{**}p < 0.01$, $^{***}p < 0.001$; Dunnett´s Test compared to controls.

Body size

At 60 dph, zebrafish of the control groups had an average length of ca. 21 mm and weighed ca. 74 mg. Both parameters were negatively influenced by the exposure to EE2. Fish of the highest exposure groups (3 and 10 ng/L EE2) were significantly smaller (Fig. 4.4). The same effect was observed at 100 dph, independent of a recovery phase. Interestingly, fish exposed to 1 ng/L EE2 were significantly heavier at 100 dph, both with and without recovery phase.

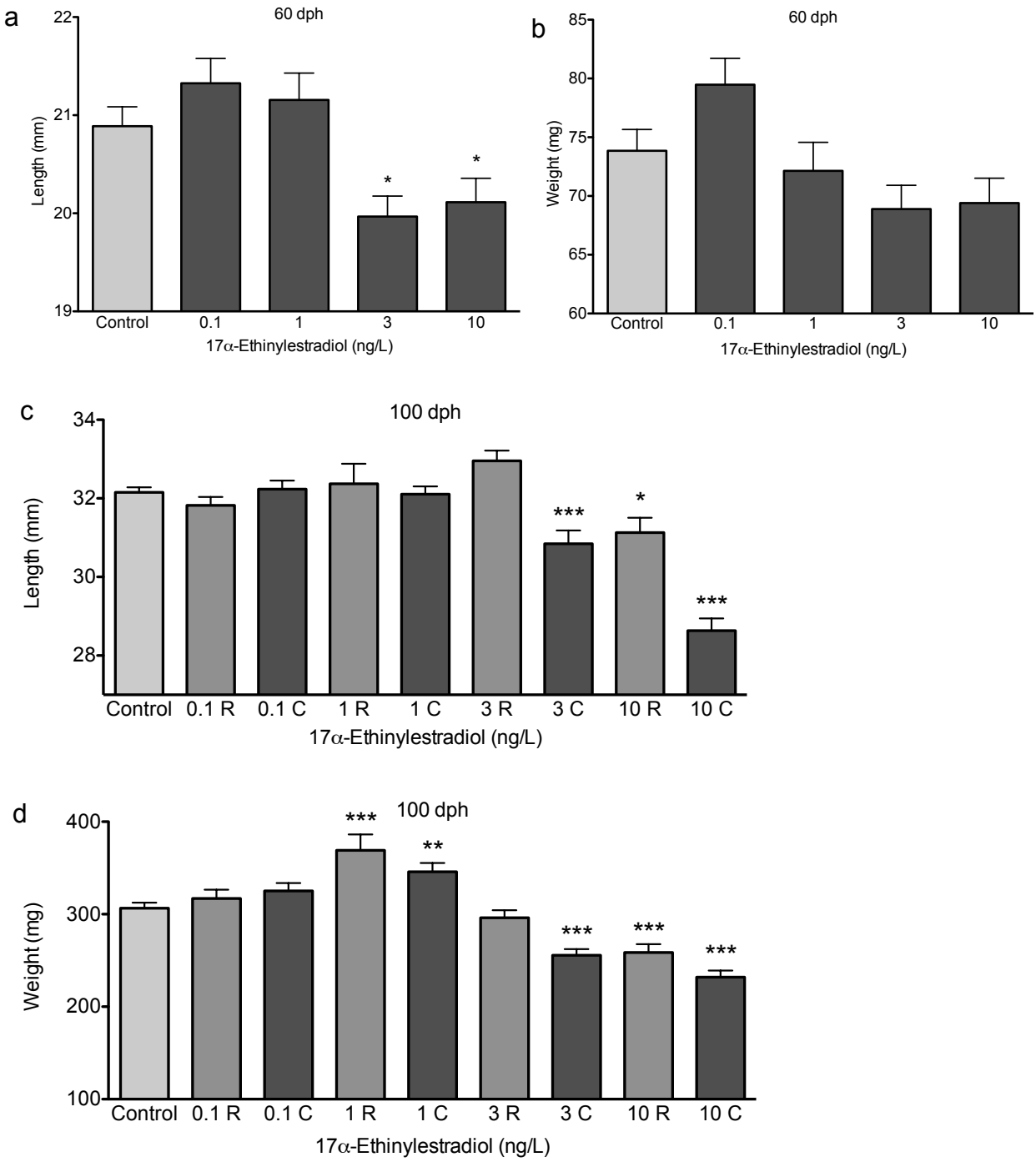


Fig. 4.4: Length and weight of zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol at 60 dph (a, b) and 100 dph (c, d), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Dunnett´s Test compared to controls.

Sex ratio

At 60 dph, 30 individuals were randomly picked from each exposure group of 70 zebrafish. The sex ratio (females:males) in the control groups was 38:60, one individual with undifferentiated gonads was found (Tab. 4.1). Comparable values were found at 0.1 and 1 ng/L EE2; from 3 ng/L, a significant decrease of males was found. No males were picked at 10 ng/L and only 10 % at 3 ng/L. The occurrence of intersex or undifferentiated fish increased dose-dependently, reaching a maximum of 50 % at 10 ng/L EE2.

At 100 dph, the remaining 40 fish were sampled from the tanks. In the negative control, the ratio (females:males) was 47:53 (Tab. 4.1). The sex ratios at 0.1 and 1 ng/L EE2 were similar to controls. Exposure to 3 and 10 ng/L EE2 resulted in an increased percentage of females in the groups that were continuously exposed (3 ng/L: 69 %, 10 ng/L: 74 %), but no effect on sex ratio could be observed in the corresponding recovery groups.

Tab. 4.1: Sex ratio (%) of zebrafish (*Danio rerio*) exposed to 17α-ethinylestradiol at 60 and 100 days post hatch (dph), IS = intersex, UD = undifferentiated, C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Dunnett´s Test compared to controls.

	60 dph			**100 dph (C)**		**100 dph (R)**	
	Females	Males	IS / UD	Females	Males	Females	Males
Control	38	60	2	47	53	47	53
0.1 ng/L	50	50	0	50	50	41	59
1 ng/L	40	45	15**	52	48	48	52
3 ng/L	50	10**	40***	69*	31*	50	50
10 ng/L	50	0***	50***	74**	26**	49	51

4.5 Discussion

The feminizing effect of 17α-ethinylestradiol (EE2) is a well-established fact in research on EDCs in fish. It can provoke sex-reversal of males, resulting in all-female populations (Kidd et al. 2007, Nash et al. 2004) and has been reported to inhibit gonad maturation in both genders (Baumann et al. 2012, Hill & Janz 2003). These massive alterations in gonad development depend on developmental stage and duration of the exposure, as well as the fish species. Not all fish are able to recover from exposure to EE2: estrogen exposure during the sensitive period of sexual differentiation in gonochorists, for example, fathead minnow (*Pimephales promelas*), leads to irreversible effects (Länge et al. 2001). Protogynic fish species like zebrafish have a more plastic gonad development and are able to develop according to their genetic sex once EDC exposure stops (Fenske et al. 2005, Maack & Segner 2004). This, in turn, is also dependent on the specific mode of action of the EDC. In a previous study, zebrafish proved not able to recover from strong masculinization caused by the anabolic steroid 17β-trenbolone (chapter 3). This effect was persistent at all biological organization levels, from mRNA to population. The present study was designed to compare the two substances and to try to elucidate the basic mechanisms underlying the different outcomes of estrogenic and androgenic disruption.

Starting at the lowest level of organization, the mRNA of biomarkers, a down-regulation of brain aromatase (cyp19b) could be observed after EE2 exposure. This effect was only significant in the highest exposure group (10 ng/L) without recovery. All other exposure groups only showed tendencies to decreased cyp19b mRNA levels. Surprisingly, these results are not in accordance with most recent studies about the effects of estrogens on aromatase activity in fish. Exposure to estrogens or aromatizable androgens normally increases the levels of cyp19b mRNA, which is thought to be due to the presence of estrogen-responsive elements in the promoter region of the aromatase gene (Andersen et al. 2006, Brion et al. 2004 & 2012, Hinfray et al. 2006). As aromatase activity is influenced by several factors such as social status, reproductive cycles and behavior (Cheshenko et al. 2008), the reason for this contradiction in our results is probably more indirect and complex. Behavioral alterations were not specifically evaluated in the present study, but it might be presumed that a group of zebrafish consisting of only 26 % males, but 74 % females with both sexes being immature, would probably not show normal sex-specific behavior typical of their age (100 days). Filby et al. (2012) found significant differences in cyp19b expression between dominate and subordinate male zebrafish after EE2 exposure, showing that behavior is an essential factor for the hormonal status of an individual. Moreover, it can be suggested that the developmental

stage of the individual has to be considered for the interpretation of the effect on aromatase expression: In the present experiment, zebrafish of the highest continuous exposure group (10 ng/L EE2) were not only less mature than in other exposure groups or the controls, but also significantly smaller. These two parameters are closely correlated to each other (Baumann 2008). In zebrafish, aromatase expression is known to be comparably low in individuals with undifferentiated gonads, whereas it increases before and during the differentiation of gonads (Fenske & Segner, 2004). Thus, in our experiment, the strong inhibiting effect of EE2 on growth and gonad maturation probably covers the expected up-regulation of cyp19b by the estrogen. These two effects might compensate each other at 0.1 ng/L EE2, so that no effect on cyp19b was seen. Since the highest exposure group with recovery period did not show any alterations concerning gonad maturity or cyp19b expression, it might be concluded that zebrafish were able to recover from EE2 exposure at cyp19b mRNA level.

The most striking reversibility of EE2 effects was seen at the protein level: increased VTG values were only found in zebrafish that were continuously exposed to high concentrations of EE2, whereas the recovery groups had values similar to controls or even lower. This was especially striking for male zebrafish and can probably be interpreted as a compensatory mechanism against the feminizing effect of EE2. Other studies report similar observations (Brion et al. 2004), whereas Fenske et al. (2005) only report a partial reversibility of VTG levels. Again, it is essential to consider both time-point and duration of exposure, as well as the mode of action of the EDC in the interpretation of results. In the previous study with 17β-trenbolone, zebrafish were not able to recover from the inhibitory effect on VTG production (chapter 3). The same difference between the two EDCs was observable at the population level, where zebrafish were able to recover from the exposure to EE2, but not from 17β-trenbolone. While zebrafish continuously exposed to 3 and 10 ng/L EE2 had a sex ratio of females:males of approx. 70:30, the corresponding recovery groups had a ratio of 50:50. Exposure to 30 ng/L 17β-trenbolone resulted in all-male populations, regardless of recovery. The reversibility of the effects of EE2 exposure on sex ratio has been reported before and can be explained by the hypothesis that the high proportion of females after 60 days of exposure is probably partly due to males arrested in their normal development, which includes an ovary gonad stage (Fenske et al. 2005). This assumption is supported by the fact that EE2 exposure during the period of sexual differentiation results in strong inhibition of gonad maturity (Baumann et al 2012, Hill & Janz 2003). Once the estrogen exposure is stopped, the arrested males can develop according to their genetic sex. Interestingly, exposure to androgens results

in permanent masculinization, which is not reversible even after long depuration periods (chapter 3, Larsen & Baatrup 2010, Morthorst et al. 2010).

In summary: an arrest is reversible, a real development is irreversible. It can only be speculated about the underlying mechanisms of this observation. Larsen and Morthorst (2010) raised the hypothesis that primordial germ cells (PGC) may be the key factor in the gonadal development of zebrafish. The normal transition of ovary-like tissue to testis tissue in juvenile males is induced by the loss of PGCs (Uchida et al. 2002). In contrast, the presence of PGCs is required for ovary development in zebrafish (Siegfried & Nüsslein-Volhard 2008). Since the exposure to 17β-trenbolone caused testis-development in genetic females, it is likely that PGCs were lost and thus later development of ovaries was impossible. This would explain why estrogen-induced sex-reversed males are able to recover and build up testis-tissue, whereas androgen-induced sex-reversed females are not able to develop ovaries after depuration.

Since in both of the presented studies, the disruptive effects of the investigated EDCs were observable at almost all effect levels from mRNA to population-relevant endpoints like sex ratio, there is probably a more complex mechanism underlying the ability to recover - or not - from different EDC exposure. However, it is clear that different parameters like species, time point, duration, concentration and mode of action are important for the capacity to recover. More research on the sexual differentiation in zebrafish is needed, as this species is very popular in research on EDCs and results can only be appropriately interpreted with knowledge of the underlying molecular and genetic mechanisms.

5 Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after periodic exposure to prochloraz

5.1 Abstract

Exposure of wildlife and humans to endocrine disrupting chemicals represents a serious hazard for reproductive capacities of individuals and, thus, population fitness. Agriculture continuously and significantly contributes to the pollution of the environment *via* the use of pesticides. Especially aquatic organisms are massively affected, either by high periodic peak exposure or low-dose chronic exposure. The aim of the present study was to investigate the persistence of endocrine effects by the popular fungicide prochloraz, which is known to have multiple effects on the endocrine system of vertebrates. Periodic exposure is particularly relevant for aquatic organisms; therefore, an exposure scenario including an exposure phase and a subsequent recovery period was chosen to assess the potential for reversibility of the effects of prochloraz on sexual development of zebrafish (*Danio rerio*). Zebrafish were exposed to environmental relevant concentrations of prochloraz (10-300 µg/L) during their sexual development until 60 days post hatch (dph). For the following 40 days, fish were either held in clean water for depuration or under further continuous exposure. Different organization levels, from mRNA to the population-relevant endpoints, were investigated with respect to adverse endocrine effects: Sex ratio was skewed towards males and significantly more intersex individuals were found after exposure to prochloraz. High concentrations of prochloraz persistently inhibited growth. Gonad maturation was slightly affected, accompanied by lowered VTG values in both genders, again without recovery. Likewise, lowered brain aromatase (cyp19b) mRNA expression did not show any sign of reversibility. Prochloraz thus irreversibly affects sexual development of zebrafish at all levels of biological organization. The underlying mechanisms however, are not yet fully understood.

5.2 Introduction

Prochloraz is a fungicide with multiple modes of action. It inhibits the biosynthesis of ergosterol, which is a constituent of the cell membrane in many fungi (Johnston 1996), but absent in other cells; therefore prochloraz has become very popular in agricultural plant treatment and industrial usages. Through these applications it regularly reaches surface waters; yet, data on environmental concentrations are not available. There are only predicted environmental concentrations, which are supposedly very low (EFSA 2011). In vertebrates, different endocrine disrupting properties of prochloraz are known: it antagonizes the androgen

and the estrogen receptor (depending on the concentration), agonizes the aryl hydrocarbon receptor and inhibits aromatase activity (reviewed by Vingaard et al. 2006). There are studies that indicate a causal relation between human exposure to pesticides and poor sperm quality (Swan et al. 2003) or increased incidence of cryptorchidism in sons of female gardeners (Weidner et al. 1998). There are also indications that prochloraz has adverse effects on the thyroid system of vertebrates (Brande-Lavridsen et al. 2010, Liu et al. 2011).

In zebrafish (*Danio rerio*), masculinization and inhibited VTG production have been reported, as well as increased numbers of intersex individuals and inhibited gonad maturation (Baumann et al. 2012 & 2008, Holbech et al. 2012, Kinnberg et al. 2007, Thorpe et al. 2011).

The aim of the present study was to investigate the adverse effects of prochloraz on zebrafish at different effect levels, from mRNA modulation to population effects. Additionally, the ability of zebrafish to recover from those effects was tested. Since exposure of wildlife fish populations to pesticides depends on seasonal changes, as well as agricultural activities, periodic exposure is much more likely than continuous exposure. Therefore, an exposure scenario including prolonged exposure followed by a recovery period was chosen and different endpoints, such as cyp19b mRNA expression, VTG induction, growth and gonad staging were recorded. Results are compared to those from previous experiments with 17α - ethinylestradiol and 17β-trenbolone under the same exposure scenario (Chapter 3 & 4).

5.3 Material and Methods

Test substance

Prochloraz (CAS-No.: 67747-09-5) was obtained from Sigma-Aldrich (Deisenhofen, Germany). The following test concentrations were used for the exposure of zebrafish in the 100 days assay, 10, 30, 100 and 300 µg/L. Fresh stock solutions were produced every second day before in light-isolated glass reservoirs, from which they were added to the exposure tanks *via* peristaltic pumps (Minipuls 3, Gilson, Wiesbaden, Germany).

Exposure and sampling

The exposure of zebrafish (*Danio rerio*, Westaquarium strain) to prochloraz started at latest 1 hour post fertilization (hpf) and ended at 100 days post hatch (dph). Fish were held in aerated 12 L flow-through glass tanks at 26 – 27 °C and a dark-light cycle of 10/14 hours. Temperature and flow-through rate (complete water exchange every 8 hours) were controlled twice daily. Hardness (200-280 mg/L), conductivity (600-750 µS), pH (8.0-8.2) and oxygen

saturation (90-95 %) were tested at least once weekly. Feces and food leftovers were removed daily. From day 4 to 14, larvae were fed with powdered dry food (Staubfutter, Sera Micron, Heinsberg, Germany), after that with granular flake food (TetraMin™, Tetra-Werke, Melle, Germany) and newly hatched nauplii of *Artemia* spec. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Ogden, USA).

The exposure to prochloraz was carried out until 60 days post hatch, followed by a recovery period in clear water of 40 days. Additionally, control groups with and without any continuous exposure over the whole time of the experiment (100 days) were analyzed. Each treatment was run in two replicates. 100 Eggs per replicate were used at the beginning. After 30 and 60 days, randomly 30 individuals from each tank were removed. In case of slight differences in the amount of individuals per tank, more or less fish were removed to avoid density-dependent effects. Fish were euthanized with a saturated solution of benzocaine (ethyl-*p*-aminobenzoate, Sigma-Aldrich, Deisenhofen, Germany). Length and wet weight of each individual was documented. Head and tail (for ELISA) or only the head (for qPCR) were cut off with a razor blade behind the operculum and behind the anal fin, weighed and then immediately frozen in liquid nitrogen for subsequent quantification of VTG or cyp19b mRNA. Half of the fish were used for VTG, the other half for cyp19b. Trunks were placed in embedding cassettes (Histosette, Neolab, Heidelberg, Germany) and fixed in modified Davidson´s fixative (Romeis & Böck 2001) for subsequent histological analyses.

Histology

Samples were incubated in modified Davidsons's fixative (Romeis & Böck 2001) at 4°C for at least 24 h before embedding into paraffin by a tissue processor (TP 1020, Leica Microsystems, Nussloch, Germany). Embedding into blocks was performed with a heated paraffin embedding module (EG 1140 H, Leica Microsystems, Nussloch, Germany) with trunks orientated ventrally to the cutting surface. Sections of the gonads with a thickness of 4 - 5 µm were cut with a HN 40 microtome (Reichert-Jung, Heidelberg, Germany), mounted on glass slides (Langenbrinck, Langenseibold, Germany) and stained with hematoxylin-eosin (Romeis & Böck 2001) the next day. Light microscopical evaluation of the tissue sections was performed according to the OECD Histopathology Guidance Document (OECD 2010). Each fish was identified either as female, male or intersex or recorded as undifferentiated. Additionally, the maturation stages of the gonads were categorized as relative proportions of various gametogenic cell types into a numerical staging system (ovary: stages 0 - 5, testis: stages 0 - 4) according to the OECD Histopathology Guidance Document (OECD 2010).

Maturity index

As an enhancement to the regular staging system in the OECD Histopathology Guidance Document (OECD 2010), each stage of maturity was given a fixed maturity value, increasing with the maturity of the fish (Stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The sum of those values from all individuals of each replicate was divided by the number of fish. The sex-specific mean values for each treatment were calculated from the replicates and termed the maturity indices for females or males (Baumann et al. 2012). Completely undifferentiated or intersex individuals were not included into this assessment, since they could not be classified as female or male.

ELISA

The measurement of the VTG concentration in head and tail homogenates of zebrafish was performed as described by Holbech et al. (2006). In short, the frozen tissues were homogenized with a plastic pistil in 1,5 ml centrifuge tubes and mixed with 10 times the weight of homogenization buffer (50 mM Tris-HCl, pH 7.4: 1 % protease inhibitor cocktail, Sigma-Aldrich). The homogenate was centrifuged for 30 min at 25,000 × g at 4°C (Multifuge 1 S-R, Heraeus, Hanau, Germany), the supernatant was collected and stored at -80 °C. The VTG concentration in the supernatant was measured by a direct non-competitive sandwich ELISA, based on polyclonal affinity purified antibodies against zebrafish lipovitellin developed by Holbech et al. (2001).

qPCR

Total RNA isolation of zebrafish heads was performed with TriReagent (guanidine thiocyanate and phenol, Sigma-Aldrich) according to the manufacturer's instructions. The tissue was homogenized with the aid of Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at a frequency of 18 beats per second. RNA concentration and purity was measured with the NanoVueTM Plus Spectrophotometer (General Electric, Fairfield, USA).

Subsequent cDNA synthesis was performed with following reagents per reaction with 1 µg RNA: 1 µg hexanucleotide random-primer-mix (Roth, Karlsruhe, Germany), 2.5 µl M-MLV RT reaction buffer (Sigma-Aldrich), 1.25 µl deoxynucleotide (dNTP) Mix (Sigma Aldrich), 1 µl RiboLock RNase inhibitor (40 U; Fisher Scientific, Schwerte, Germany), 1 µl M-MLV reverse transcriptase (Sigma-Aldrich), 4.25 µl RNase-free water.

The qPCR reaction with the cDNA was performed using the StepOne^{TM} real-time PCR System (Applied Biosystems, Foster City, USA) and the Fast SYBR Green master mix

(Applied Biosystems, Foster City, USA). Expression of 18S-rRNA (endogenous control) and cyp19b mRNA were measured using following primers (5`-3`):

18S Forward-Primer: CACTTGTCCCTCTAAGAAGTTGCA
18S Reverse-Primer: GGTTGATTCCGATAACGAACGA

Cyp19b Forward-Primer: GCTCCAGACACGCTCTCCAT
Cyp19b Reverse-Primer: CATCCTCCAGAGACTGCCTCA

In each qPCR run, cDNA of the pooled control fishes (negative control) was measured in duplicates with 18S and cyp19b as targets. For all other samples, measurement of 18S cDNA was performed in duplicates, and measurement of cyp19b cDNA was performed in triplicates. In addition, there were duplicates of water controls for each master mix that did not contain any template cDNA. A cDNA pool of 5 female or male unexposed fish was used as negative control for the measurement of female and male fish respectively.

Statistics

For statistical evaluation, data were analyzed by one-way ANOVA and Dunnett´s Test using SigmaStat 12.0 (Statsoft-Jandel Scientific, Erkrath, Germany).

Chemical analyses

No chemical analyzes were conducted, as previous experiments with the same substance in the same facility revealed high stability and small deviations from nominal concentrations after chemical analyses (Baumann 2008).

5.4 Results

Cyp19b (brain aromatase) mRNA expression

Cyp19b mRNA expression revealed very high variability between individuals of both genders. No gender-specific differences could be assessed, and therefore females and males were analyzed together. Cyp19b abundance tended to decrease after exposure to prochloraz but no statistical significance could be assessed (Fig. 5.1).

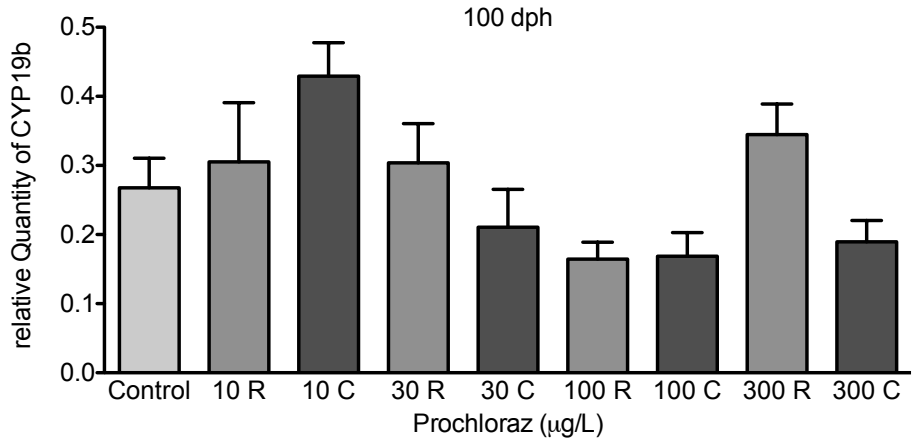


Fig. 5.1: Cyp19b mRNA expression of zebrafish (*Danio rerio*) exposed to prochloraz at 100 dph, C = continuous exposure, R = 60 d exposure + 40 d recovery

Vitellogenin (VTG)

VTG production of zebrafish was affected in an inverted U-shaped manner after 60 days of exposure to prochloraz (Fig. 5.2). No effect could be observed at 10 µg/L prochloraz, whereas at 30 µg/L a strong increase of VTG production was obvious in both genders. At high concentrations of prochloraz, an inhibition of VTG production could be determined, even though it was not statistically significant.

At 100 dph, no significant effects could be determined in females, even though it was obvious that all exposure groups had lowered VTG values compared to the controls (Fig. 5.2). Moreover, recovery groups tended to have higher VTG-levels compared to their corresponding continuous exposure group. For males, no clear differences could be determined between continuous and shortened exposure. Both exposure groups of the highest concentration (300 µg/L prochloraz) displayed the lowest VTG levels, which was statistically significant.

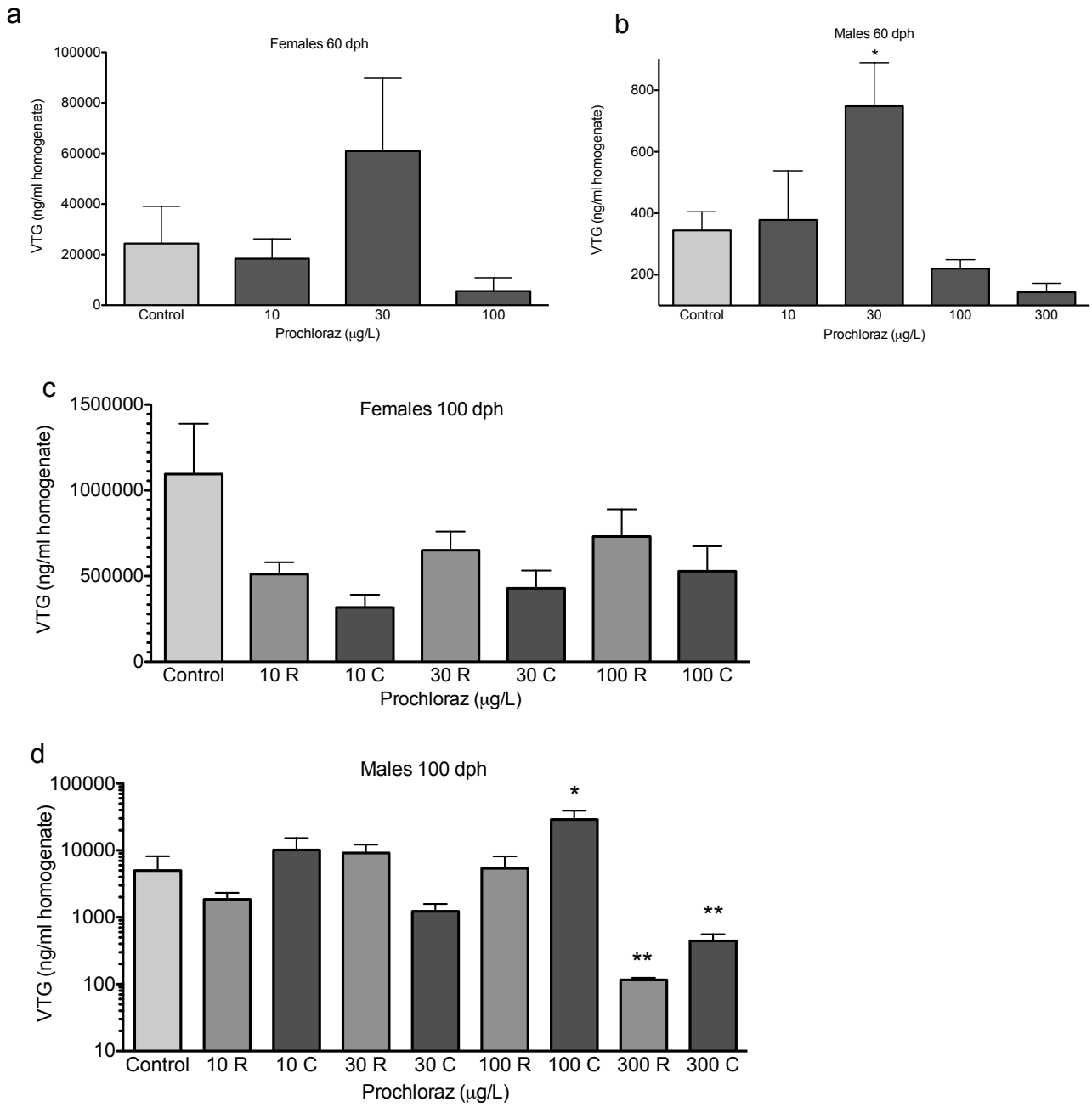


Fig. 5.2: Vitellogenin levels of zebrafish (*Danio rerio*) exposed to prochloraz at 60 dph (a,b) and 100 dph (c, d), C = continuous exposure, R = 60 d exposure + 40 d recovery (*$p < 0.05$, **$p < 0.01$; Dunnett´s Test, compared to control). Note different scaling for females and males

Maturity index

At 60 dph, both genders showed their highest maturity indices at a concentration of 30 µg/L prochloraz (Fig. 5.3). No clear dose-dependent effects on gonad maturity could be observed; Gonad maturity in females was slightly decreased at 10 µg/L and slightly increased at 30

µg/L, whereas males tended to be more mature after exposure to prochloraz. In any case, effects were statistically not significant.

At 100 dph, males of the continuous exposure groups showed elevated maturity indices at concentrations of 10, 100 and 300 µg/L prochloraz, whereas the corresponding recovery groups had lower maturity indices (Fig. 5.3).

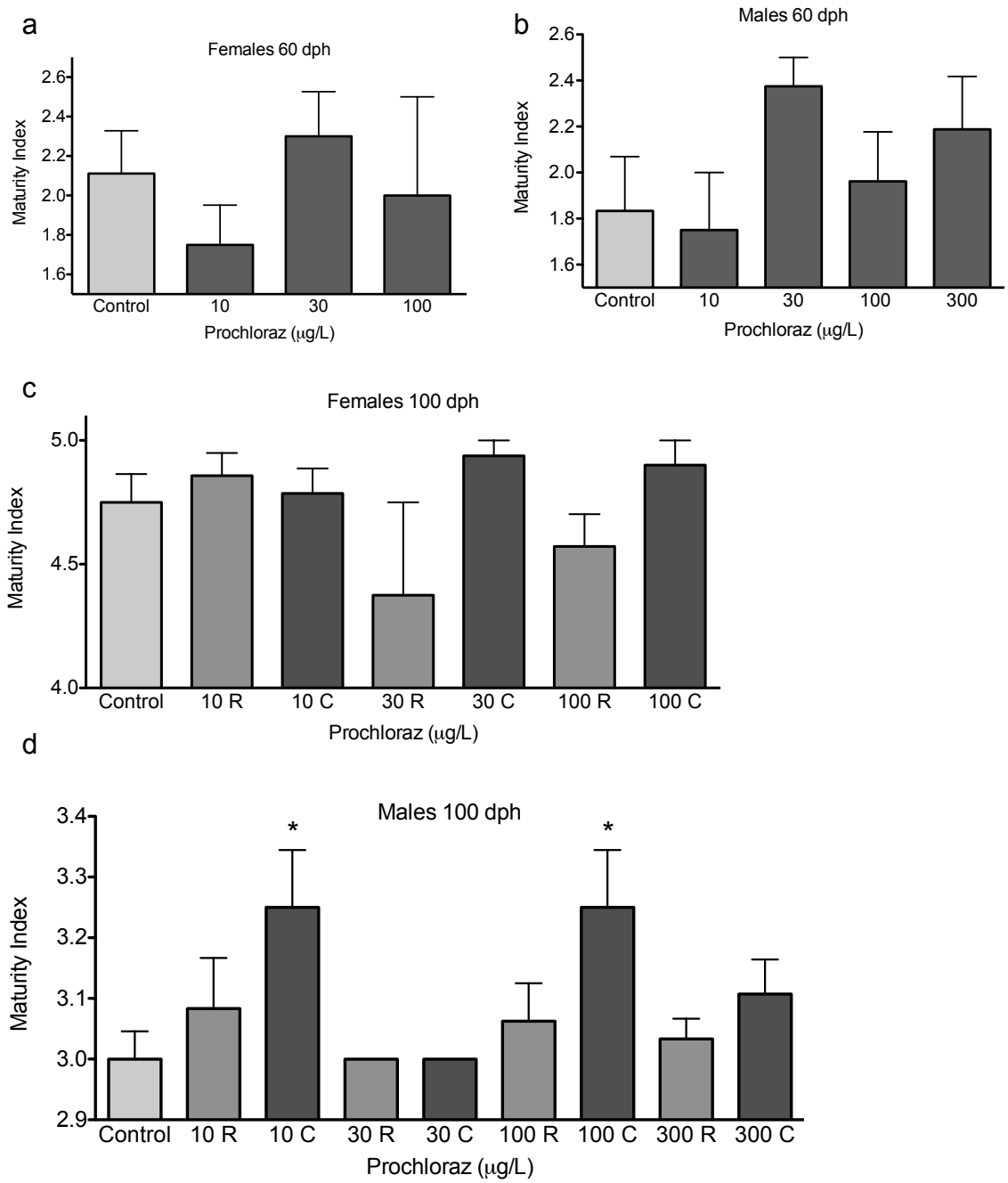


Fig. 5.3: Maturity index of zebrafish (*Danio rerio*) exposed to prochloraz at 60 dph (a,b) and 100 dph (c, d), C = continuous exposure, R = 60 d exposure + 40 d recovery (*$p < 0.05$, Dunnett´s Test, compared to control).

Body size

At 60 dph, zebrafish of the control groups had an average length of 22 mm and weighed 95 mg (Fig. 5.4). Growth was a slightly increased at low concentrations of prochloraz (30 µg/L), but a clear inhibition of growth was significant at the highest concentration (300 µg/L). At 100 dph, no clear differences in growth could be determined between continuous and shortened exposure (Fig. 5.4). Both exposure groups of the highest concentration (300 µg/L prochloraz) were significantly smaller and lighter than the controls.

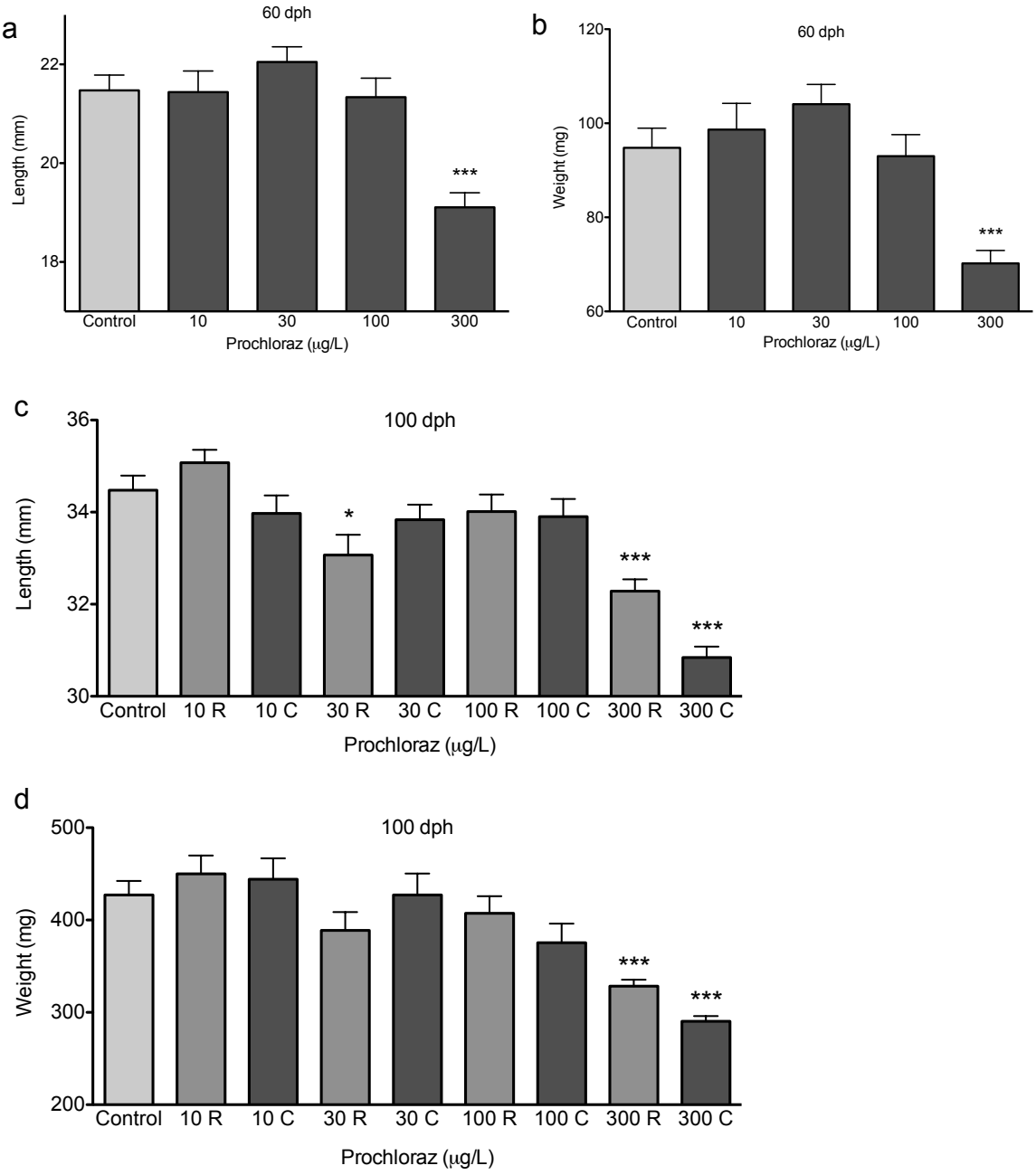


Fig. 5.4: Length and weight of zebrafish (*Danio rerio*) exposed to prochloraz at 60 dph (a, b) and 100 dph (c, d), C = continuous exposure, R = 60 d exposure + 40 d recovery (*$p < 0.05$, ***$p < 0.001$; Dunnett´s Test, compared to control).

Sex ratio

At 60 dph, 30 individuals were randomly picked out of an exposure group of 70 zebrafish. The ratio of females:males:intersex in the control groups was 45:45:10 (Tab. 5.1). The relative amount of females decreased dose-dependently, resulting in 0 % females at 300 µg/L prochloraz. Interestingly, while at 10 µg/L prochloraz significantly more females developed, the number of male zebrafish did not change remarkably, expect in the lowest concentration of 10 µg/L prochloraz. The most striking effect was the increased occurrence of intersex individuals after exposure to 100 and 300 µg/L prochloraz.

At 100 dph, no intersex individuals were found. The sex ratio (females:males) was 39:61 in the controls. A sex ratio of 50:50 was seen consistently at 10 µg/L prochloraz. A masculinizing effect was observable from 30 µg/L prochloraz, without significant differences between continuous and discontinuous exposure.

Tab. 5.1: Sex ratio (%) of zebrafish (*Danio rerio*) exposed to prochloraz at 60 and 100 days post hatch (dph), C = continuous exposure, R = 60 d exposure + 40 d recovery, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; Dunnett´s Test, compared to control

	60 dph			**100 dph (C)**		**100 dph (R)**	
	Females	Males	Intersex	Females	Males	Females	Males
Control	45	45	10	39	61	39	61
10 µg/L	70*	20*	10	51	49	50	50
30 µg/L	50	40	10	46	54	32	68
100 µg/L	10**	65*	25*	20*	80*	23*	77*
300 µg/L	0***	50	50**	11**	89**	6***	94***

5.5 Discussion

Prochloraz exposure during the first 100 days of development of zebrafish had strong influence on their sexual development at different effect levels.

Brain aromatase (cyp19b) mRNA expression

Even though prochloraz is an aromatase-inhibitor (Andersen et al. 2002), there was no significant effect at the mRNA level. Slightly elevated expression levels in the low exposure groups and slightly decreased levels in the high exposure groups were measured, but not statistically significant in any treatment. These results are not in accordance with previous studies: a comparable exposure scenario with zebrafish was performed by Fenske & Segner (2004) with the aromatase inhibitor fadrozole. 35 days old zebrafish were fed with fadrozole-contaminated food for 36 days. Cyp19b expression in the brains was slightly but not significantly lowered directly after the treatment. After 35 days of exposure, zebrafish were reared in clean water with uncontaminated food for another 90 days. Interestingly, cyp19b expression in the brains of fadrozole-treated zebrafish was significantly increased after the depuration period. Moreover, the gonads of these fish were irreversibly masculinized, as in our experiment with prochloraz. Ankley et al. (2009) and Liu et al. (2011) found an association of prochloraz-induced inhibition of steroid synthesis with an up-regulation of cyp19 mRNA in adult fathead minnow and zebrafish. In contrast, Marca Pereira et al. (2011) exposed brown trout to prochloraz and found only slight, but not significant down-regulation of cyp19b mRNA expression. It may be possible that the aromatase-inhibiting effect of prochloraz, that was clearly visible at other effect levels (VTG, sex ratio), is not expressed at genomic level. Laier et al. (2006) exposed juvenile rats to prochloraz and also found strong effects at protein, but not at the genomic level, indicating a post-translational regulation Furthermore, it has to be taken into account that several factors like gonad maturity, social status or behavior play important roles in the individual expression level of aromatase (Cheshenko et al. 2008). E.g., Filby et al. (2012) found significant differences in cyp19b expression between dominant and subordinate male zebrafish after EE2 exposure. As zebrafish of the highest exposure groups were significantly smaller, less mature and produced less VTG, their overall physiology and behavior was obviously impaired by prochloraz exposure, which could also be represented in their aromatase activity. For a clear verification of this theory, it would be interesting to measure not only aromatase mRNA expression, but also the enzyme activity.

Body size

Prochloraz not only affects the endocrine system, but is also a toxic substance. The LC50 for prochloraz in rainbow trout after 96 h of exposure is 1.43 mg/L (EFSA 2011). Even though the chosen concentration range in the present experiment (10 - 300 µg/L) was far below acute toxicity, the chronic long-term exposure had probably negative effects. The combination of endocrine disruption and chronic toxicity becomes obvious from the impaired growth of zebrafish exposed. Treatment with 300 µg/L significantly inhibited growth at 60 and 100 dph. The recovery group was less affected, but still significantly smaller than controls. Those results are not in accordance with those of Kinnberg et al. (2007), who performed an FSDT with zebrafish and prochloraz in a slightly lower concentration range and found no effects on growth. On the other hand, Thorpe et al. (2011) observed inhibited growth in zebrafish and fathead exposed to 320 µg/L prochloraz in an FSDT. These and the present results are consistent with reports that prochloraz inhibits the synthesis of both testosterone and estradiol (Ankley et al. 2005, Marca Pereira et al. 2011), which are involved in the regulation of growth in fish (Bhatta et al. 2012). Moreover, prochloraz was shown to reduce muscle growth in juvenile rainbow trout by reducing food intake (Fauconneau & Paboeuf, 2001). In the present experiment, the effects on growth only occurred in the highest exposure groups, but were persistent after depuration. Consequently it can probably be concluded that zebrafish were not able to recover from the toxic and/or endocrine disrupting properties of prochloraz that were inhibiting growth.

Maturity index and VTG

Gonad maturity and VTG induction are discussed together, as those two parameters generally correlated to each other (chapter 2). The results at 60 dph are similar to those discussed for prochloraz in chapter 2. In short: the highest VTG values and maturity indices were observed at 30 µg/L prochloraz, which we interpreted as hormesis-like effect, since at higher concentrations aromatase-inhibition and toxic effects of prochloraz prevailed and caused under-development and masculinization of females as well as a remarkable dose-dependent decrease in the maturity index of males.

At 100 dph, VTG production was clearly inhibited in males at the highest concentration of prochloraz, and the same tendency was observable in females. Nevertheless, a clear correlation with gonad maturity was not visible in both genders. Females were not affected at all, whereas males had significantly increased gonad maturity at 10 and 100 µg/L prochloraz (continuous exposure). Data for females can only be seen as a trend, since only a total of 14

individuals were left for analyses at 100 µg/L in both replicates together. Nevertheless, ovary maturation did not seem to be affected by prochloraz, once female differentiation was possible at all. The same phenomenon was observed after exposure to the androgen 17β-trenbolone under the same exposure conditions: The small number of female individuals left in high exposure groups displayed comparably high gonad maturity if compared to the controls. This was interpreted as a compensatory mechanism against the androgenic treatment(chapter 3). Another interpretation for prochloraz could be a specific mechanism: In rainbow trout cell cultures, oocyte maturation was triggered by prochloraz by the induction of the expression of luteinizing hormone (LH)-related genes (Rime et al. 2010). In the same study, synergistic effects of prochloraz and LH on oocyte maturation were discovered. For males instead, elevated gonad maturity was observed in only 2 exposure groups. This seems to be in conflict with a previous study, which reported a significant inhibition of spermatogenesis due to exposure to prochloraz in rainbow trout (Le Gac et al. 2001). From this point of view, higher maturity indices without permanent exposure would be expected, but the opposite was the case in the present experiment.

Sex ratio

At 60 dph, a dose-dependent increase of intersex individuals was striking after exposure to prochloraz. This agrees with the findings of Kinnberg et al. (2007) and Thorpe et al. (2011), who also observed high occurrence of intersex gonads after prochloraz exposure during sexual differentiation of zebrafish. Zebrafish is a protogynic fish species, i.e. all fish first develop immature female gonads, of which approx. 50 % will later be transformed into testes in genetic males. This transformation is a simultaneous process of degeneration of immature oocytes and development and maturation of testis tissue. Consequently, intersex gonads are part of the normal sexual differentiation of males, which is called “juvenile hermaphroditism” (Maack & Segner, 2003, Takahashi 1977). Therefore, the high percentage of intersex gonads after 60 days of exposure to prochloraz can be interpreted as retardation in the development of genetic males, due to a surplus of testosterone resulting from the inhibition of aromatase. In fact, at the highest concentration of prochloraz, no female individual was found. Since genetic sex identification in zebrafish is not yet possible, even though several genes have been correlated to sexual differentiation of zebrafish (Anderson et al. 2012, Jørgensen et al. 2008, Orban et al. 2009), this interpretation remains uncertified. Another hint, however, is that no intersex individuals were found at 100 dph. This shows that the development of intersex gonads due to prochloraz exposure at 60 dph was not permanent and that these individuals probably further developed to be functional males.

This also becomes obvious in the sex ratio: a concentration-related effect could be determined after 100 days of exposure to 100 and 300 µg/L prochloraz, where 77-94 % of the fish were males. This is in line with results from other studies (Holbech et al. 2012, Kinnberg et al. 2007) and was not reversible through a recovery period.

Prochloraz has multiple modes of action, but the dominant effect is the inhibition of aromatase (reviewed by Vingaard et al. 2006). The enzyme catalyzes the conversion of testosterone, or 11-keto-testosterone in fish, to 17β-estradiol (Giuguen et al. 1999). Consequently, an inhibition leads to a lack of estradiol and a surplus of testosterone, resulting in masculinization, which is reflected in the sex ratio at the highest concentrations. On the other hand, at 10 µg/L prochloraz, a tendency to feminization could be observed. This could be due to the ability of prochloraz to act as an androgen antagonist (Laier et al. 2006). Interestingly, the masculinizing effect of high doses of prochloraz was persistent after a depuration period of 40 days. The same effect was found in a previous study with 17β-trenbolone (chapter 3), where the same exposure scenario was studied. Zebrafish were not able to recover from the androgenic effect, which resulted in all-male populations in the highest exposure groups with or without recovery period. As a conclusion, treatment with androgenic or aromatase-inhibiting EDCs during sexual differentiation of zebrafish leads to irreversible masculinization of gonad development. This effect has already been observed in other studies with androgens (Larsen & Baatrup 2010, Morthorst et al. 2010) or aromatase-inhibitors (Fenske & Segner 2004). In contrast, exposure to estrogens like 17α-ethinylestradiol is mostly reversible (Fenske et al. 2005, Nash et al. 2004, Schäfers et al. 2007). The difference between the underlying modes of action and the adverse outcome will be discussed in chapter 6 in detail.

6 Conclusions

6.1 Adverse outcomes of endocrine disruption at different effect levels

The three different EDCs investigated within the present study had strong impact on the sexual development of zebrafish, which was obvious at all effect levels of biological organization from mRNA to sex ratio. This observation raises the question about the relevance of biomarkers and starts the discussion about the most predictive endpoint for the evaluation of adverse outcomes at the population level.

The adverse effects of the chosen EDCs were already detectable at mRNA level, except for prochloraz, where surprisingly no significant effects could be observed. Moreover, for all three experiments, brain aromatase (cyp19b) expression did not show the expected results and made interpretations difficult. Even underestimation of an otherwise very potent EDC (prochloraz) could have been possible with exclusive analyses of cyp19b mRNA. Likewise, exposure to 17β-trenbolone did not result in a clear dose-response of the genetic regulation of the endocrine system: only low-dose effects could be identified, which are difficult to interpret. Exposure to EE2 caused an inhibition of cyp19b expression, even though the opposite had to be expected, since estrogens normally up-regulate aromatase expression (Brion et al. 2012, see Chapter 4 for details). In summary, none of the three experiments gave clear results concerning cyp19b mRNA expression and, thus, genetic results alone failed to predict the adverse outcome at the population level. The question remains if this can generally be expected from a biomarker. Nevertheless, especially the results for the prochloraz experiment show that aromatase has been regulated, respectively disrupted, posttranslational, resulting in adverse effects of the hormonal balance. This observation has also been made by Laier et al. (2006), who found effects of prochloraz at protein level, but not at genetic level. A more in-depth analysis of current literature on aromatase underlines this impression: The mechanism underlying disruption of aromatase are not yet fully understood, as is the normal function of the enzyme in different physiological processes of fish development (reviewed by Diotel et al. 2011). Multiple parameters like behavior, social status or seasonal changes are associated with aromatase function in fish (Cheshenko 2008, Diotel et al. 2010, Filby et al. 2012), and the consequences of impairment are very complex and highly depend on age, strain and species of the fish exposed. Moreover, the exact mechanisms for sexual differentiation of zebrafish are not fully understood (Anderson et al. 2012), and, therefore, the examination of one single gene in a complicated multifactorial system like the hormonal system, can only provide a small hint of the overall effect on the organism. From a scientific

point of view, aromatase is a very interesting field of research, where many components are still unknown; however from a regulatory point of view, aromatase expression is far from application as a reliable biomarker for the detection of endocrine effects.

However, one step higher in the hierarchy of organization, at the protein level (vitellogenin (VTG) induction), all three substances showed significant effects that could be correlated to the effects at organism and even population level. VTG induction and maturity index, e.g., showed an excellent correlation for different EDCs after 60 days of exposure. Consequently, the investigation of both parameters provides detailed insight into the hormonal and reproductive status of an individual. Nevertheless, the investigation of both parameters together is necessary, since the way of correlation can differ depending on the mode of action of an EDC and therefore the investigation of only one parameter could even lead to misinterpretations. VTG has been used as an important and reliable biomarker for several years and has proven to be a very sensitive tool to detect endocrine disruptive effects in fish (Matozzo et al. 2008). The protein represents a key element in the maturation of eggs in female fish, but can also be synthesized by males, especially under estrogenic exposure (Holbech et al. 2001, Rose et al. 2002). This parameter gives very convincing information about the hormonal status of the individual. Consequently, numerous publications over the last years have used VTG induction as biomarker, either alone or as supportive information in parallel with, e.g., histological investigations (see Scholz & Klüver 2009 for review).

Histopathology has been an established and valuable tool in natural sciences and medicine since the 19th century, which has continuously been modernized with special techniques like immuno-staining or *in-situ*-hybridization. It has proven to be irreplaceable for the detailed and specific assessment of pathological changes of tissues and organs. In the research field of endocrine disruption in fish, histological investigations of the gonads are an irreplaceable method for the detection of specific effects on the reproductive organs (Maack & Segner 2004, Van der Ven et al. 2003). In chapter 2, the maturity index is presented, a method for the quantification of gonad maturity, as a valuable tool for the detection of gonad-specific effects in fish. This is especially important, as the reproductive capacities of a population not only depend on the presence of males and females, but also on their sexual maturity. Thus, the investigation of gonad stages is a very important method to predict the reproductive fitness of a population. Moreover, the identification of intersex gonads, which was the main effect after 60 days of exposure to prochloraz in the present study (Chapter 5), cannot be detected from external examination or with molecular biomarkers. Consequently, gonad histology represents an indispensable method for solid evaluation of EDC-related effects on sexual development,

especially in research with zebrafish, as its sex cannot yet be identified with genetic markers (Anderson et al. 2012).

The fast, easy and cheap use of biomarkers has resulted in extensive use of fish embryos in exposure experiments in the field of ecotoxicology. On the one hand, this is economic and important for the reduction of animal testing, since most countries accept this method as non-animal testing as long as the embryo is inside the chorion or is still nourishing on its yolk-sack. Most genetic and physiological mechanisms can already be examined in embryos, but, on the other hand, results observed in embryos are not completely transferable to adults or even whole populations. Exposure experiments with EDCs on embryos often need much higher concentrations of the chemical to show significant effects. In the present experiments, elevated cyp19b expression after 100 days of exposure to trenbolone was significant at concentrations of 1 ng/L. In embryos, at least the 100 000-fold (100 µg/L) is needed to see an induction of the mRNA expression (Brion et al. 2012).

For many toxic substances, the opposite is the case and intensive studies of the last years have shown that the fish embryo toxicity test (FET) can replace acute toxicity tests with adult fish, since equal or even higher sensitivity was found (Lammer et al. 2009). However, effects on the endocrine system are much more complex than e.g. lethality, and mostly concern the sexual development and reproductive capacities of fish. These endpoints cannot be assessed in embryos and make extrapolation to adults very difficult, if not impossible. However, the use of fish embryos in EDC research can be a useful help for preliminary testing, e.g. for unknown substances.

Another very popular method for the reduction of animal tests is the use of cell cultures, i.e. true *in vitro* assays. This approach has not been used within the present study, but for the detection of e.g. receptor-binding affinities of a potential EDC, this method is very useful and offers insight into the basic molecular mechanism underlying effects at higher levels of organization. Especially when it comes to endocrine disruption, the knowledge about the basic molecular properties of a compound is essential for further experiments and the interpretation of results. The three EDCs that were used in the present study have been extensively investigated concerning their binding-affinities to different hormone-receptors in previous studies (e.g. Segner et al. 2003, Vingaard et al. 2006, Wilson et al. 2002). This offered the possibility to use the current knowledge for the interpretation of results; EE2 e.g. is known to have a twofold higher affinity to the estrogen-receptor than natural estradiol (Blair et al. 2000). This fact helped interpreting the extreme increase in VTG values in

exposed zebrafish, compared to a weaker estrogen, 4-*tert*-pentylphenol (Chapter 2). Additionally, if an unknown substance is to be examined in a complex and expensive test system, *in vitro* assays offer the opportunity to define a reasonable concentration range in which further animal tests could be performed. This, in turn, avoids high animal throughput.

The complete opposite of *in vitro* assays is a multi-generation test with animals. When it comes to the detection of population-relevant endpoints, such as impaired reproductive capacities, skewed sex ratios or altered sexual behavior, this test system definitely yields the most conclusive data. Nevertheless, time- and cost-considerations have to be taken into account, as well as animal welfare. Therefore, short but sophisticated tests like the Fish Sexual Development Test FSDT (OECD 2011) represents a good compromise for the solid assessment of EDC-related effects in fish that have high predictive value for effects of population-relevance. 60 Days of exposure are short compared to approx. 6 – 7 months in a multi-generation test with zebrafish, but as the exposure period takes place within the sexual development and maturation, very meaningful and predictive endpoints can be investigated (Chapter 1 & 2).

Regarding the variety of test systems and effect levels, it can be concluded that the use of biomarkers and the analyses of different effect levels are very important and meaningful for research on endocrine disruption, but "classic" endpoints like gonad histology or effects on sex ratio and growth should implicitly be examined to get an overview of the adverse outcome pathway of an EDC. Only a detailed analyses from the first binding of an EDC to a receptor, over the synthesis of different proteins, up to the effects on single organs and finally to the population fitness offers the possibility to get enough information to make decisions on regulatory basis.

6.2 Reversibility of endocrine disruption in zebrafish (*Danio rerio*)

Within the scope of the present study, three different endocrine-disrupting chemicals (EDCs) were studied with respect to the persistence of their adverse effects on the sexual development of zebrafish (*Danio rerio*). The underlying modes of action were either estrogenic (17α-ethinylestradiol, EE2), androgenic (17β-trenbolone) or aromatase-inhibiting (prochloraz). The comparison between the three different substances was assessed by performing exposure experiments with zebrafish during their sexual differentiation, followed by a depuration period (see chapter 3-5 for details).

The effects of the strong semi-synthetic estrogen EE2 were reversible, whereas the masculinization caused by 17β-trenbolone or prochloraz was not. This was especially striking at the population level, where skewed sex ratios were still found after recovery from prochloraz and 17β-trenbolone exposure. Zebrafish that were exposed to EE2 showed normal sex ratios of 1:1 after depuration. This phenomenon has already been published in single experiments with different EDCs (Fenske et al. 2004 & 2005, Larsen et al. 2009 & 2010, Morthorst et al. 2010), but a copious comparison with three different modes of action examined at the same time with the same exposure scenario has not been performed before. Thus, existing data could be confirmed and new interesting data concerning the sexual development of zebrafish under exposure to different EDCs could be provided in the present study.

Even though zebrafish is a very popular model organism in scientific research, its sexual development still remains partially unexplained. A lot is known about the morphological development of zebrafish gonads, which includes an "all-female" state of immature ovaries in both genders (Maack & Segner 2003, Takahashi 1977, Van der Ven et al. 2003). This special development is called protogyny and can be influenced by several environmental factors, resulting in altered differentiation of gonads (reviewed by Baroiller et al. 2009). Nevertheless, comparably little is known about the genetic mechanisms of sexual development in zebrafish as undifferentiated gonochorists. Recently, a report has been published about a putative sex chromosome in zebrafish (Anderson et al. 2012), but further research is needed to confirm this hypothesis, as differences among strains were found.

Primordial germ cells (PGCs) are progenitors of gametes and differentiate either into eggs or sperms during sexual development (Saito et al. 2008). If PGCs are absent or ablated, only testis development is possible, whereas ovaries cannot develop without the presence of PGCs (Slanchev et al. 2005, Uchida et al. 2002). Germ line-deficient zebrafish even display male coloration and male behavior, indicating that steroid hormones produced by the testis are sufficient for masculinization of the whole organism (Siegfried & Nüsslein-Volhard 2008). This phenomenon may offer an explanation for the observations made in the present study: Feminization of zebrafish is reversible, but masculinization is not. As the exposure to 17β-trenbolone and prochloraz caused testis development in genetic females, it is likely that PGCs were lost in these individuals, and, thus, later development of ovaries after depuration was impossible. In contrast, cessation of estrogenic exposure allowed normal development of testis tissue in genetic males. Consequently, masculinization of genetic females represents a one-way development, whereas feminization of genetic males can be reconverted. The

underlying mechanism is difficult to assess without measurement of different molecular markers, but the basic reason for this phenomenon can probably be found in the protogyny of zebrafish. The juvenile "all-female" state of the zebrafish gonad has basically two options: either the endogenous (or exogenous) estrogen level is high and the gonad remains an ovary. Or: if the estrogen-level is low and the androgen level high, the oocytes lack the signal to mature and are degraded. This degradation of oocytes, respectively PGCs, represents an irreversible one-way development that cannot be reversed.

This hypothesis is probably the best explanation for the results of the present study, especially concerning the effects on sex ratio. Nevertheless, it can only be part of more complex mechanisms underlying the persistence of effects, since they were observable at all effect levels from mRNA to population-relevant endpoints. Further research is needed to detect more details.

6.3 Environmental relevance of the results of the present study

The three different substances investigated within the present study are well known for their endocrine disrupting properties (Ankley et al. 2003, Le Gac et al. 2001, Scholz & Gutzeit 2000) and are of high relevance for humans and the environment. EE2 is the most popular compound of oral contraceptives, which are used by over 100 million women worldwide (UN 2009). Trenbolone is an anabolic steroid that is intensively used in industrial animal farming and in sport doping as muscle growth promoter. Prochloraz is a fungicide, which is commonly used in agriculture and industry against fungi. Reports about measured concentrations in the environment differ widely, but the presence of those three chemicals is an undisputed fact (Bartelt-Hunt et al. 2012, EFSA report 2011, Wise et al. 2011).

In order to increase the environmental relevance of the present study, a realistic exposure scenario was chosen to investigate EDC-related effects on zebrafish (*Danio rerio*). Exposure of wildlife populations to pollutants is mostly not as constant as under laboratory conditions, since the input of chemicals to surface waters is more likely to occur periodically or in mixtures, due to different influences like agricultural activities, seasonal changes or industrial processes or even accidents. Therefore, an exposure scenario with subsequent depuration period was chosen to investigate the effects of three different EDCs on the sexual development of zebrafish. Nevertheless, this simple approach is by far not sufficient to simulate realistic conditions in the environment, and more research is needed to satisfy this need.

Our analyses show that the three investigated EDCs have massive influence on the sexual development of zebrafish. Those results can, to a certain extent, be transferred to other animals or humans, as the hormonal system of zebrafish is basically similar to other vertebrates (reviewed by Löhr & Hammerschmidt 2011). Regarding the presence of the three EDCs in the environment, it can be concluded that especially aquatic organisms may suffer from exposure and that fish populations could be impaired in their reproductive fitness, even after short periodic exposure, especially if this occurs during their sexual differentiation. Multiple other studies have been published that show that the three EDCs not only affect fish in their sexual development but also other vertebrates and humans (see chapter 1 for details). Consequently, our results additionally emphasize that EDCs represent a serious hazard for wildlife populations and humans.

The health threat resulting from chronic exposure to EDCs has increasingly come into the focus of public interest. Even non-scientific popular journals regularly report on adverse effects of EDCs on human health (e.g. Kristof 2012). Especially plastic softeners have aroused the awareness of consumers and even led to banning of certain substances, like bisphenol A, from the food market (see Flint et al. 2012 for review). The population apparently becomes more aware of the risks of exposure to pollutants. E.g. the fear of pesticide contamination of food is widespread in the public. The consumption of organically and ecologically grown food has massively increased over the last years (EU 2010). Another red-hot topic, more and more reaching public discussion, is the strong estrogen EE2, which is the most important compound in oral contraceptives. Recent publications suggest that the impact of EE2 on human health is highly underestimated and that the level of exposure should be drastically lowered (Owen & Jobling 2012). As the consumption of oral contraceptives cannot, and should not, be restricted, the only possibility to reduce EE2 input to the environment is to use active carbon filters in water treatment systems (Clouzot et al. 2008). This method is very effective, but also very expensive and would thus result in increased costs for water purification. Unfortunately, the public does not yet seem to be willing to take this financial burden. However, regarding all the experimental results and documentation of EDC-related effects on wildlife, it can be concluded that if the continuous EDC exposure is not restricted by regulatory forces, e.g. by optimizing water purification with active carbon filters, massive effects on wildlife populations and humans are likely to increase during the next years.

7 References

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