Effects of endocrine disruptors in zebrafish (*Danio rerio*) as revealed with the fish sexual development test

Diplomarbeit

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Hiermit erkläre ich, dass ich die vorliegende Diplomarbeit selbständig unter Anleitung verfasst und keine anderen Hilfsmittel als die angegebenen Quellen benutzt habe.

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Index

Index I

1. Introduction 1

1.1 Endocrine disrupting chemicals (EDCs) 1

1.2 Test substances 2

1.2.1 Prochloraz 2

1.2.2 4-*tert*-pentylphenol 3

1.3 The Zebrafish (Danio rerio) 4

1.3.1 Development and reproduction 4

1.3.2 Suitability of zebrafish (*Danio rerio*) for the evaluation of EDCs 5

1.4 Vitellogenin 6

1.4.1 Endocrine regulation in fish 6

1.4.2 Vitellogenin as a biomarker for EDCs 6

1.5 Pigmentation in zebrafish (Danio rerio) 7

1.5.1 Development of pigmentation in zebrafish (*Danio rerio*) 7

1.5.2 Use of transparent zebrafish (*Danio rerio*) in science 8

1.6 Aims of the present study 8

1.6.1 Evaluation of the “fish sexual development test“ as an OECD guideline 8

1.6.2 Establishment of a simple method to quantify pigmentation in zebrafish (*Danio rerio*) 9

2. Materials and methods 10

2.1 The Zebrafish (Danio rerio) 10

2.1.1 General description 10

2.1.2 Maintenance 11

2.1.3 Spawning 11

2.2 Test substances 12

2.2.1 Prochloraz 12

2.2.2 4-*tert*-Pentylphenol 13

2.3 The "Fish Sexual Development Test" (FSDT) 13
2.3.1 Test design FSDT 13
2.3.2 Test setup FSDT 14
2.4 Histology 15
2.4.1 Fixation 15
2.4.2 Dehydration and embedding 15
2.4.3 Sectioning and staining 16
2.4.4 Analysis of histology 18
2.5 Measurement of vitellogenin concentrations via ELISA 24
2.6 Acute fish embryo toxicity assay 25
2.6.1 Exposure design fish embryo assay 26
2.6.2 Analysis of standard parameters 26
2.6.3 Analysis of pigmentation in zebrafish (*Danio rerio*) 27
3. Results prochloraz 29
3.1 Water analysis of FSDT with prochloraz 29
3.1.1 Water parameters of FSDT with prochloraz 29
3.1.2 Chemical analysis of FSDT with prochloraz 29
3.2 Mortality of zebrafish (Danio rerio) exposed to prochloraz 30
3.3 Sex ratio of zebrafish (Danio rerio) exposed to prochloraz 30
3.4 Staging of zebrafish (Danio rerio) exposed to prochloraz 31
3.4.1 Staging of female zebrafish (*Danio rerio*) exposed to prochloraz 31
3.4.2 Staging of male zebrafish (*Danio rerio*) exposed to prochloraz 32
3.4.3 Overall maturity index of zebrafish (*Danio rerio*) exposed to prochloraz 33
3.5 Histopathology of zebrafish (Danio rerio) exposed to prochloraz 34
3.5.1 Histopathology of female zebrafish (*Danio rerio*) exposed to prochloraz 34
3.5.2 Histopathology of male zebrafish (*Danio rerio*) exposed to prochloraz 34
3.6 Length und weight of zebrafish (Danio rerio) exposed to prochloraz 36
3.6.1 Length and weight in correlation to concentration and gender 36
3.6.2 Length and weight as correlated to stages of maturity 37
3.7 ELISA with zebrafish (Danio rerio) exposed to prochloraz 39

3.7.1 Vitellogenin synthesis in female zebrafish (*Danio rerio*) exposed to prochloraz 39
3.7.2 Vitellogenin synthesis in male zebrafish (*Danio rerio*) exposed to prochloraz 40

3.8 Acute fish embryo toxicity assay with zebrafish (Danio rerio) exposed to prochloraz 41
3.8.1 Fish embryo assay 24 hours post-fertilization 41
3.8.2 Fish embryo assay 48 hours post-fertilization 42
3.8.3 Fish embryo assay 72 hours post-fertilization 44

4. Results 4-*tert*-Pentylphenol 46

4.1 Water analysis of FSDT with 4-tert-pentylphenol 46
4.1.1 Water parameters of FSDT with 4-*tert*-pentylphenol 46
4.1.2 Chemical analysis of FSDT with 4-*tert*-pentylphenol 46

4.2 Mortality of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 46

4.3 Sex ratio of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 47

4.4 Staging of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 48
4.4.1 Staging of female zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol 48
4.4.2 Staging of male zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol 48
4.4.3 Overall maturity index of zebrafish (*Danio rerio*) exposed to 4–*tert*-pentylphenol 49

4.5 Histopathology of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 50

4.6 Length and weight of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 50
4.6.1 Length and weight as correlated to gender and concentration 50
4.6.2 Length and weight as correlated to stage of maturity 52

4.7 ELISA with zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 54
4.7.1 Vitellogenin synthesis in female zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol 54
4.7.2 Vitellogenin synthesis in male zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol 55

4.8 Acute fish embryo toxicity assay with zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 56
4.8.1 Fish embryo assay 24 hours post-fertilization 56
4.8.2 Fish embryo assay 48 hours post-fertilization 57
4.8.3 Fish embryo assay 72 hours post-fertilization 58
4.8.4 Fish embryo assay 96 hours post-fertilization 61
4.9 Pigmentation of zebrafish (Danio rerio) eyes exposed to 4-tert-pentylphenol 62

5. Discussion prochloraz 66
5.1 Sexual differentiation and vitellogenin synthesis in zebrafish (Danio rerio) within the scope of a FSDT with prochloraz 66
5.2 Maturation and growth of zebrafish (Danio rerio) exposed to prochloraz 68
5.3 Histopathology of zebrafish (Danio rerio) exposed to prochloraz 70
5.4 Toxic effects of prochloraz 71

6. Discussion 4-*tert*-pentylphenol 72
6.1 Sex ratio of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 72
6.2 Maturation and growth of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol ... 73
6.3 Vitellogenin synthesis in zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 75
6.4 Histopathology of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol 76
6.5 Toxic effects of 4-tert-pentylphenol 76
6.6 Effects of 4-tert-pentylphenol on pigmentation in zebrafish (Danio rerio) embryos 77

7. Correlation between maturation and growth in zebrafish (*Danio rerio*) 79

8. Evaluation of the FSDT 80

9. Summary 83

10. References 84

1. Introduction

1.1 Endocrine disrupting chemicals (EDCs)

Industrial progress developed a variety of inventions, which may be comfortable and useful, however, the disadvantages cannot be ignored. As seen in the past, chemical pollution of land, air and water can cause very complex damage at all levels of organization from subcellular systems to ecosystems, and in every species, including humans. Since species under concern mostly show no obvious signs of impairment or mortality, effects of these so-called endocrine disrupting chemicals (EDCs) may not be striking at first sight. However, research on EDCs has a comparably short history and even today the impact on the environment is probably underestimated.

The first postulation of the technical term "endocrine disruptor" was made in 1992 after a meeting concerning wildlife populations in the North American Great Lakes (Colborn & Clement 1993). As a consequence, the issue was picked up especially the problem of estrogens in the environment. Disturbance of hormonal balance between estrogens and androgens is a central target of EDCs. Especially during sexual differentiation the consequences of such a misbalance can be severe and lead to effects at the population level. The estimated number of EDCs is in the range of approximately 70,000 substances (Zacharewski 1998), which mainly interact with the gonadal and the thyroidal axes. They affect the endocrine system of species in very different manners:

1. direct interaction with the estrogen receptor (PCB, DDT, alkylphenols, pesticides)
2. impact as an receptor agonist, blockage of hormone binding and function (tamoxifen, DDE, linuron)
3. binding to plasma proteins (alkylphenols, bisphenol A)
4. direct impact on hormonal metabolism (TCDD, fadrozol, coplanar PCBs)

The effects on the gonadal axis caused by these mechanisms depend on the gender and the type of species (most of the following examples apply to zebrafish):

1. estrogenic effects: compound mimics estradiol and acts like it (e.g. sex ratio drifts to females, males produce vitellogenin)
2. androgenic effects: compound mimics natural androgens, such as testosterone, and acts like it (e.g. sex ratio drifts to males, females produce less vitellogenin)

3. anti-estrogenic effects: compound impairs the normal function of estradiol and therefore inhibits its reactions (e.g. sex ratio drifts to males, females produce less vitellogenin)
4. anti-androgenic effects: compound interferes with the normal function of androgens and therefore their reactions (e.g. sex ratio drifts to females, males produce vitellogenin)
5. aromatase inhibitors: special form of anti-estrogenic effects, compound inhibits the transformation of testosterone to estradiol (e.g. sex ratio drifts to males, females produce less vitellogenin)

Negative effects of EDCs can be found in different classes of vertebrates, including humans. Populations of alligators in Lake Apopka, e.g., suffered from decline in the 1990`s because of exposure to pesticides. A reduction of penis size and plasma testosterone concentrations was due to accumulation of DDT products stored in their fats (Guillette 1999). Wild populations of white-tailed sea eagle (*Haliaeetus albicilla*) at the Baltic Sea in Sweden showed reduced reproductivity caused by organochlorine compounds in their eggs (Helander 2002). Estrogene-like substances in human food and drinking water have been correlated with reduced numbers and elevated motility of spermatozoa in men (Toppari 1996). The impact on fish will be described in the following chapters.

1.2 Test substances

1.2.1 Prochloraz

In this study, prochloraz was used as an example for aromatase-inhibiting substances. Prochloraz is a fungicide belonging to the group of imidazoles (Cravedi 2001). It inhibits ergosterol, which is part of the cell membrane in many fungi (Johnston 1996). Imidazole derivatives are widely used as antifungal agents in agriculture. Through this application, they can enter the aquatic environment and accumulate in aquatic organisms. In fish, it has been shown that several imidazole derivatives are able to modulate the CYP1A system, provoking both inhibitory and inducing effects on CYP1A expression (Sturm 2001). *In vivo*, it reduced the weights of androgen-sensitive tissues in the Hershberger rat assay (Vinggaard 2002). Interaction with cytochrome P450 inhibits the enzyme aromatase and therefore the synthesis of steroids (Hinfray 2006) in different species (Ankley 2005, Blystone 2007, Kinnberg 2007, Vinggaard 2005).

In addition to its inhibiting effects, prochloraz may also act as an androgen receptor antagonist (Laier 2006, Vinggaard 2006 & 2002). 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 2003) or increased incidence of cryptorchidism (Weidner 1998).

1.2.2 4-*tert*-pentylphenol

4-*tert*-pentylphenol belongs to the group of alkylphenols, which are widely spread in industrial use, e.g. as washing substance or for the separation of petroleum and water in offshore oil industries (Taylor 1997). Marine ecosystems are endangered by large disposals of alkylphenols in industrially produced water. In households, they occur in laundry detergents, strong surface cleaners and hair-care products. Alkylphenols are toxic for several species (Choi 2004) and have estrogenic activity (Nimrod & Benson 1996, Routledge & Sumpter 1997). Affinity studies with estrogen receptor-rich tissue homogenates (usually the uterus of rodents) have shown that some alkylphenols, especially the short-chained, can bind to the estrogen receptor, and thus displace natural estrogens (Mueller & Kim 1978). The estrogenicity of alkylphenols depends on position (*para* > *meta* > *ortho*) and branching (tertiary > secondary = primary) of the alkyl chain. The highest estrogenic activity is found in C6–C8 *para* substituted tertiary alkylphenols (1,000 to 6,000-fold less potent than estradiol), but also C5, C4 and C3 phenols are estrogenic (100,000 to 20,000,000-fold less potent than estradiol; Routledge & Sumpter 1997).

Many *in vivo* studies using fish have confirmed that these compounds cause physiological and developmental effects related to their estrogenic properties. Estrogens and pseudo-estrogens including alkylphenols are known to induce the synthesis of vitellogenin in the liver of male and immature female oviparous vertebrates (Pelissero 1991; Jobling 1996; Nimrod & Benson, 1996; Gimeno 1998). The exposure of sexually mature male rainbow trout (*Oncorhynchus mykiss*) to alkylphenols showed a dose-dependent elevation of plasma vitellogenin levels, accompanied by a reduction in testicular weight (Jobling 1996). Genetically male carp (*Cyprinus carpio*) treated with 4-*tert*-pentylphenol developed an oviduct in the gonad (Gimeno et al., 1997). Furthermore, these chemicals have produced altered sexual differentiation in male medaka (*Oryzias latipes*) as shown by macroscopic and gonadal feminization (Gray & Metcalfe 1997; Nimrod & Benson 1996; Gray 1999; Yokota 2005; Seki 2003). Other laboratory studies have shown that reproductive abnormalities such as varying

degrees of feminization of the testes can be induced in male fish exposed to various pseudo-estrogens (Gimeno 1996, 1998; Gray & Metcalfe 1997). These studies have in common that the exposure period included the early development of the gonad, a period known to be sensitive to hormonal disturbance (Yamamoto 1962).

1.3 The Zebrafish (Danio rerio)

1.3.1 Development and reproduction

Knowledge about the normal gonadal development of zebrafish is essential for the detection of any abnormal development caused by EDCs. Zebrafish are protogynic, which means, that all fish first develop ovaries, from which approximately 50% will be transformed to testis later. The following table (Tab. 1.3.1) is based on fundamental studies (Takahashi 1977, Maack 2003, Andersen 2000, Eaton & Farley 1974) that described the gonadal development of *Danio rerio* under laboratory conditions in detail. It has to be considered that some variations between zebrafish of different strains may occur. Rearing conditions, which should normally be standardized, can differ and therefore cause differences in the development of *Danio rerio*. Other variations might be due to incest or mutations. The use of a staging system referring to the physiological instead of the nominal age of the fish should facilitate a more reliable comparison between results of different laboratories.

Tab. 1.3.1: Stages of gonadal differentiation postulated in different studies; dph = days post hatch, wpf = weeks post fertilization, dpf = days post fertilization

Stage of gonadal differentiation	**Data from different studies**
Undifferentiated gonad	0-10 dph (Takahashi 1977) 0-4 wpf (Maack 2003)
Differentiation to ovary	10-12 dph (Takahashi 1977) 4 wpf (Maack 2003)
Transformation to testes (juvenile hermaphroditism)	23-25 dph (Takahashi 1977) 7 wpf (Maack 2003)
Completion of sex reversal	40 dph (Takahashi 1977)
Final maturation of gonads	60 dpf (Andersen 2000)
First spawning	75 dpf (Eaton&Farley 1974)

1.3.2 Suitability of zebrafish (*Danio rerio*) for the evaluation of EDCs

During the last years, much research has been focused on the investigation of EDCs. Many fish (e.g. japanese medaka, fathead minnow, roach, rainbow trout) have been used as models for detection of endocrine effects (Patyna 1999, Bogers 2006, Liney 2005, Schwaiger 2002). For different reasons, zebrafish have become one of the most successful established models. Its general, major advantages are easy and cheap maintenance, the relatively short reproduction cycle (maturation of zebrafish only takes 10 weeks), non-seasonal breeding, egg-production all over the year, easily observable and fast development of the embryos in fully transparent eggs. Additionally, in comparison to other established vertebrate models, e.g. the mouse, fish have simple advantages: Early developmental processes are less accessible in mammalians because they occur *in utero*. Other established systems such as *Drosophila* sp. and *Caenorhabditis elegans* can serve as powerful model systems for many biological processes and are amenable to large scale screens; however, they cannot be utilized to address the development and function of vertebrate-specific features such as kidney, multi-chambered heart, multi-lineage hematopoiesis, notochord, neural crest cells and hormonal processes and regulation (Lele & Krone 1996).

The zebrafish has some striking features that make it a popular model for endocrinology. The key to its suitability is its sexual development as described in Tab. 1.3.1. As mentioned before, the sexual reversal from ovary to testis, called protogynity, is a fragile process, which can easily be disturbed by hormone-like substances. High estrogen levels induce the differentiation of ovaries, whereas androgens lead to the differentiation of testis (Yamamoto 1962; Piferrer 2005; Nagahama 1993). This has been documented in several studies concerning the detection of EDCs (Andersen 2003; Van den Belt 2001; Orn 2003, Knörr & Braunbeck 2002; Fenske 2004). These studies have also shown that it is important to expose zebrafish to potential EDCs in the critical window of gonadal development, i.e. the transition stage (Maack 2003). The critical window is of particular relevance for understanding the effects of EDCs on fish, since even short-term exposure during this life stage may have lasting, irreversible effects on phenotypic sex and reproductive functions. Disturbance of the endogenous androgen/estrogen ratio during the critical period, for instance by exposing the fish to exogenous steroids, can therefore lead to altered phenotypic sex. In practice, this knowledge has been applied in aquaculture where sex steroid treatment during critical developmental stages is routinely used to produce monosex populations of fish.

1.4 Vitellogenin

1.4.1 Endocrine regulation in fish

Endocrine regulation in fish is based on complex interactions between hypothalamus, pituitary, gonads and liver. Substances with hormone-like characteristics included in this regulatory framework can influence one component of this network and thus disturb it (Arukwe 2001). Other external stimuli like temperature, feeding, social factors and photoperiod may also influence the endocrine network by stimulation of the hypothalamus (Hoar 1979). In turn, the hypothalamus is regulated by the central nervous system and controls the synthesis of hormones and chemical messengers. These messengers induce the synthesis of pituitary hormones, including adrenocorticotropin, prolactipatinon, growth hormone, thyroid-stimulating hormone and gonadotropins (GtH). GtH play an essential role in reproductive processes of fish. Two forms of GtH have been isolated from fish (Swanson 1991): GtH I, which is analogous to mammalian follicle-stimulating hormone (FSH) and GtH II, which is analogous to the mammalian luteinizing hormone (LH). GtH I is mainly responsible for gametogenesis and steroidogenesis, whereas GtH II is involved in the final maturation stages of gametogenesis. The major consequence of stimulation through GtH is the synthesis of steroids (i.e. androgens, estrogens and progestins) in target tissues like the gonads. Steroids are the main messengers to regulate gametogenesis, reproduction, sexual phenotype and behavior. During early oocyte development, GtH I binds to receptors on the thecal and granulosa cell layer of the follicle. The thecal cells synthesize testosterone and allow aromatization to result in the formation of estradiol in the granulosa layer prior to plasma secretion (Cyr & Eales 1992). The major target of estradiol is the liver, were it triggers the production of vitellogenin, which is taken up by oocytes under control of GtH 1 (Tyler 1991) for yolk production.

1.4.2 Vitellogenin as a biomarker for EDCs

Vitellogenin is an estrogen-dependent precursor of yolk proteins in oviparous vertebrates. Yolk consists of lipovittelin and phosvitin, which result from cleavage of vitellogenin. Due to its function, vitellogenin is normally found in females, but in case of exposure to estrogenic substances it can also be synthesized by males (Copeland 1986, Tyler 1999). Thus, measurement of vitellogenin concentrations in male fish is an established biomarker to detect EDCs (Tyler 1999). It can be used *in vitro* (e.g. hepatocyte culture) and *in vivo*. A very useful feature of vitellogenin induction is the variability of the changes in the concentrations of

plasma vitellogenin in response to estrogen exposure (up to a thousand fold, from ng/ml to µg/ml concentrations; Tyler 1999).

1.5 Pigmentation in zebrafish (Danio rerio)

1.5.1 Development of pigmentation in zebrafish (*Danio rerio*)

The investigation of pigment development during embryogenesis has a long history in zebrafish research. Investigations on this issue are of medical relevance, because pigment abnormalities correlate with different disorders of neural crest origin (Nakamura 1987) and some common forms of cancer. Pigmentation of zebrafish derives from two different sources: the pigment cells of the dermis and epidermis derive from the neural crest and the pigment cells of the retina originate from the optic cup (Lister 2002). The zebrafish neural crest is induced during gastrulation at the edge of the neural plate, at the border between neural and non-neural ectoderm. It produces 3 different types of pigment cells (Kelsh 1996), melanophores, xanthophores and iridophores. Melanophores are analogous to melanocytes in higher vertebrates and produce dark melanin. Xanthophores appear yellow because of pteridine pigments. The silver reflecting parts of the zebrafish are based on platelets of guanin and other purines produced by the iridophores. Melanophores appear from 24 h post-fertilization laterally on each side of the ears. Subsequently, xanthophores appear dorsally on the head. Finally, iridophores appear in the tail. The basic pigment pattern is completed by 48 h post-fertilization. During the following larval development of the zebrafish, the 3 pigment cell types begin to spread all over the body. This biological attribute is easy to observe and, therefore, one of the most interesting features of zebrafish to understand cell fate and migration.

The retinal pigment cells located in the retinal pigment epithelium (RPE) originate from the optic cup. The optic cup consists of 2 layers; the neural retina comes from the lateral one and the RPE from the medial one (Li 2000). Pigment synthesis is dependent on the enzyme tyrosinase (Camp & Lardelli 2001), which catalyses the oxidation of phenols. First pigmentation of the eyes is visible after 24 hours post-fertilization. The RPE has maintenance function for the neural retina, through the phagocytosis of shed outer segments of photoreceptors and transport of retinoids. It also plays a structural role by forming the blood-retina barrier (Zhao & Overbeek 1999). The main function seems to be the protection of the neural retina against light-induced damage (Neuhauss 1999).

1.5.2 Use of transparent zebrafish (*Danio rerio*) in science

The natural transparency of zebrafish in early embryonal stages makes it an interesting object to observe cellular mechanisms, development of organs or gene expression. The increase of pigmentation in larvae during their development may disturb some optical techniques, e.g. confocal microscopy or detection of green fluorescent protein (GFP). Inhibition or even arrest of pigmentation can facilitate the scientific use of zebrafish. Different techniques to generate longer-lasting transparency have been developed: albino strains through genomic manipulation (Kelsh 1996), postfixative bleaching with hydrogen peroxide (Inohaya 1995), or inhibition of melanin pigmentation by use of compounds such as hydroquinone (Palumbo 1992) or 1-phenyl-2-thiourea (PTU) (Karlsson 2001). Inhibition of pigmentation by higher concentrations of chemical compounds may cause problems due to high incidence of embryo mortality, reduced hatching frequency and teratogenesis.

1.6 Aims of the present study

1.6.1 Evaluation of the "fish sexual development test" as an OECD guideline

The "Organization for Economic Co-operation and Development" (OECD) is working on the development of test guidelines for the detection of endocrine disrupting chemicals (EDCs). In 1997, the "Endocrine Disruptors Testing and Assessment" (EDTA) was established. Ever since, three "Validation Management Groups" (VMG-mammalian, VMG-ecology and VMG-non-animal) are working on EDCs. The present study was made possible by cooperation between the University of Southern Denmark at Odense and the University of Heidelberg. The aim of the work is to evaluate the "fish sexual development test" FSDT (an extension of the existing OECD test guideline 210, fish early life stage toxicity test) as an OECD guideline. The main idea is to establish an *in vivo* test for EDCs that is relatively easy, cheap and more rapid than full-life cycle tests. The EDTA defined the major endpoints of endocrine disruption in fish as gross morphology, gonad histology and biological markers such as vitellogenin. As test organisms, the zebrafish (*Danio rerio*), the Japanese medaka (*Oryzias latipes*) and the fathead minnow (*Pimephales promelas*) were selected (Rose 2002). An open question is whether the obtained results are sensitive enough and whether they can be correlated with results from other test systems and results of other working groups. The FSDT focuses on the period of sexual development and differentiation in zebrafish, which has been reported to be to be particularly sensitive to EDCs, the transition stage (Koger 2000; VanAerle 2002; Andersen 2000; Maack 2003). A major motivation for the FSDT is to prove

that the comparably short time of exposure (60 days post-fertilization) to a potential EDC is sufficient to assess its endocrine activity (compared to a full life-cycle test).

1.6.2 Establishment of a simple method to quantify pigmentation in zebrafish (*Danio rerio*)

Pigmentation is an endpoint not frequently used in fish egg assays. No technical method has yet been established to quantify the pigmentation of zebrafish embryos. The use of numerical systems, based on optical inspection, might be sufficient in some cases, but a more objective method should help to improve the quality of results. This is even more important when the only effect of a chemical substance in studies, including the early life stage, is the disturbance of pigmentation. Basic research on development of pigmentation could also be specified. Other testing strategies might profit from a new method, as well: the permeability of the eggs` chorion can be visualized by staining toxic compounds. Exact measurement of color gradients could facilitate statistical analysis of otherwise subjective observations. Important investigations are made on painting compounds in chemical industry, these studies could also use a technical method to measure color intensity. All these domains of research have in common that the techniques used have to be straightforward, rapid, cost- and labor-effective as well as reproducible and specific.

2. Materials and methods

2.1 The Zebrafish (Danio rerio)

2.1.1 General description

The zebrafish (*Danio rerio*, Hamilton-Buchanan 1822; Fig. 2.1.1) is a small benthopelagic freshwater representative of the family of cyprinids. This species originates from the Ganges River system, Burma and Sumatra (Eaton & Farley 1974). The ideal temperature for zebrafish maintenance usually averages around 26 °C. Within this range, it grows quickly and reaches maturity within three months. Zebrafish are easily obtainable, inexpensive and readily maintainable. The fish has an elongated, slightly compressed shape and reaches a mean adult length between 3 and 5 cm. Both male and female fish are brownish-olive colored with a yellow-white waist and five uniform pigmented, horizontal lateral lines, all extending onto the end of caudal fin rays. Particularly during spawning maturity, females can be distinguished easily from the males by their swollen abdominals. Male fish are more slender and show an orange to reddish tint in the silvery bands along the body. One female produces 50-200 eggs per day and only needs a few days for regeneration. Thus, under appropriate conditions, a large number of non-adherent, fully transparent eggs can be obtained all-year-round (Laale 1977). The embryonic development is short and respectively has been described in detail (Kimmel 1995, Westerfield 2000). It can be observed easily due to the transparency of the eggs. Thus, zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology.

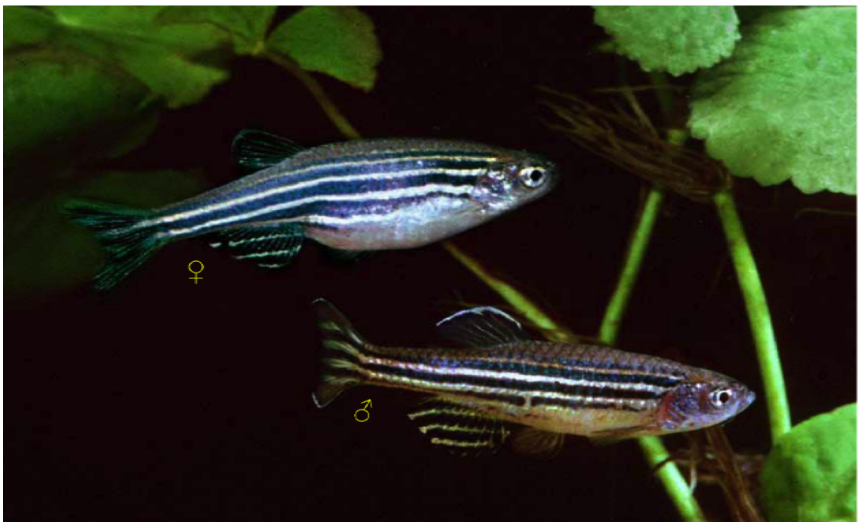


Fig. 2.1.1: Adult zebrafish - upper individual: female; lower individual: male

2.1.2 Maintenance

The Department of Zoology, University of Heidelberg, rears several stocks of zebrafish for regular egg production. Each group consists of 7 females and 7 males. These are kept in 13 L tanks under flow-through conditions at 26 ± 1 °C and continuous aeration. The room is light-isolated, and an artificial dark-light cycle of 10/14 hours is maintained. The animals are fed once a day with dry flake food (TetraMin™, Tetra-Werke, Melle) and additionally fed with fresh larvae from *Artemia* spec. (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Ogden, USA) the day before spawning.

2.1.3 Spawning

For egg production, the fish (7 females, 7 males) were transferred into a special spawning facility (Fig. 2.1.3). The constant temperature of 26°C was guaranteed by a heating rod (3602, Aquarium heater, Eheim); oxygen supply was regulated *via* external aeration by pressured air. The basins were coated with black silicone except for the front to avoid interference with adjacent aquaria. The top was covered with a grid to prevent fishes from jumping out of the tank. The base of the aquaria was made of grids with a mesh size of 1.25 mm, to allow the passage of eggs into a separate spawning tray. This was necessary to avoid the predation of eggs by the adults. The ground of the spawning trays was coated black to facilitate identification of the eggs. A green plant dummy made of plastic was used for spawning stimulation. Courtship and spawning took place in the “artificial morning” period after the onset of light. After retransferring the adult zebrafish into the maintenance tanks, the eggs could be collected from the spawning trays. For relocation of the eggs, these were transferred into petri dishes and kept, until usage, in a heating cabinet with circulating air at 27 ± 1 °C. Each spawning-group is usually able to produce 200-400 eggs. The females need at least 4 days of regeneration to spawn again.

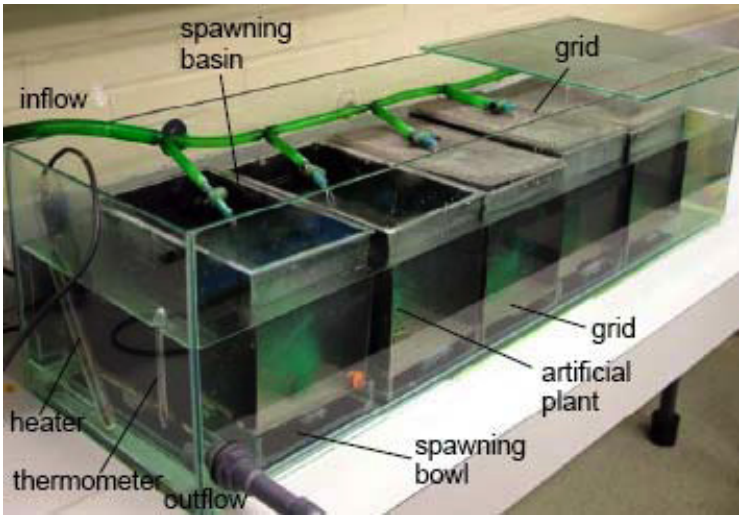


Fig. 2.1.3: Spawning facility.

2.2 Test substances

2.2.1 Prochloraz

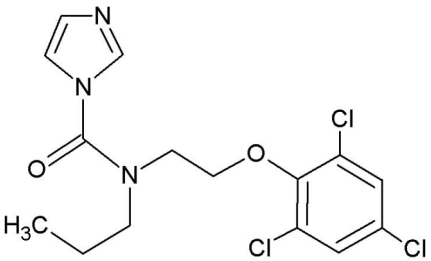


Fig. 2.2.1: Molecular structure of prochloraz.

CAS-No.: 67747-09-5
Product number: 45631
Lot no.: 2226X
Supplier: Sigma Aldrich, US
Molecular formula: $C_{15}H_{16}Cl_3N_3O_2$
Molecular weight: 376.6693
Purity: 99.5 %

The test substance was obtained from Sigma-Aldrich Company, Saint Louis, USA. It was stored at room temperature. A stock solution of 15 mg/L prochloraz in Aqua bidest was produced and stirred at 50 °C over night. The solution was produced every third day and then distributed to the different flasks in the test setup, where it was diluted with double-distilled water to the final exposure concentrations.

2.2.2 4-*tert*-Pentylphenol

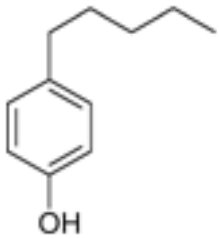


Fig. 2.2.2: Molecular structure of 4-*tert*-pentylphenol.

Product name: 4-*tert*-amylphenol
CAS-No.: 80-46-6
Lot no.: 13625LB
Product number: 15,384-2
Molecular formula: $C_{11}H_{16}O$
Supplier: Sigma Aldrich, US
Molecular weight: 164.2
Purity: 99.9 %

The test substance was obtained from Sigma-Aldrich, Saint Louis, USA, and shipped by a central depository. It was stored at room temperature. Two stock solutions were produced every second day and then distributed to the different flasks in the test setup, where they were diluted with double-distilled water to the final exposure concentrations. For the production of stock solution no.1 with a concentration of 16 mg/L, 80 mg of the test substance were dissolved in 5 L Aqua bidest. For the 8.475 mg/L concentration (stock solution no.2), 42.375 mg were dissolved in 5 L Aqua bidest. To dissolve the test substance, the stock solutions were heated up at 50 °C and stirred over night.

2.3 The "Fish Sexual Development Test" (FSDT)

2.3.1 Test design FSDT

In the fish sexual development test, zebrafish (*Danio rerio*) are exposed to potential endocrine disrupting chemicals (EDCs) over 60 days post-hatch. Exposure starts in the first 3-4 hours of embryonic development just after fertilization of eggs. Eggs were removed from the spawning tanks and immediately exposed to the different concentrations of 4-*tert*-pentylphenol and prochloraz as described in chapter 2.1.3. 100 fertilized eggs per concentration were stored in 250 ml basins in a heating cabinet with circulating air at 27°C for 4 days. Afterwards, the hatched larvae were transferred to 10 L tanks with a flow-through system. 4 - 30 days post-hatch, the larvae were fed with powdered dry food (Staubfutter, Sera Micron, Heinsberg,

FRG). 30-60 days post-hatch, they were fed with granular food (Flockenfutter TetraMin™, Tetra-Werke, Melle) as well as larvae of *Artemia* spec. After 30 days of exposure, the number of fish per tank was reduced to 40 individuals. The rate of mortality could easily be determined at this occasion by counting the remaining fish. To ensure good water quality, regular water-analyses were carried out. In order to guarantee constant exposure conditions, water temperature and the flow-through rates were controlled daily. Feces and leftovers of food were removed from the tanks every day. After 60 days post-hatch, fish were anaesthetized with a saturated solution of benzocain (ethyl 4-aminobenzoate, Sigma Aldrich, Saint Louis, USA). Each fish was numbered, measured and weighed. Head and tail were cut off with a razor blade and immediately frozen in liquid nitrogen for subsequent measurement of vitellogenin at Odense. Each trunk was put into 5 ml of Davidson`s fixative for at least 1 week and then prepared for the histological examination.

2.3.2 Test setup FSDT

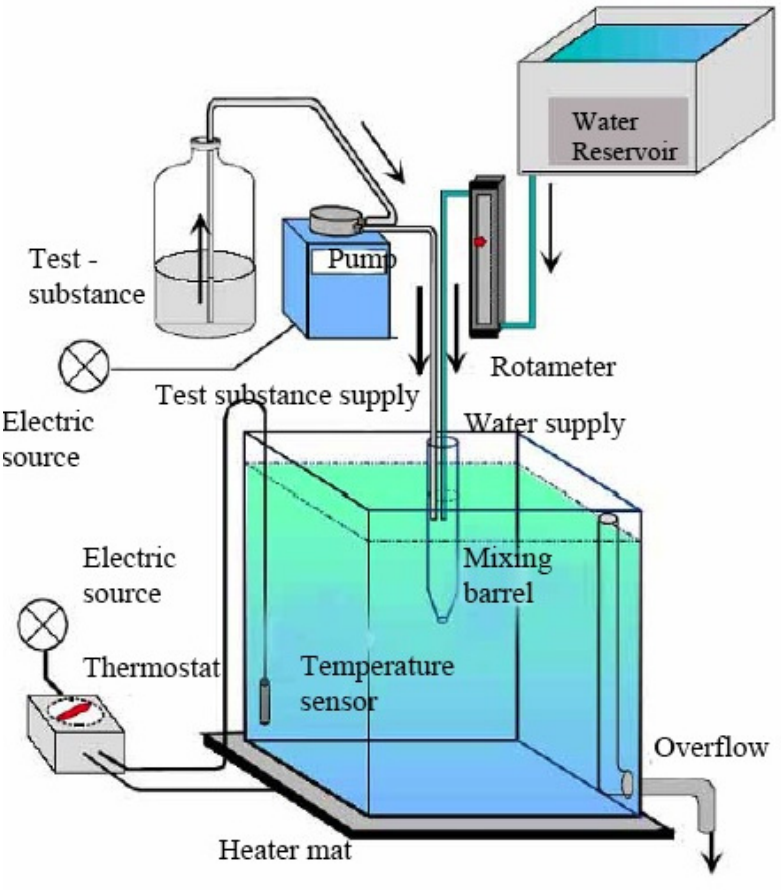


Fig. 2.3.2: Test setup of the FSDT (Source: S. Knoerr)

The fish were kept in 12L tanks (40 cm x 40 cm x 20 cm, filled up to 15 cm). To ensure a continuous exposure to the test substances, the fish were kept in a flow-through system. Well-defined volumes of water and test substance were supplied to the tanks by a mixing barrel. To avoid the accumulation of test substance in certain areas of the tanks, these were continuously aerated and thus mixed with pressured air, which also guaranteed a saturated level of oxygen. An overflow-system caused an exchange of water 3 times a day. Heater mats controlled by a thermostat ensured the constant water temperature of 27 °C. The room was light-isolated and an artificial photoperiod of 10/14h dark/light was maintained.
5 different concentrations of prochloraz were supplied in 2 replicates (37.5 75, 150, 300 and 600 µg/L) and the water control in 4 replicates. Approximately 40 fish per tank were kept, which made a total of 607 zebrafish.
4-*tert*-pentylphenol was supplied in 4 different concentrations. 32, 100 and 320 µg/L and the water control were run in 4 replicates. 75 µg/L was run in 2 replicates. Approximately 40 fish per tank were kept, which made a total of 717 zebrafish.

2.4 Histology

2.4.1 Fixation

After the 60 days of exposure, the trunks of the fish were incubated in 5 ml of Davidson`s fixative for at least 1 week. Fixation with Davidson`s conserves the tissue and maintains their structure. Additionally, it decalcifies the tissue, which simplifies subsequent sectioning.

Composition of Davidson`s fixative:
220 ml formaldehyde (37 – 40 %)
115 ml glacial acetic acid
330 ml ethanol (95 %)
335 ml H_2O dest.

2.4.2 Dehydration and embedding

Samples were embedded in Histoplast S (Serva, Heidelberg) for the histological examination of the gonads. Since paraffin is a very hydrophobic embedding material, tissues had to be dehydrated in a graded series of ethanol. The gradually increasing concentrations of ethanol slowly remove the water from the tissues without destroying or shrinking it.

Process of dehydration:

1. 3x 20 min 70 % ethanol
2. 3x 20 min 80 % ethanol
3. 3x 20 min 90 % ethanol
4. 3x 20 min 96 % ethanol
5. 2x 30 min 100 % isopropanol

The next intermedium, methylbenzoate, serves to facilitate paraffin intrusion into the tissues. It also hardens the tissues, which eases sectioning. As methylbenzoate has a higher density than isopropanol, the samples float at the surface first. The sinking of the sample indicates the complete soaking of methylbenzoate in the first step.

Process:

1. Methylbenzoate I at least 1h
2. Methylbenzoate II over night
3. Methylbenzoate III 3h

To produce paraffin blocks with homogenous hardness, the tissues were soaked completely with Histoplast over night in a heating cabinet at 60°C. The next day, the Histoplast was changed, and the samples were again incubated over night. Embedding was performed in an automat (Leica EG 1140 H, Leica, Tempe, USA). The carcasses of the fish were orientated ventrally to the cutting surface. Since the animals were relative small, it was possible to embed 2 fish per block. The blocks were cooled down on a cooling plate type EG1140C (Leica) and then stored at room temperature.

2.4.3 Sectioning and staining

4-5 µm thick sections of the gonads were prepared by using a microtome (HN 40-Schlittenmikrotom, Reichert-Jung, Heidelberg, FRG). Sections were cut at 12 µm intervals. The slices were transferred to a water bath at a temperature of 40 °C using a fine brush. They were stretched and transferred to a glass slide coated with a protein-glycerin (Serva, Heidelberg, FRG) solution to improve adhesion. Slides were dried at 38 °C over night. As the following staining solutions are water-based, the Histoplast needed to be removed with orange terpene (Roth, Karlsruhe, FRG). After that, a descending ethanol series rehydrated the tissues. The hematoxylin erythrosine staining (HE-staining, Romeis 1989) was chosen to visualize the different tissues of the gonads according to the OECD protocol.

Mayer's acid hemalaun:

1 g/L hematoxylin

0.2 g/L sodium iodate

50 g/L potash alum

50 g/L chloral hydrate

1 g/L citric acid

Process:

1. 3x 10 min orange terpene
2. 2x 5 min 100 % isopropanol
3. 3 min each, descending ethanol series: 96 %, 90 %, 80 %, 70 % ethanol
4. 1 min Aqua dest.
5. 15 min Mayer's acid hemalaun
6. 10 min blueing under flowing tap water
7. 1 min Aqua dest.
8. 5 min 0.1 % erythrosine
9. 1 min Aqua dest.
10. 1 min each ascending ethanol series: 70 %, 80 % 90 %, 96 % ethanol
11. 2x 5 min 100 % isopropanol
12. 3x 10 min orange terpene

Finally, the slides were coverslipped in DePeX (Serva, Heidelberg, FRG) and dried for at least one day at room temperature. For investigation by microscopy, surplus DePeX was removed with a razor blade, and the slides were cleaned with alcohol.

2.4.4 Analysis of histology

The sections were examined with a Leitz Aristoplan Microscope (Leitz, Wetzlar, FRG) and photographed with an associated digital camera (ColorView, Soft Imaging Systems, Münster, FRG).

The following parameters were analyzed:

a) **Gender of the fish**: 5 types of gonad development were distinguished from each other (female, male, hermaphrodite, testis-ova, undifferentiated). The ratio of these different types was correlated to the concentration of test substance.

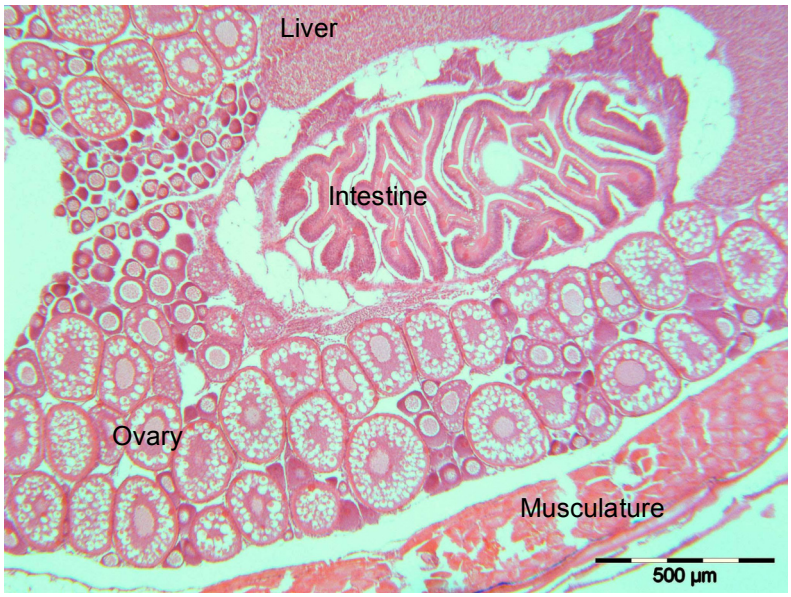


Fig. 2.4.4a: Female gonad of a zebrafish (*Danio rerio*) from a control group, HE-staining.

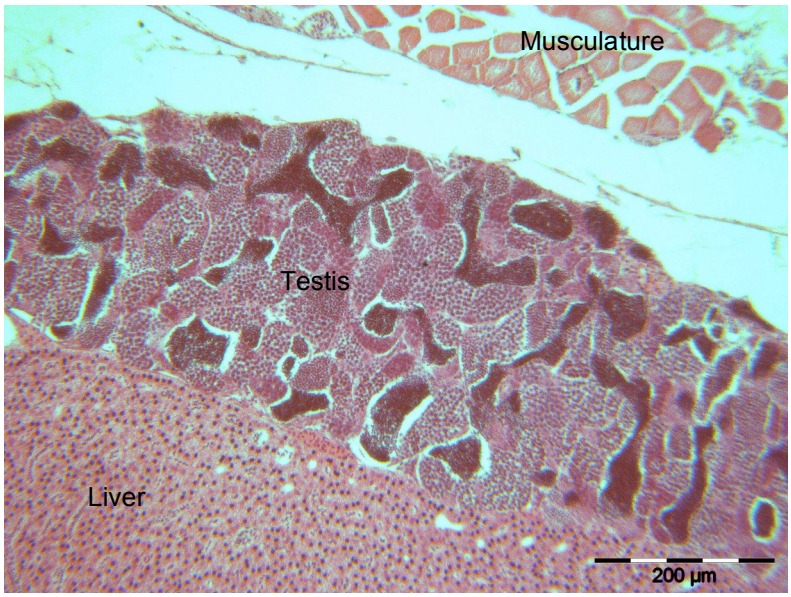


Fig. 2.4.4b: Male gonad of a zebrafish (*Danio rerio*) from a control group, HE-staining.

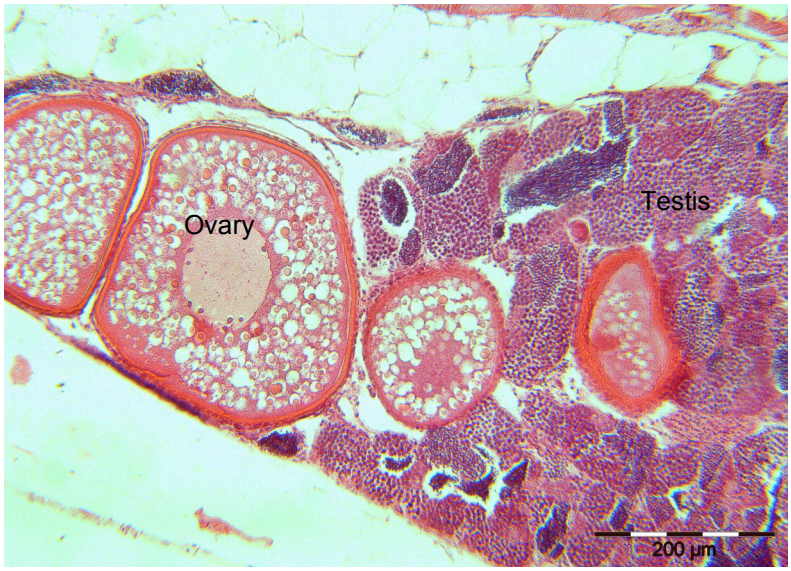


Fig. 2.4.4c: Hermaphroditic zebrafish (*Danio rerio*) exposed to 75 µg/L 4-*tert*-pentylphenol, HE-staining.

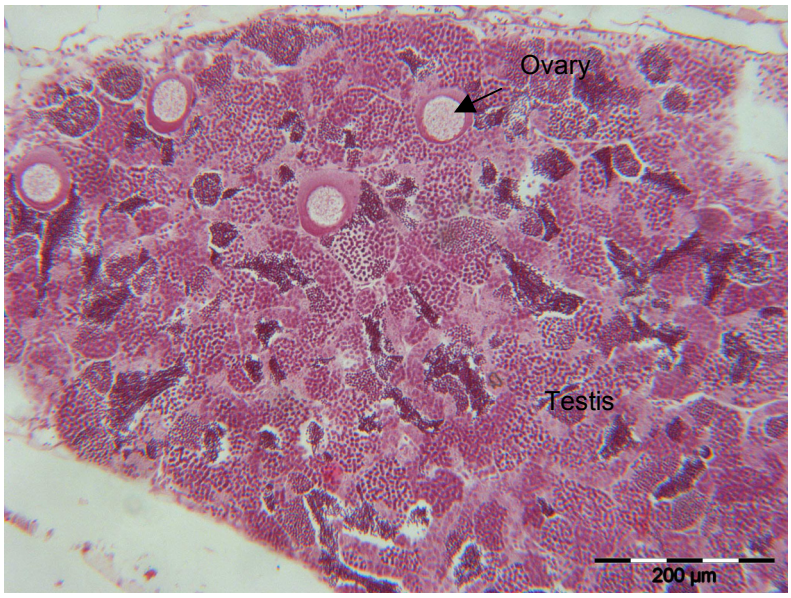


Fig. 2.4.4d: Testis-ova in zebrafish (*Danio rerio*) exposed to 100 µg/L prochloraz, HE-staining.

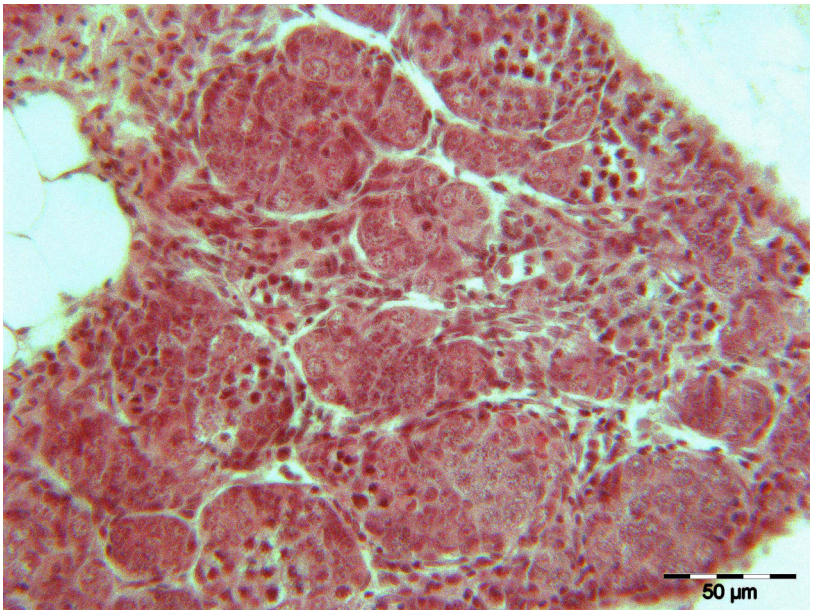
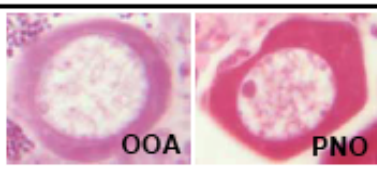
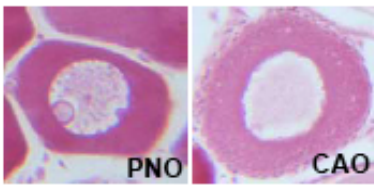
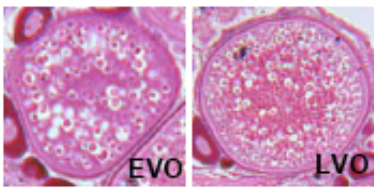
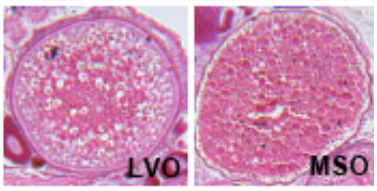
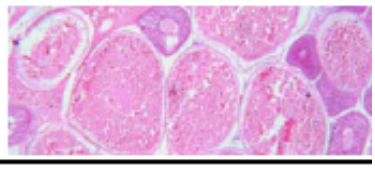


Fig. 2.4.4e: Undifferentiated gonad of a zebrafish (*Danio rerio*) exposed to 320 µg/L 4-*tert*-pentylphenol, HE-staining.

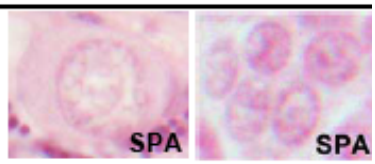
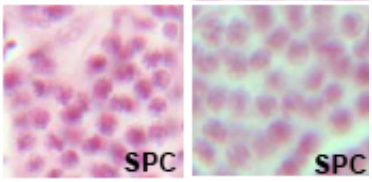
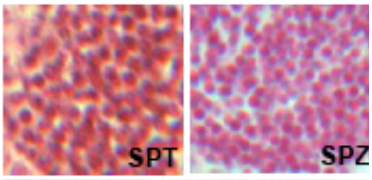
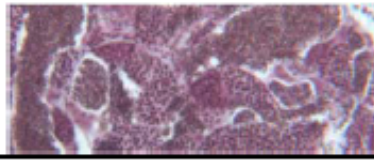
b) **Maturation stages:** the relative proportions of various gametogenic cell types were assessed and categorized into a numerical staging system (ovary: 0 - 4, testis: 0 - 3). The ratios of these stages were correlated to the concentration of test substance.

Tab. 2.4.4a: Categorization of gametogenic cell types (Source: M. Böttcher)

Females

Stage (Stg) 0 entirely immature	Oogonia (OOA) up to perinucleolare oocytes (PNO)	
Stage (Stg) 1 early vitellogenic phase	Main part of the germ cells are pre-vitellogenic follicles; mostly perinucleolare oocytes (PNO) and alveolare oocytes (CAO)	
Stage (Stg) 2 middle vitellogenic phase	At least half of the follicles are early (EVO) to late vitellogenic (LVO)	
Stage (Stg) 3 late vitellogenic phase	The majority of follicles in the ovary are late vitellogenic (LVO) and mature/spawning follicles (MSO).	
Stage (Stg) 4 late vitellogenic/ hydrated	The majority of follicles are late vitellogenic (LVO) and mature/spawning follicles (MSO). Follicles are larger compared to Stg 3.	

Males

Stage (Stg) 0 entirely immature	Spermatogonia (SPA), spermatocytes (SPC) and spermatids (SPT)	
Stage (Stg) 1 early spermatogenic phase	immature phase is dominating; spermatozoa are present	
Stage (Stg) 2 middle spermatogenic phase	Spermatocytes (SPC), spermatids (SPT) and spermatozoa (SPZ) are present in roughly equal portion	
Stage (Stg) 3 late spermatogenic phase	All maturation grades are present, though the spermatozoa are dominating	

c) **Maturity index:** An amplification of the regular staging system from the OECD guideline was developed. Each stage of maturity was accorded to a fixed value, which is higher the more mature the fish is (Stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc.). The values of each replicate were summed up, divided by the number of female respectively male fish and the mean value of each exposure group was calculated. This method enables to summarize the stage of maturity of all fish from one exposure group.

d) **Histopathological effects:** Histopathological alterations were compared to control fish according to the following list (Tab. 2.4.4b; OECD guideline). All effects were categorized in different grades of severity (1-minimal, 2-mild, 3-moderate, 4-severe).

Tab. 2.4.4b: Diagnosis codes for histopathological effects (OECD guideline)

Diagnosis codes, testis		**Diagnosis codes, ovary**	
M01	Increased cells (+ cell type code)	**F01**	Increased cells (+ cell type code)
M02	Decreased cells (+ cell type code)	**F02**	Decreased cells (+ cell type code)
M03	Testis-ova	**F03**	Ovarian spermatogenesis
M04	Hermaphroditism, male	**F04**	Hermaphroditism, female
M05	Testicular degeneration	**F05**	Oocyte atresia, increased, immature
M06	Async. development, spermatocyst	**F06**	Oocyte atresia, increased, mature
M07	Async. development, gonad	**F07**	Async. development, gonad
M08	Async. development, left & right gonad	**F08**	Async. development, left & right gonad
M09	Proteinaceous fluid, intravascular	**F09**	Proteinaceous fluid, intravascular
M10	Proteinaceous fluid, interstitial	**F10**	Proteinaceous fluid, interstitial
M11	Interstitial fibrosis	**F11**	Interstitial fibrosis
M12	Sertoli cell hypertrophy	**F12**	Postovulatory follicles increased
M13	Hepatocyte basophilia increased	**F13**	Hepatocyte basophilia increased
M14	Nephropathy	**F14**	Nephropathy
M15	Granulomatous inflammation	**F15**	Granulomatous inflammation
M16	Histolytic cells, intraluminal	**F16**	Macrophage aggregates increased
M17	Retained peritoneal attachments	**F17**	Oocyte membrane folding
M18	Germinal epithelium atrophy	**F18**	Egg debris oviduct
M19	Germinal epithelium hypoplasia	**F19**	Liquid filled follicular epithelium
M20	Stromal tumors	**F20**	Cysts
M21	Sperm necrosis	**F21**	Decreased yolk formation
M22	Germinal epithelium hypoplasia	**F22**	Germ cell neoplasm
M23	Interstitial (Leydig) cell hypertrophy	**F23**	Stromal tumors
		F24	Empty follicle
		F25	Perifollicular cell hyperplasia
		F26	Perifollicular cell hypertrophy
Cell Type Codes			
SPA	Spermatogonia	**PNO**	Perinucleolar oocytes
SPC	Spermatocysts	**CAO**	Cortical alveolar oocytes
SPT	Spermatids	**EVO**	Early vitellogenic oocytes
SPZ	Spermatozoa	**LVO**	Late vitellogenic oocytes
STC	Sertoli cells	**MSO**	Mature/spawning oocytes
ISC	Interstitial cells (Leydig cells)	**PFC**	Perifollicular (Granulosa/Thecal) cells

2.5 Measurement of vitellogenin concentrations via ELISA

The content of vitellogenin in head and tail of zebrafish was analyzed by Dr. Henrik Holbech from the University of Southern Denmark, Odense. To quantify vitellogenin concentrations in zebrafish, a direct non-competitive sandwich ELISA (enzyme-linked immunoabsorbent assay) was performed with a zebrafish-specific anti-lipovitellin polyclonal antiserum.

The frozen samples were transferred into liquid nitrogen and then crushed with a small pistil in a mortar. Ice-cold homogenate buffer (50 mM Tris-HCl pH 7.4, 1 % protease inhibitor cocktail) was added. This homogenate was centrifuged for 60 min. at 50,000 g and 4°C. The supernatant was filtered through glass wool and stored in appropriate portions at -80°C until further analysis. Microtiterplates (certified Maxisorp F96, Nunc, Roskilde Denmark) were coated with 150 µl/well of anti-zebrafish lipovitellin-IgG diluted to 5 µg/ml in coating buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated over night at 4°C on a shaker. Plates were thawed and washed 3 times with PBS (160.0 g NaCl, 4.0 g KH_2PO_4, 26.6 g Na_2HPO_4, $2H_2O$, 4.0 g KCl; adjusted to pH 6.8 and filled with milipore H_2O to 2 L).

The free sites were blocked by adding 250 µl PBS and 3 % BSA. Again, the plates were incubated over night at 4°C on a shaker, and after that, washed 3 times with washing buffer (500 ml PBS-stock, 5 g BSA, 5 ml TWEEN, pH adjusted to 7.3, and filled to 5 L with millipore water). The plates were dried by knocking them into a towel and then stored at -20°C until use. Battelle zebrafish standard AP4.6.04 (1.18 mg/ml) was serially diluted to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml in dilution buffer (100 ml PBS-Stock, 3.0 g BSA, 1.0 ml Tween 20; pH adjusted to 7.3, and filled to 1 L with milipore water) and samples were diluted at least 200 times (to prevent a matrix effect) in dilution buffer and applied to the plates.

An internal standard was applied in duplicate. 150 µl were applied to each well. Standards were run in duplicate and samples in triplicate. The plates were incubated over night at 4 °C on a shaker. The next day, they were washed 5 times with washing buffer. AMDEX (Horseradish peroxidase coupled to a dextran chain (AMDEX A/S, Denmark)) -conjugated antibodies were diluted in washing buffer; the actual dilution differed with batch and age. 150 µl were applied to each well, and the plates were incubated for 1 hour at room temperature on a shaker. The plates were washed 5 times with washing buffer. 150 µl TMB plus (TMB plus is a ”ready-to-use” substrate, KemEnTec (Denmark), a substrate that is sensitive to light and should be stored in the fridge) were applied to each well. The plates were protected against light with tinfoil, and the color developing on a shaker was observed. The enzyme activity was stopped by adding 150 µl 0.2 M H_2SO_4 to each well. The bottom of the plate was

carefully cleaned with ethanol, and the absorbtion was measured at 450 nm on Molecular Devices Thermomax plate reader. Data were analyzed on the associated software (Softmax).

2.6 Acute fish embryo toxicity assay

The acute fish embryo toxicity assay was conducted according to DIN 38415-6. A negative control with artificial water corresponding to ISO 7346/3 and one solvent control with artificial water and 1 % DMSO (dimethyl sulfoxide) were tested. Furthermore, as a positive control, a solution of 3,4-dichloranilin (DCA) with a concentration of 3.7 mg/L was tested. The positive control should ensure overall the test sensitivity.

Before eggs were collected, test solutions were prepared. For the dilutions, artificial water was used instead of tap water to exclude the influence of different water qualities on the embryonic development of *Danio rerio* and to maintain the comparability with other studies. The test water was always produced freshly the day before use and aerated overnight in an Erlenmeyer flask until saturation. By using a stereomicroscope, only fertilized, regularly dividing eggs in the 8-32-cell stage were selected. For each concentration, 24 eggs were directly transferred to separate wells of 24-well-multiplates (TPP; Trasladingen, Switzerland, ISO 9001), which were previously filled with 2 ml of the test solutions, respectively. To transfer the eggs, plastic pipettes with a wide diameter/opening were used to prevent any mechanical damage. The multiplates were then covered with self-adhesive foil (TPP; Renner, Dannstadt, FRG) to inhibit evaporation-caused changes in the concentration. Sufficient oxygen supply was guaranteed by oxygen saturation of the artificial water, a remaining airspace in the well and oxygen-permeability of the covering foil. The eggs were incubated at 27 ± 1 °C and examined for lethal and sublethal endpoints as described in chapter 2.6.2 at 24, 48, 72 (prochloraz) and 96 (4-*tert*-pentylphenol) hours post fertilization, respectively, using an inverted microscope (CKX41, Olympus Hamburg, FRG).

Results were documented with an associated digital camera (Camedia C-5060, Olympus, Hamburg, FRG) and the software Analysis 5.0 (Soft Imaging Systems GmbH, Münster, FRG). A test was classified as valid according to DIN 38415-6, if 90 % of the embryos in the negative control treatments showed neither sublethal nor lethal effects, and the positive control delivered anticipated results, as described below:

After 24 hours, only in individual cases a minor retardation of the development should be found. After the second exposure day, heartbeat and blood circulation of embryos should be visible. Furthermore edema formation, if any, should be detectable. These effects should

enhance during the exposure period and be accompanied by an increase in the number of deformations and the coagulation rate. Each compound was tested in 4 replicates.

Artificial water:

960 ml H_2O bidest.

10 ml calciumchloride-2-hydrate (20 mM)

10 ml magnesium-7-hydrate (5 mM)

10 ml sodium hydrogene carbonat (7.5 mM)

10 ml potassium chloride (0.37 mM)

2.6.1 Exposure design fish embryo assay

Prochloraz			**4-*tert*-pentylphenol**		
Stock solution: 10 mg prochloraz solved in 1 ml DMSO and 99 ml artificial water			***Stock solution:*** 10 mg 4-*tert*-pentylphenol solved in 1 ml DMSO and 99 ml artificial water		
wells	**concentration**	**replicates**	**wells**	**concentration**	**replicates**
24	5 mg/L	4	24	5 mg/L	4
24	4 mg/L	4	24	4 mg/L	4
24	3 mg/L	4	24	3 mg/L	4
24	2 mg/L	4	24	2 mg/L	4
24	1 mg/L	4	24	1 mg/L	4
24	0.5 mg/L	4	24	0.5 mg/L	4
24	1 % DMSO	4	24	0.1 mg/L	4
24	3.7 mg/L DCA	4	24	1 % DMSO	4
24	artificial water	4	24	3.7 mg/L DCA	4
			24	artificial water	4

2.6.2 Analysis of standard parameters

Toxicological endpoints: The embryos were examined at 24, 48, 72 (prochloraz) and 96 (4-*tert*-pentylphenol) hours post fertilization. As endpoints for toxicity, the following endpoints were used according to DIN 38415-6 (DIN 2001).

Lethal effects: According to DIN 38415-6, coagulated eggs, appearing macroscopically opaque by view and dark under the microscope, were recorded at each time point. Underdeveloped embryos arrested in the developmental stage of epiboly missing heartbeat and retarded somite stage.

Sublethal effects: Incomplete tail detachments from the yolk or severe deformations (e.g. edemas) were recorded at any time point. Additionally, at 72 hours, embryos were examined with respect to hatching success. Also the development of pigmentation was observed (see chapter 2.6.3). Attention was paid to blood circulation and heartbeat. Spinal deformations after hatch were also registered. Embryos, which showed any of the aberrations mentioned above, were recorded as sublethal to facilitate the statistics.

2.6.3 Analysis of pigmentation in zebrafish (*Danio rerio*)

Since embryos of *Danio rerio* exposed to 4-*tert*-pentylphenol showed a distinct retardation in pigmentation, a correlation between dose of 4-*tert*-pentylphenol and the degree of pigmentation was drawn at 48, 72 and 96 hours after fertilization. A categorization by a numerical system was not satisfying, so a technical method for measurement of the pigmentation had to be found. The embryos were photographed and were analyzed with Adobe Photoshop, CS2 (Adobe systems inc.) as described below.

First attempts of measuring the degree of pigmentation of the whole body were not successful, because it was impossible to get comparable photos. The positions of the fish in the wells were always different. Thus, zebrafish are mainly transparent with sporadic melanophores on them, overlapping parts of their body may appear darker than they truly are. For this reason, only the eyes were used for investigation. The eyes are easy to extract in "Photoshop" program, and after hatch they are mainly orientated in the same way, so that the results are comparable. Most fish were not hatched after 48 hours, and it was very difficult to take pictures of their eyes without overlapping each other. So the following procedure is supposed to be most successful after 72 and 96 hours.

Procedure:

Embryos of zebrafish were photographed with a digital camera (Camedia C-5060, Olympus, Hamburg, FRG) under an inverted microscope (CKX41, Olympus, Hamburg, FRG) after 72 and 96 hours of exposure to 4-*tert*-pentylphenol. Illumination of the room and the microscope were kept constant. The software Analysis 5.0 (Soft Imaging Systems GmbH, Münster, FRG)

was used to produce pictures of the .jpg format. The subsequent image processing was carried out with Adobe Photoshop CS2 (Adobe Systems Inc.). The pictures were converted to grayscales. Zooming on them and selecting them with the “magnetic lasso” isolated the eyes of the fish. The selected area could now be “homogenized” with the “average”-filter. This was necessary for the subsequent measurement of brightness with the “pipette”-tool, because the eye of the fish does not appear homogenous on the pictures. The “pipette”-tool scans and scales the brightness of one pixel between 0 (white) and 100 (black), and therefore it was important to homogenize the eye to detect its mean brightness.

One source of error is the position of the fish in the well. When the eyes are lying near to the side of the well, they appear darker because the side casts a shadow. Measuring the brightness of the background just next to the eye could compensate for this. The values were related to each other. The brightness of 2 eyes in 24 fish per concentration was scaled between 0 and 100 by this procedure. Data are presented as means calculated by MS Excel (Microsoft Inc.).

3. Results prochloraz

3.1 Water analysis of FSDT with prochloraz

3.1.1 Water parameters of FSDT with prochloraz

During the entire period of exposure, different water quality parameters were measured:

- pH-values: 7.8 - 8.0
- Oxygen concentrations: 5.6 - 6.4 mg/L
- Total hardness: 16 - 18 °dH
- Carbonate hardness: 10.4 - 11.6 °dH

3.1.2 Chemical analysis of FSDT with prochloraz

The chemical analysis of the water used in the FSDT with prochloraz was conducted by Dr. Henrik Holbech. Results are summarized in Tab. 3.1.2. The concentrations in 7 out of 11 tanks were slightly higher than the nominal concentrations. 2 tanks out of 11 were slightly lower than the nominal concentrations. Only two samples (tanks no. 2 and 5) differed by over a factor 2 from the nominal concentration.

Tab. 3.1.2: Chemical analysis of water used in FSDT with prochloraz

Tank no.	Nominal concentration (µg/L prochloraz)	Measured concentration (µg/L prochloraz)
1	37.5	56.19
2	75	171.33
3	150	209.22
4	300	215.41
5	600	1440.02
6	0	2.28
8	37.5	63.24
9	75	98.47
10	150	156.15
11	300	251.43
12	600	892.46

3.2 Mortality of zebrafish (Danio rerio) exposed to prochloraz

After 30 days of exposure, the mortalities of zebrafish (*Danio rerio*) exposed to prochloraz were determined (Fig. 3.2). The number of dead fish varied between replicate tanks; however, the mortality was significantly increased at the 600 µg/L prochloraz exposure groups, where 49.5 % of the fish died. In the control, less than half as many fish (19.8 %) died.

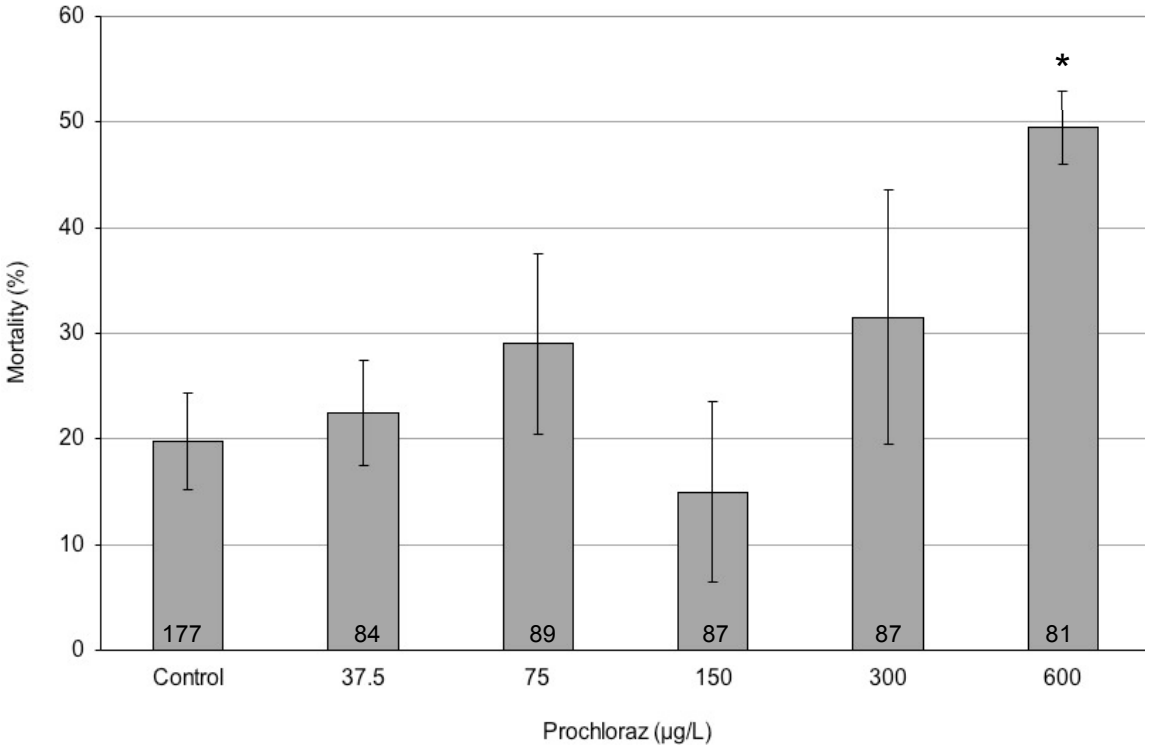


Fig. 3.2: Overall mortality (%) of zebrafish (*Danio rerio*) 30 days post hatch (dph) exposed to different concentrations of prochloraz. Asterisk: Value differs significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Data at the base of the columns indicate the total number of fish in each exposure group.

3.3 Sex ratio of zebrafish (Danio rerio) exposed to prochloraz

60 days post-hatch, the sex ratio of zebrafish (*Danio rerio*) was determined by histological investigation (Fig. 3.3). The gender of the fish was categorized into female, male, undifferentiated or testis-ova. The 4 replicates of the control showed an average sex ratio of females to males of 42.9 % to 55.4 %. In the control, 1.7 % were categorized as testis-ova; this value was exceeded by a factor 10 (16.1 %) at 300 µg/L. The number of male fish increased in a dose dependent manner. At the highest concentration of prochloraz (600 µg/L) 35 times less females were found (1.2 %) and 1.7 times more males (92.6 %). When

compared to the control, a significantly higher number of males could be found at concentrations of 300 and 600 µg/L. The reduction of females was significant in all concentrations of prochloraz. Undifferentiated fish were only found after exposure to 150, 300 and 600 µg/L prochloraz, which, however, was statistically not significant. The percentage of testis-ova increased significantly at concentrations of 150 and 300 µg/L.

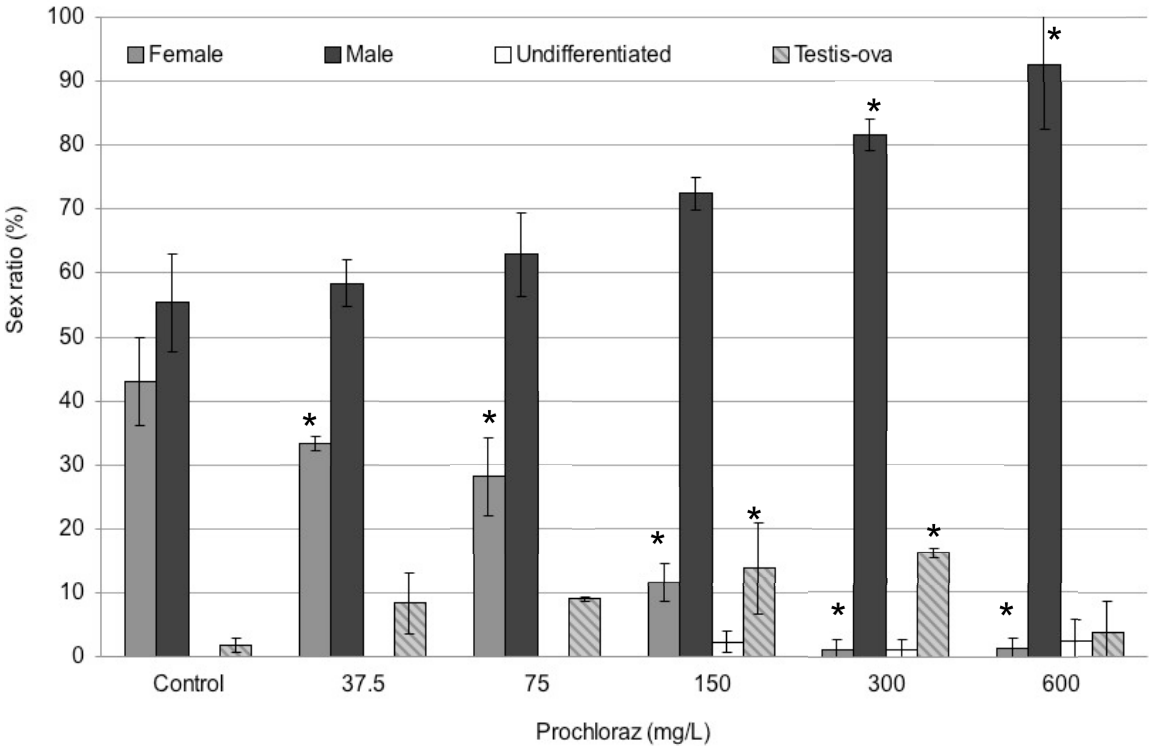


Fig. 3.3: Sex ratio (%) of zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of prochloraz. Asterisks: Values differ significantly from the corresponding values in the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls).

3.4 Staging of zebrafish (Danio rerio) exposed to prochloraz

3.4.1 Staging of female zebrafish (*Danio rerio*) exposed to prochloraz

By means of histological investigation, the stage of maturity of all female zebrafish (*Danio rerio*) was categorized (Fig. 3.4.1). Staging criteria were used as described in chapter 2.4.4. Female fish of stage 0-3 were found at 37.5, 75 and 150 µg/L prochloraz and the control. Comparison between controls and 37.5 µg/L prochloraz showed that the number of immature females of stage 1 was more than doubled from 28.4 % to 70.4 %, and the number of more mature females in stage 2 was reduced from 43.2 % to 18.5 %. As only one female fish was found in the 300 and 600 µg/L tanks respectively, these concentrations are statistically not

relevant. A decrease of mature females (stage 3) could be found at all concentrations of prochloraz, if compared to the control (21.6 %).

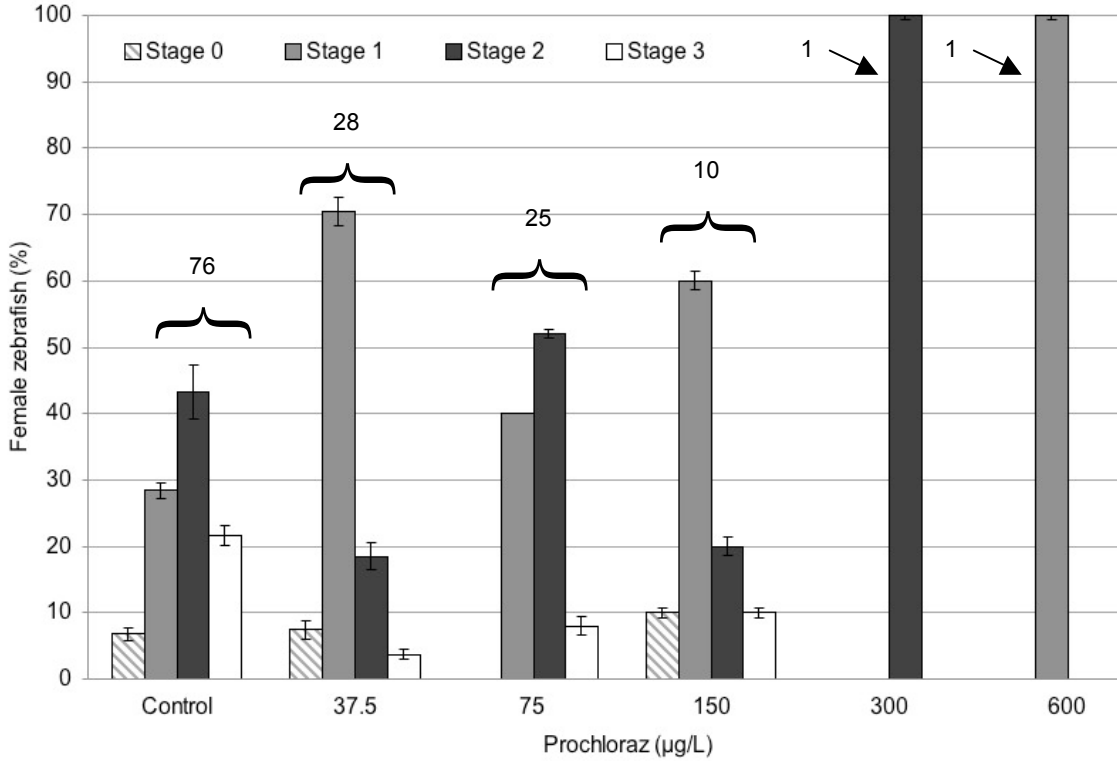


Fig. 3.4.1: Stages of maturity (%) of female zebrafish (*Danio rerio*) 60 days post-hatch (dph) after exposure to different concentrations of prochloraz. n = 2 (n = 4 in controls). Data at the top of the bars indicate the total number of female fish in each exposure group.

3.4.2 Staging of male zebrafish (*Danio rerio*) exposed to prochloraz

Male fish were categorized into stages of maturity from 0 - 3 (Fig. 3.4.2). Fish from the control tanks were composed of 16.8 % stage 1 and 83.2 % stage 2. More mature fish of stage 3 were only found in tanks of 37.5 µg/L and 75 µg/L (2.3 and 1.8 %). Immature male fish of stage 0 only appeared in tanks of 75 µg/L, 300 µg/L and 600 µg/L (< 2 %). The ratio of stage 1 to stage 2 varied at low concentrations of prochloraz but at higher concentrations (150, 300 and 600 µg/L), the number of immature males (stage 1) was nearly twice as high as in the control, and the number of mature males from stage 2 decreased by at least 15 %.

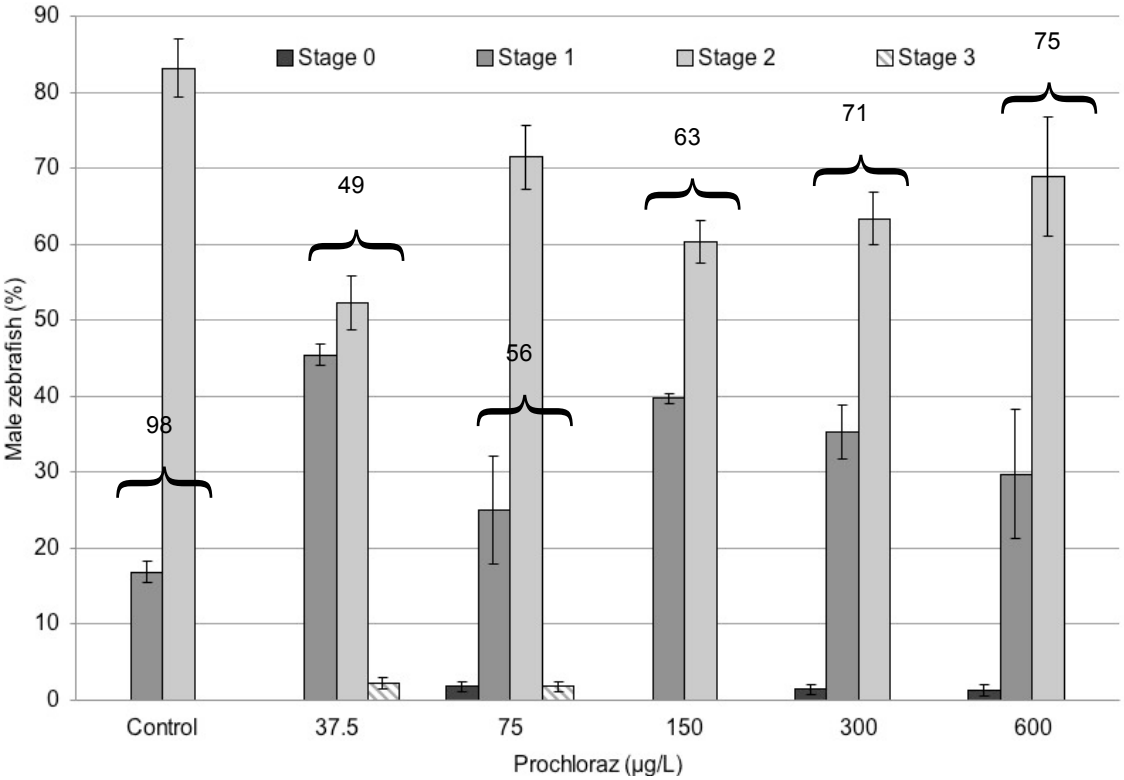


Fig. 3.4.2: Stages of maturity (%) of male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of prochloraz. n = 2 (n = 4 in controls). Figures at the top of the bars indicate the total number of male fish in each exposure group.

3.4.3 Overall maturity index of zebrafish (*Danio rerio*) exposed to prochloraz

The stage of maturity of female and male fish exposed to prochloraz was combined and described as the overall maturity index (MI; described in chapter 2.4.4). This method is an amplification of the regular staging system from the OECD guideline. Each stage of maturity was attributed to a certain fixed value, which increases with maturation of the fish. The values of each replicate were summed up, divided by the number of fish, and the mean value for the exposure group was calculated (Fig. 3.4.3). Female fish from 300 and 600 µg/L prochloraz had to be ignored since there was only one fish per concentration. The mean maturity index of female zebrafish at the control was MI 2.77. No significant difference could be determined for the concentration of 75 µg/L and 150 µg/L prochloraz (MI 2.25), but the values were significantly decreased at 37.5 µg/L (MI 2.19). Male zebrafish were slightly underdeveloped in high concentrations of prochloraz, if compared to controls (MI 2.83). A significant difference could only be determined in the exposure group of 37.5 µg/L prochloraz, where the mean maturity index was only MI 2.48. Female and male zebrafish (*Danio rerio*) did not show a clear correlation between stage of maturity and concentration of prochloraz.

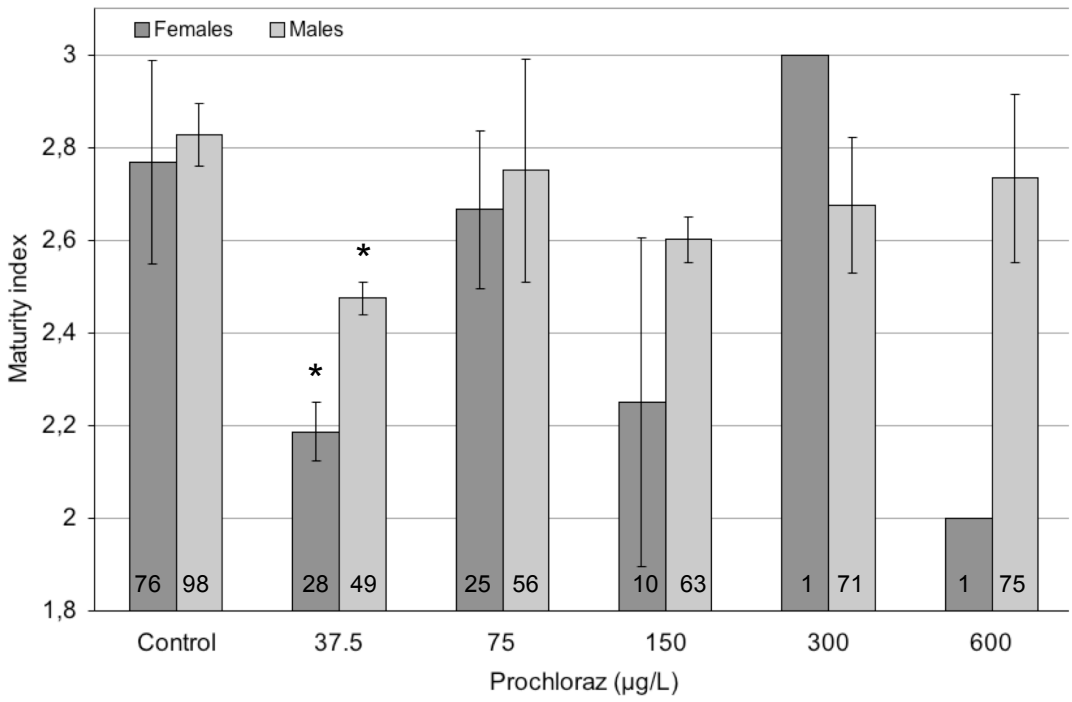


Fig. 3.4.3: Maturity index of female and male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of prochloraz. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Figures at the base of the bars indicate the total number of female and male fish in each exposure group.

3.5 Histopathology of zebrafish (Danio rerio) exposed to prochloraz

3.5.1 Histopathology of female zebrafish (*Danio rerio*) exposed to prochloraz

Only a marginal number of histopathological effects (minimal fibrosis and atresia) were found in female zebrafish from tanks with different concentrations of prochloraz (details not shown). Since these effects did occur both in controls and exposure groups, no correlation between effects and concentration of prochloraz could be determined.

3.5.2 Histopathology of male zebrafish (*Danio rerio*) exposed to prochloraz

The main histopathological effects found in male zebrafish were fibrosis and increased numbers of Sertoli cells. Both did occur in a more or less dose-dependent manner: only 3.3 % of the males at the control had slight fibrosis in their testes, but 39.4 % in the highest concentration (600 µg/L). A significant increase of Sertoli cells could also be revealed at the highest concentration (23.7 %), if compared to the control (10 %). Other observed effects like increase of Leydig cells or spermatogonia were not significant or dose-dependent.

Exposure group	Fibrosis (%)	Hypertrophy/ Hyperplasia of Sertoli cells (%)
Control	3.3	10.0
37.5 µg/L	19.6	7.8
75 µg/L	22.3	6.5
150 µg/L	13.8	17.9
300 µg/L	33,9	15.6
600 µg/L	39.4	23.7

Tab. 3.5.2: Histopathological alterations in male zebrafish (*Danio rerio*) exposed to prochloraz.

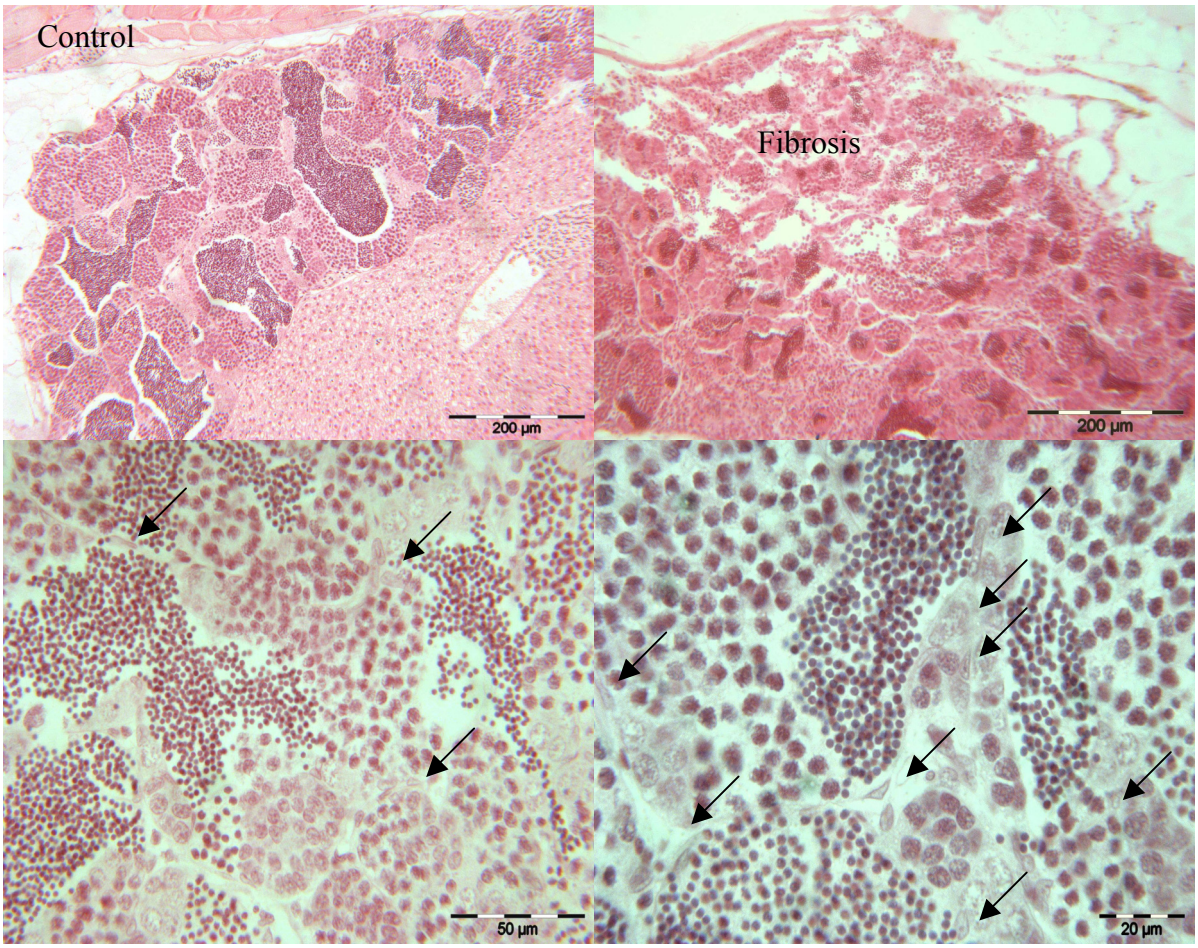


Fig. 3.5.2: Normally developed testes of zebrafish from the control group (upper and lower left) compared to those from 600 µg/L prochloraz (upper an lower right); the arrows point to Sertoli cells, note the hypertrophy as well as the hyperplasia.

3.6 Length und weight of zebrafish (Danio rerio) exposed to prochloraz

3.6.1 Length and weight in correlation to concentration and gender

Length and weight of male and female zebrafish (*Danio rerio*), as correlated to the concentration of prochloraz, were compared (Fig. 3.6.1a&b). Both sexes had a significant reduction of growth compared to the control at the highest concentration of prochloraz (600 µg/l). Females from the control had an average length of 27.0 mm and weighted 173.9 mg, whereas the female from 600 µg/L prochloraz only had a length of 20 mm and weighted 78 mg. Males from the controls had an average length of 26.0 mm and weighted 146.0 mg; both parameters were reduced at 600 µg/L to a length of 21.9 mm and a weight of 100.7 mg.
Male fish from concentrations of 37.5 µg/L prochloraz had a significantly higher weight (190.1 mg) than in the controls (146.0 mg). Female fish from concentrations of 75 µg/L prochloraz had a significantly higher weight (195.9 mg) than in the controls (173.9 mg). Only 2 females were found in 300 and 600 µg/L, which made these data statistically irrelevant.

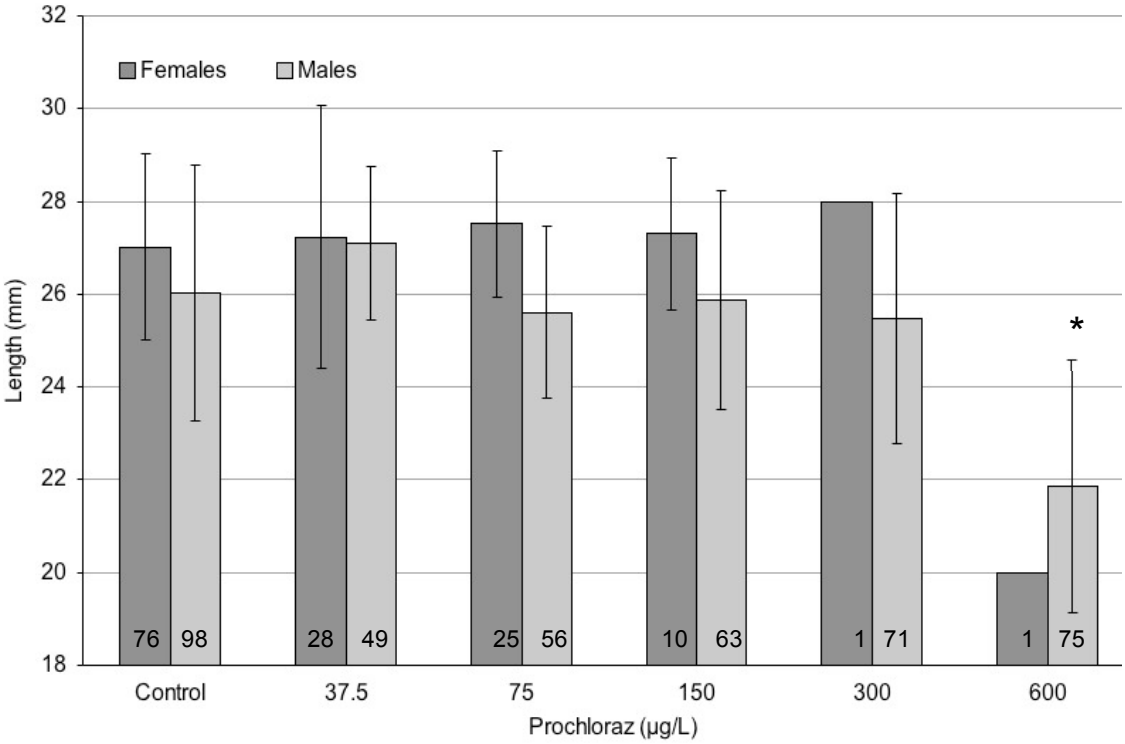


Fig. 3.6.1a: Length (mm) of female and male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of prochloraz. Asterisk: Value differs significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Data at the base of the columns indicate the total number of female and male fish in each exposure group.

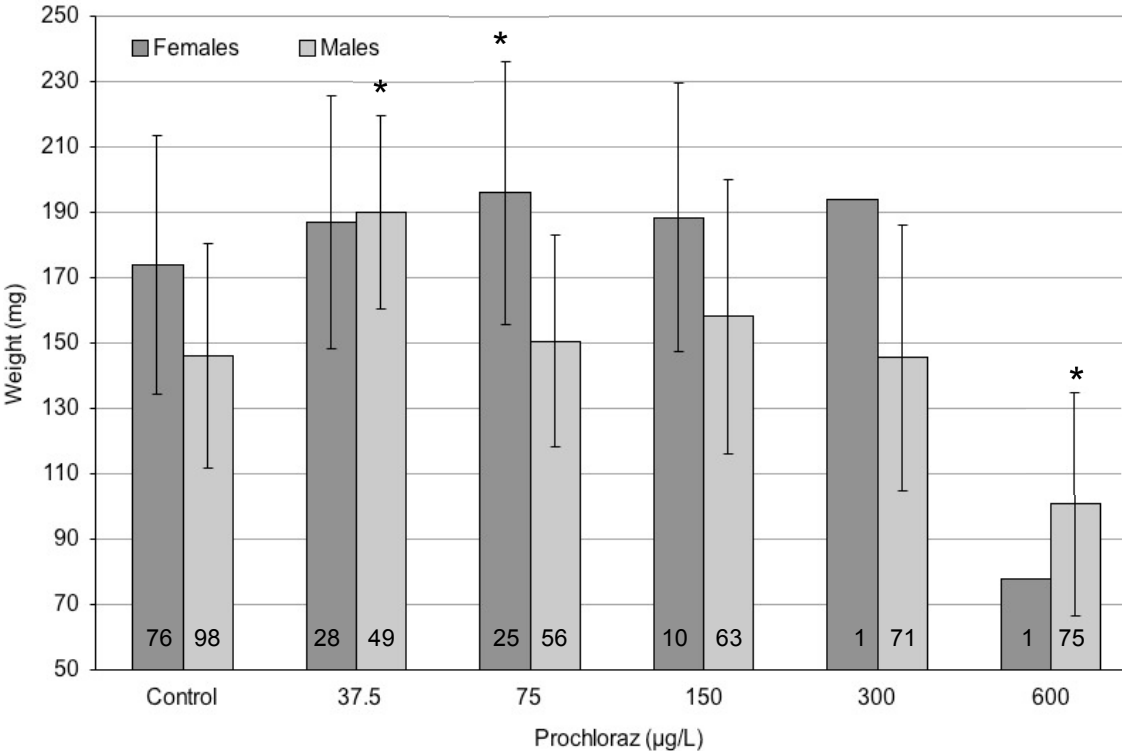


Fig. 3.6.1b: Weight (mg) of female and male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of prochloraz. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Figures at the base of the bars indicate the total number of female and male fish in each exposure group.

3.6.2 Length and weight as correlated to stages of maturity

Length and weight of male and female zebrafish (*Danio rerio*) were compared to their stage of maturity (Fig. 3.6.2a & b). Fish from exposure groups and controls were mixed up in this approach, since the same correlations between growth and stages of maturity were found in all replicates independent from exposure to prochloraz (details not shown).

For both females and males, body size and weight increased with the maturation stage. Immature females of stage 0 had an average length of 24.8 mm and a weight of 137.1 mg, whereas mature females of stage 3 were 10 % longer and 30 % heavier. Immature males of stage 0 had an average length of 19 mm and weighed 62.3 mg. Compared to that, mature males of stage 3 were 30 % longer and 65 % heavier.

The average weight of the females (169.5 mg) exceeded that of the males at all stages (148.5 mg). Similar differences were found in their length: females measured 26.18 mm and males only 25.32 mm. Stage 3 was an exception because males were longer than females.

Statistically significant differences in length compared to stage 0 were found in females from stage 2 and males from stages 2 and 3. Fish of stages 1, 2 and 3 were all heavier than fish of stage 0 (males and females).

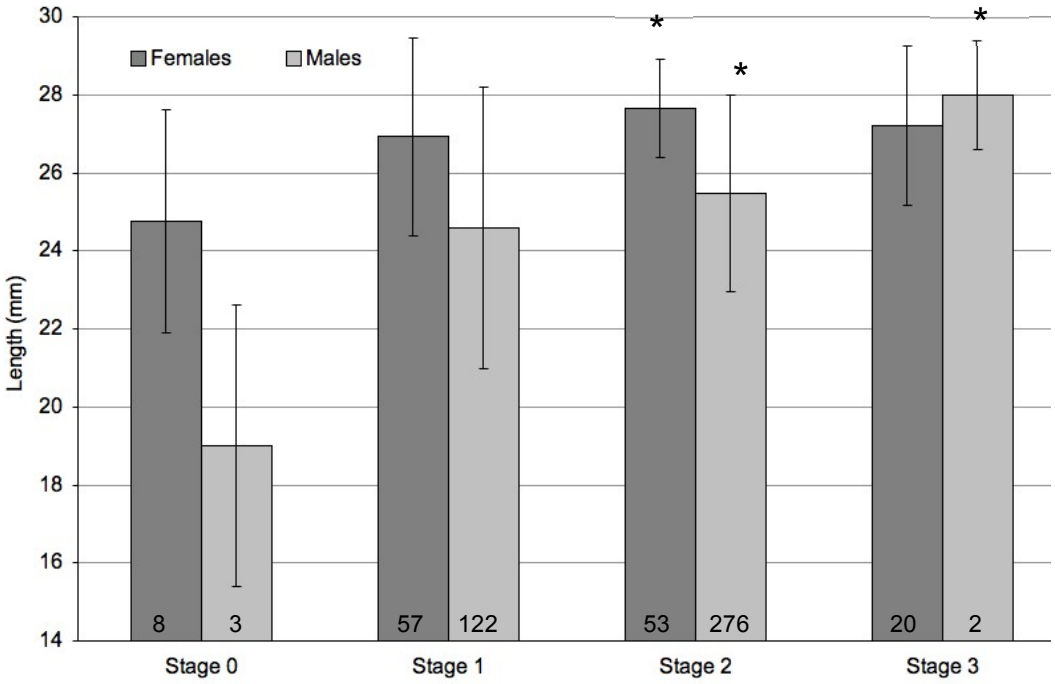


Fig. 3.6.2a: Length (mm) of female and male zebrafish (*Danio rerio*) as correlated to their stage of maturity. Asterisks: Values differ significantly from stage 0 (Anova on ranks; * $p \leq 0.05$). Data at the base of the bars indicate the total number of female and male fish in each group.

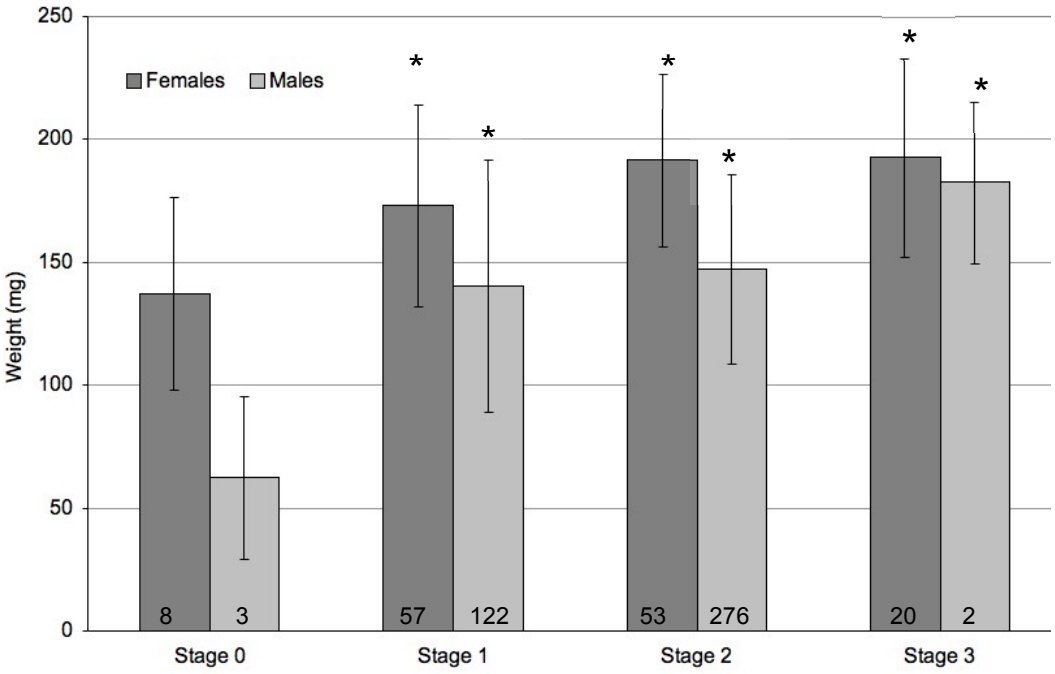


Fig. 3.6.2b: Weight (mg) of female and male zebrafish (*Danio rerio*) as correlated to their stage of maturity. Asterisks: Values differ significantly from stage 0 (Anova on ranks; * $p \leq 0.05$). Data at the base of the bars indicate the total number of female and male fish in each group.

3.7 ELISA with zebrafish (Danio rerio) exposed to prochloraz

3.7.1 Vitellogenin synthesis in female zebrafish (*Danio rerio*) exposed to prochloraz

The concentration of vitellogenin (vtg; ng vtg/g fish homogenate) in head and tail of female zebrafish exposed to different concentrations of prochloraz was measured by ELISA (Fig. 3.7.1). The concentration of vtg ranged between 5 ng/g homogenate in controls and 2 ng/g homogenate at the highest concentration of prochloraz. All female fish, except at 75 µg/L prochloraz, showed a significant decrease of vitellogenin concentration compared to the control. Since only 2 female fish were found in concentrations of 300 and 600 µg/L prochloraz, they were taken together into one group. Even though, these data are statistically irrelevant.

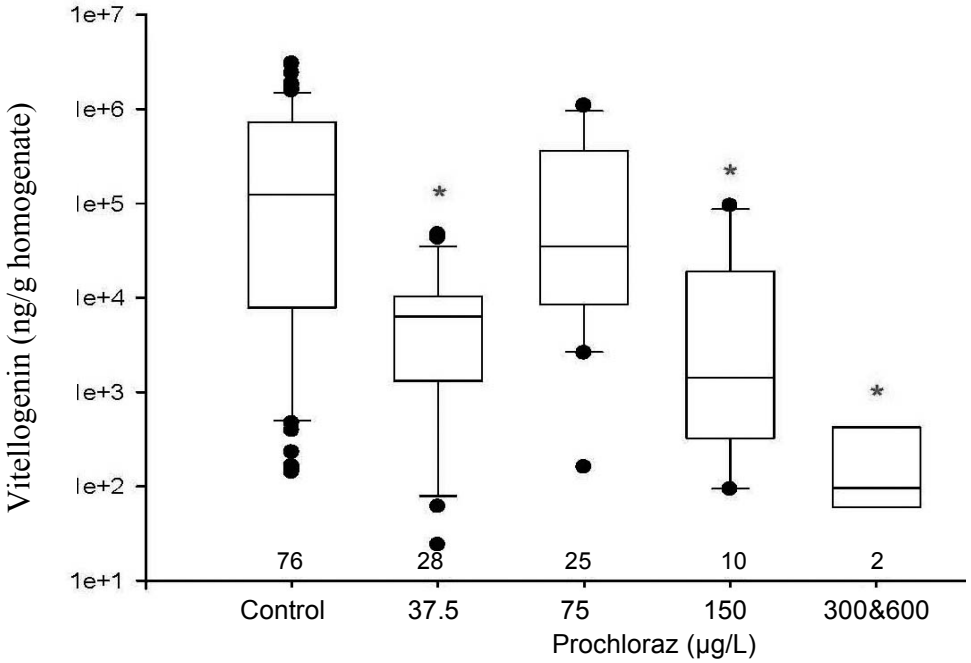


Fig. 3.7.1: Concentrations of vitellogenin (ng/g fish) in head and tail of female zebrafish (*Danio rerio*) exposed to different concentrations of prochloraz over 60 days. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Data at the base of the boxes indicate the total number of female fish in each exposure group.

3.7.2 Vitellogenin synthesis in male zebrafish (*Danio rerio*) exposed to prochloraz

The concentration of vitellogenin (vtg; ng vtg/g fish homogenate) in head and tail of male zebrafish exposed to different concentrations of prochloraz was measured by ELISA (Fig. 3.7.2). The mean concentration of vitellogenin ranged between 3 ng vtg/g homogenate at the control and 2 ng vtg/g homogenate at the highest concentration. The concentration of vitellogenin decreased significantly at all concentrations of prochloraz compared to the control.

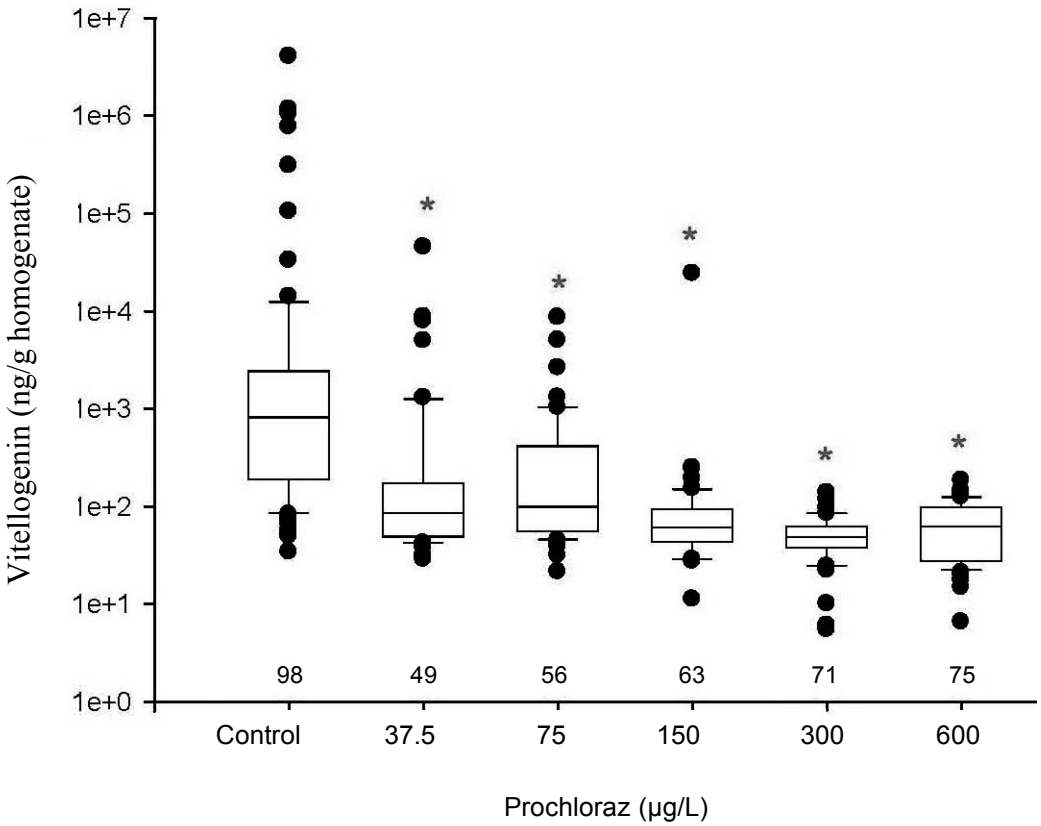


Fig. 3.7.2: Concentration of vitellogenin (ng/g fish) in head and tail of male zebrafish (*Danio rerio*) exposed to different concentrations of prochloraz during 60 days. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 2 (n = 4 in controls). Data at the base of the bars indicate the total number of male fish in each exposure group.

3.8 Acute fish embryo toxicity assay with zebrafish (Danio rerio) exposed to prochloraz

3.8.1 Fish embryo assay 24 hours post-fertilization

The following endpoints were recorded after 24 hours of exposure to different concentrations of prochloraz, dimethyl sulfoxid (DMSO; solvent), 3.4-dichloranniline (DCA; positive control) and artificial water (negative control): the number of coagulated eggs (lethal effects) and the number of normal and abnormally (sublethal effects) developed embryos (% of surviving embryos). Four replicates were run with 24 eggs per concentration (Fig. 3.8.1). 1.2 % of the zebrafish from the negative control (artificial water) were coagulated, 9.5 % at the positive control (DCA) and 4.8 % in the solvent control (DMSO). The percentage of coagulated eggs increased constantly and significantly in a concentration dependent manner. At the highest concentration, 5 mg/L prochloraz, only 13.9 % of the embryos survived, which

is 7 times less than in the negative control. Abnormally developed embryos were only found at the positive control (2.6 %) and with 3 mg/L prochloraz (5.3 %). No additional core endpoints of the fish embryo assay were found. The LC_{50} after 24 hours of exposure was 2.9 mg/L.

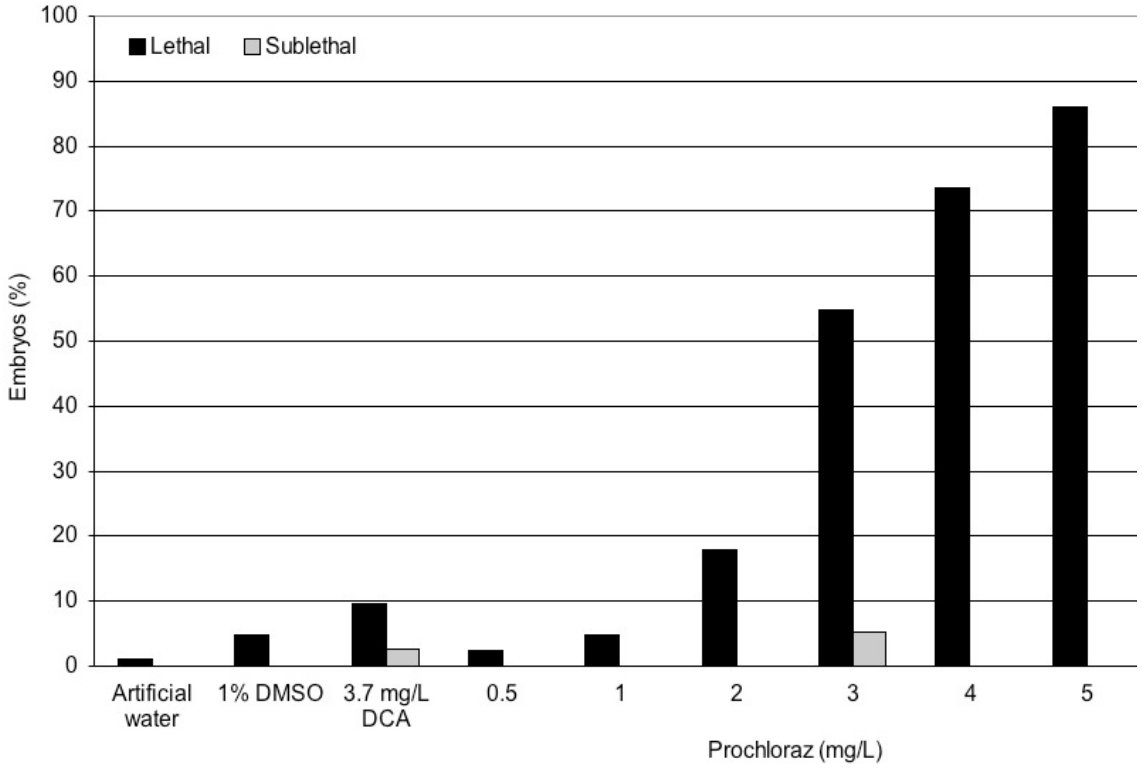


Fig. 3.8.1: Mortality and development of zebrafish (*Danio rerio*) embryos after 24 hours of exposure to prochloraz; n = 4; 24 eggs per replicate.

3.8.2 Fish embryo assay 48 hours post-fertilization

The same endpoints as after 24 hours post fertilization were recorded after 48 hours (Fig. 3.8.2). No additional eggs coagulated in the negative and the solvent controls. In the positive control, the number of coagulated eggs increased from 9.5 % to 10.7 %, and the number of abnormally developed embryos increased from 2.6 % to 69.3 %. Most abnormally developed embryos due to prochloraz were found at the 5 mg/L exposure group, with 11.1 % of the surviving embryos. The LC_{50} after 48 hours of exposure was 2.9 mg/L.

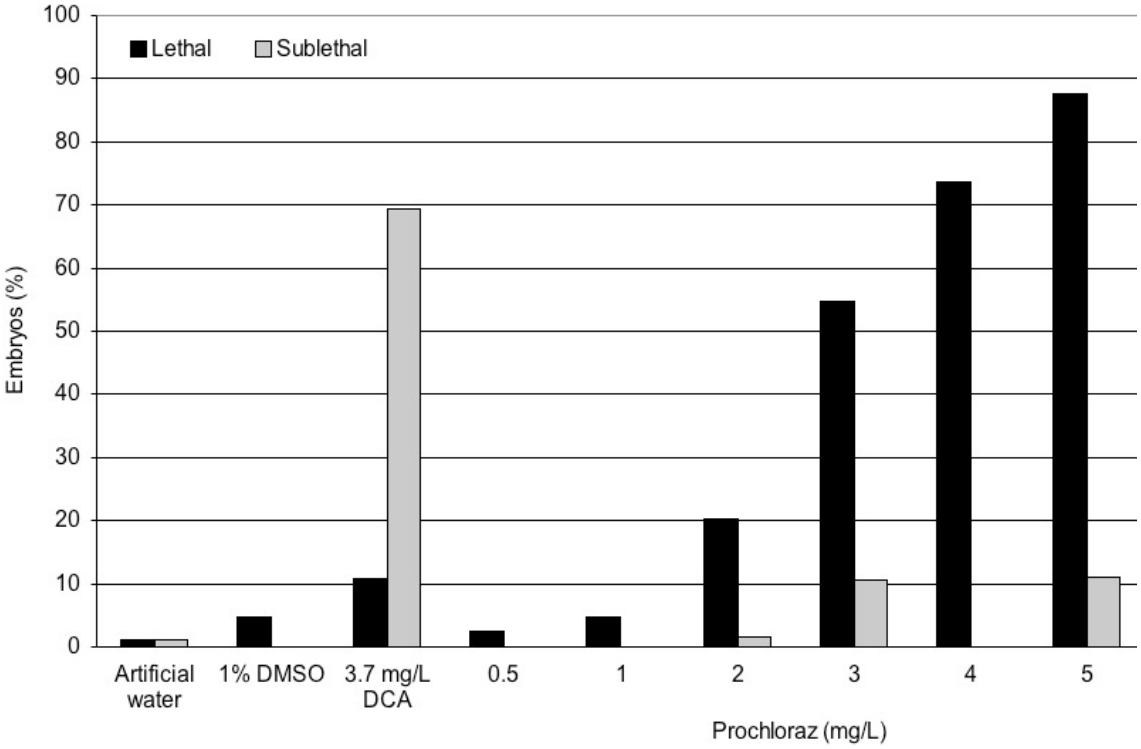


Fig. 3.8.2a: Mortality and development of zebrafish (*Danio rerio*) embryos after 48 hours of exposure to prochloraz; n = 4; 24 eggs per replicate.

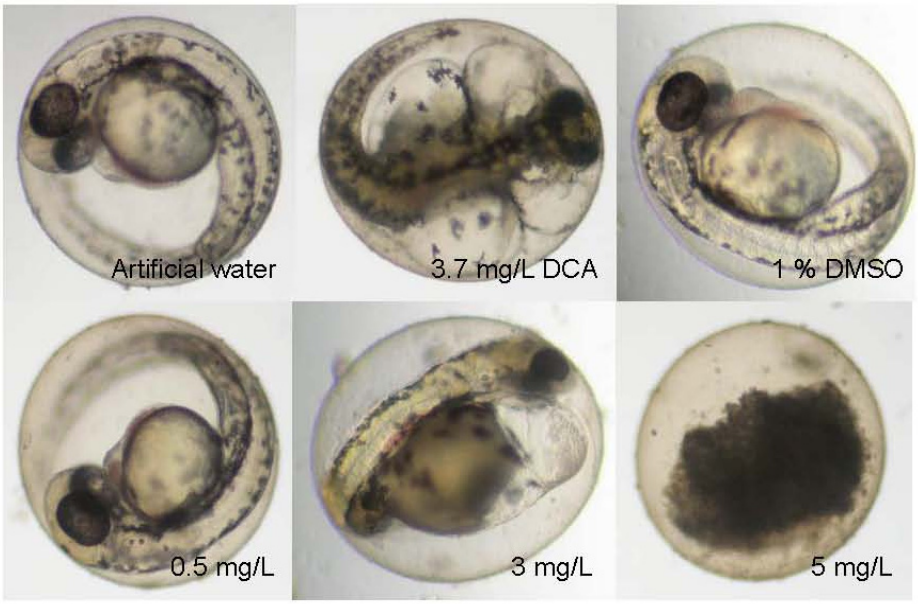


Fig. 3.8.2b: Zebrafish (*Danio rerio*) embryos 48 hours post-fertilization. Prochloraz caused development of edema and mortality (coagulation) at high concentrations.

3.8.3 Fish embryo assay 72 hours post-fertilization

After 72 hours of exposure, the hatching success was determined in addition to the endpoints after 24 and 48 hours post-fertilization (Fig. 3.8.3). 44.6 % of the embryos of the negative control were hatched, whereas only 5.3 % at the concentration of 4 mg/L prochloraz were hatched. More than 13 times more embryos were hatched at a concentration of 0.5 mg/L prochloraz (70.4 %). Lethal and sublethal effects did not increase, if compared to 48 hours post-fertilization. The EC_{50} for delay of hatch after 72 hours of exposure was 1 mg/L.

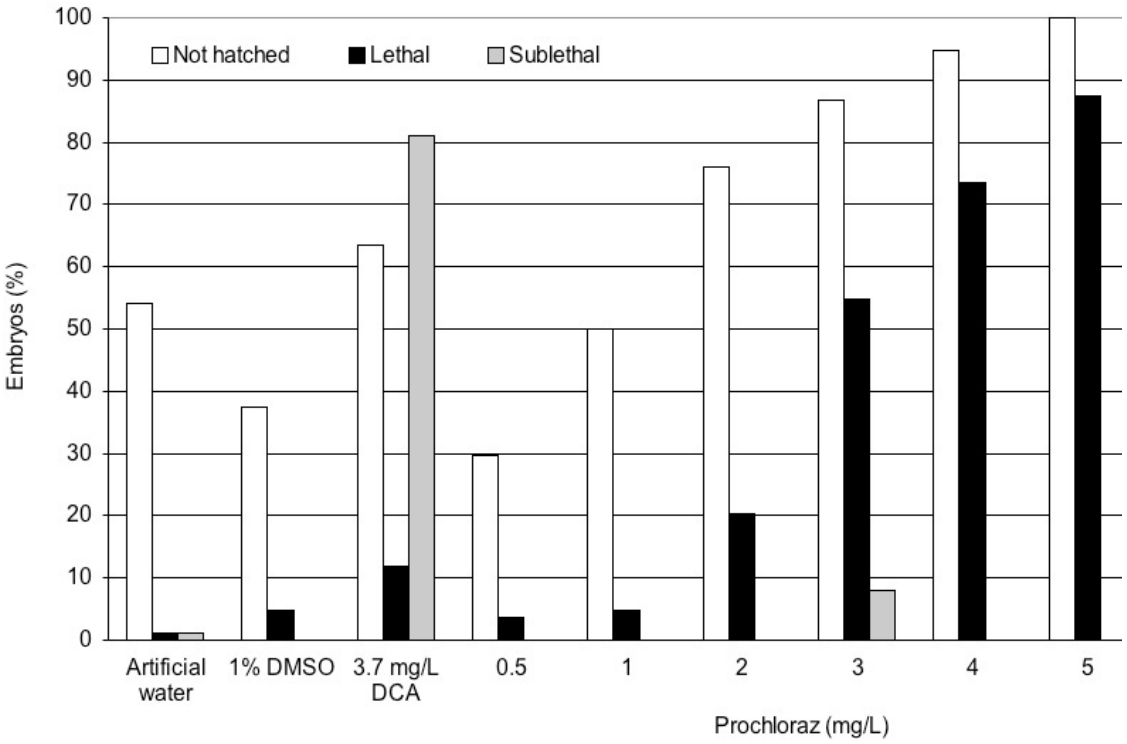


Fig. 3.8.3a: Mortality, hatch and development of zebrafish (*Danio rerio*) embryos after 72 hours of exposure to prochloraz; n = 4; 24 eggs per replicate.

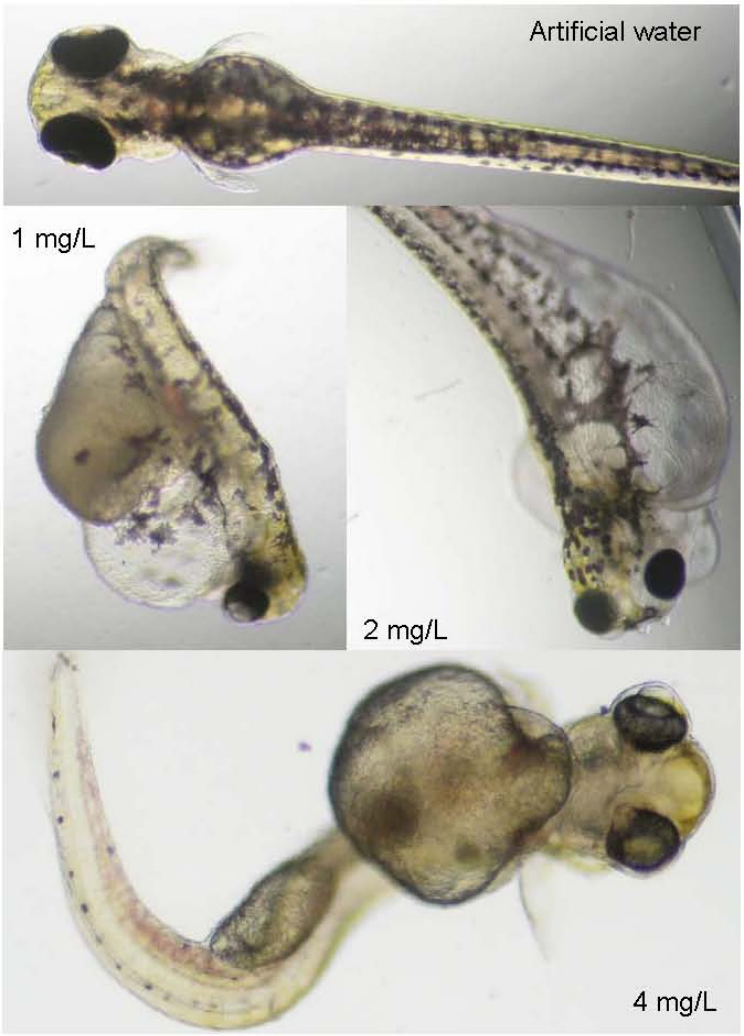


Fig. 38.3b: Zebrafish (*Danio rerio*) embryos 72 hours post-fertilization. Exposure to different concentrations of prochloraz caused developmental abnormalties, like edema and spinal distortions.

4. Results 4-*tert*-Pentylphenol

4.1 Water analysis of FSDT with 4-tert-pentylphenol

4.1.1 Water parameters of FSDT with 4-*tert*-pentylphenol

Over the entire period of exposure, different parameters of the water quality were measured in all tanks. Following results were recorded: pH-values: 7.8 - 8.0; Oxygen concentrations: 5.6 – 6.4 mg/L; Total hardness: 16 - 18 °dH; Carbonate hardness: 10.4 – 11.6 °dH

4.1.2 Chemical analysis of FSDT with 4-*tert*-pentylphenol

During the transport to Denmark, water samples were damaged, and, therefore, no results could be achieved.

4.2 Mortality of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

After 30 days of exposure, the mortality of zebrafish (*Danio rerio*) was determined (Fig. 4.2). No correlation to the concentration of 4-*tert*-pentylphenol could be found, since data varied very much. 27.25 % of the fish from the control tanks died, but only 17 % at the concentration of 75 µg/L and 39 % at the concentration of 320 µg/L.

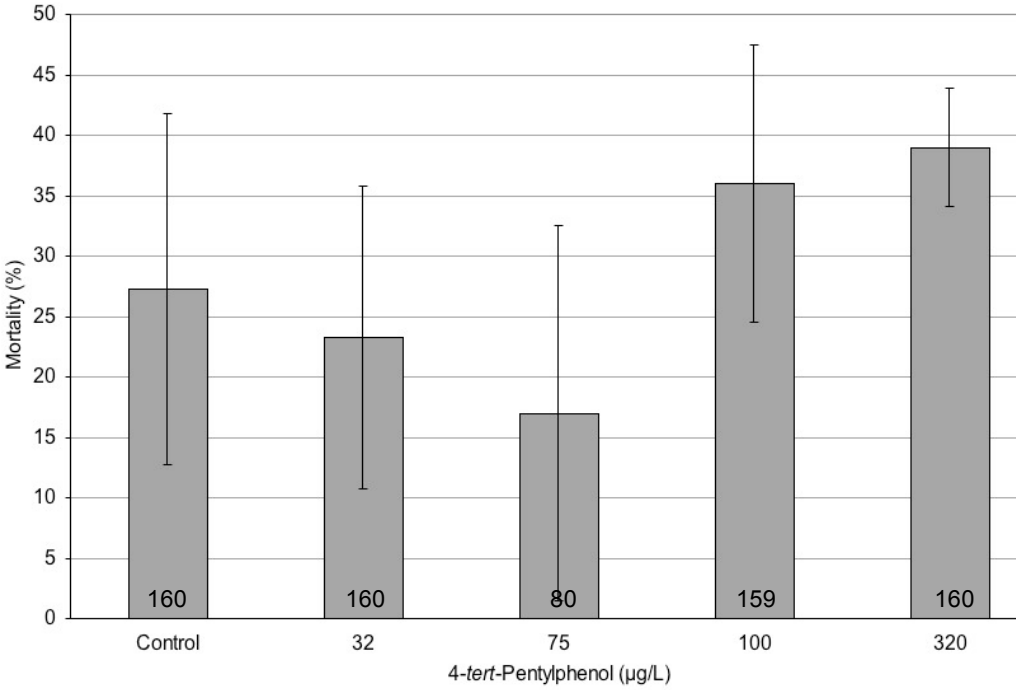


Fig. 4.2: Mortality (%) of zebrafish (*Danio rerio*) 30 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol; n = 4 (n =2 at 75 µg/L). Data at the base of the bars indicate the total number of fish in each exposure group.

4.3 Sex ratio of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

60 days post-hatch the sex ratio of zebrafish (*Danio rerio*) was determined *via* histological investigations (Fig. 4.3). The gender of the fish was categorized into female, male, undifferentiated or testis-ova. After 60 days of exposure to different concentrations of 4-*tert*-pentylphenol, a feminization and underdevelopment of the zebrafish could be determined. Fish from the control tanks were composed of 38.75 % males and 55.63 % females, whereas at a concentration of 320 µg/L 4-*tert*-pentylphenol the male ratio decreased to 10.63 % and the females represented 62.5 %. The remaining fish at this concentration were undifferentiated (18.25 %) or revealed testis-ova (8.75 %). Two hermaphrodites were determined at the control and one at the concentration of 32 µg/L 4-tert-pentylphenol. A significant reduction of males was determined at the 320 µg/L treatment groups. Additionally, undifferentiated fish were only found at the 320 µg/L concentration.

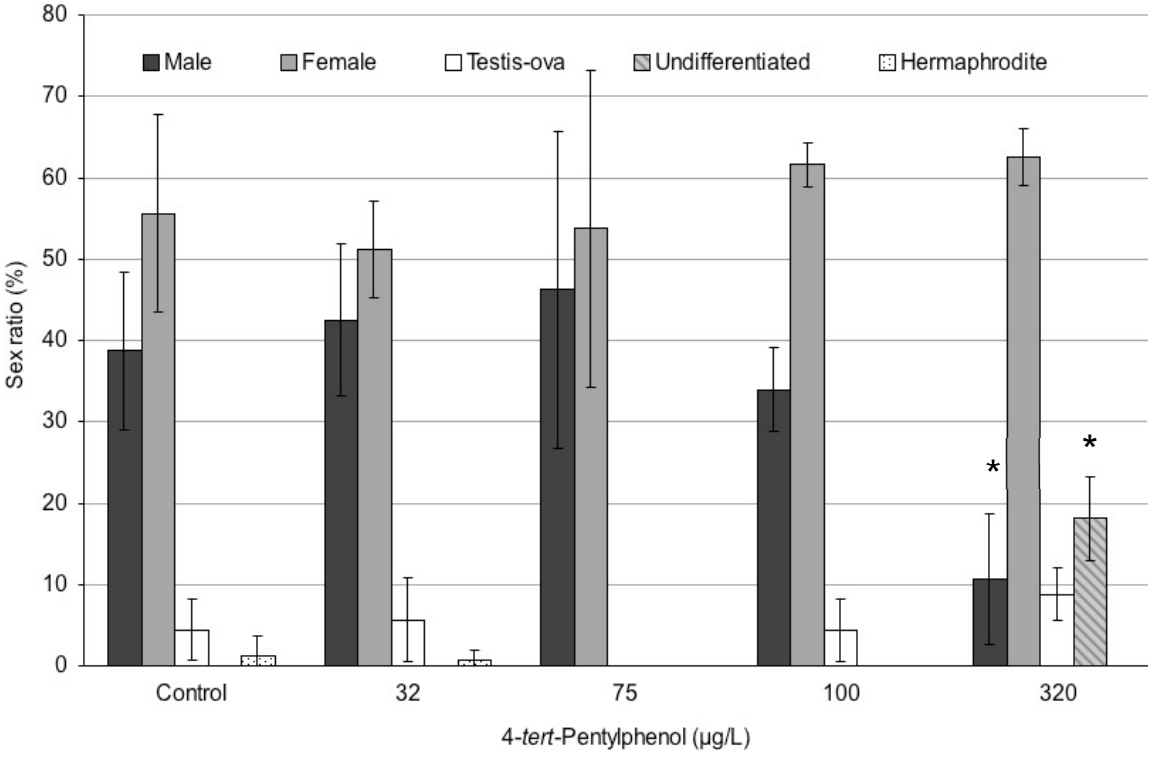


Fig. 4.3: Sex ratio (%) of zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L).

4.4 Staging of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

4.4.1 Staging of female zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol

Female zebrafish (*Danio rerio*) were categorized into stages of maturity from 0 - 4 (Fig. 4.4.1). Fish from the control tanks were composed of 26.9 % stage 1, 60.6 % stage 2 and 12.5 % stage 3 fish. The number of relatively mature female fish belonging to stage 2 was 20 times lower at the concentration of 320 µg/L 4-*tert*-pentylphenol (3 %). Only 1 % of the evaluated females were in stage 3. The percentage of immature females belonging to stage 0 increased from 0 % at the control to 65 % at 320 µg/L.

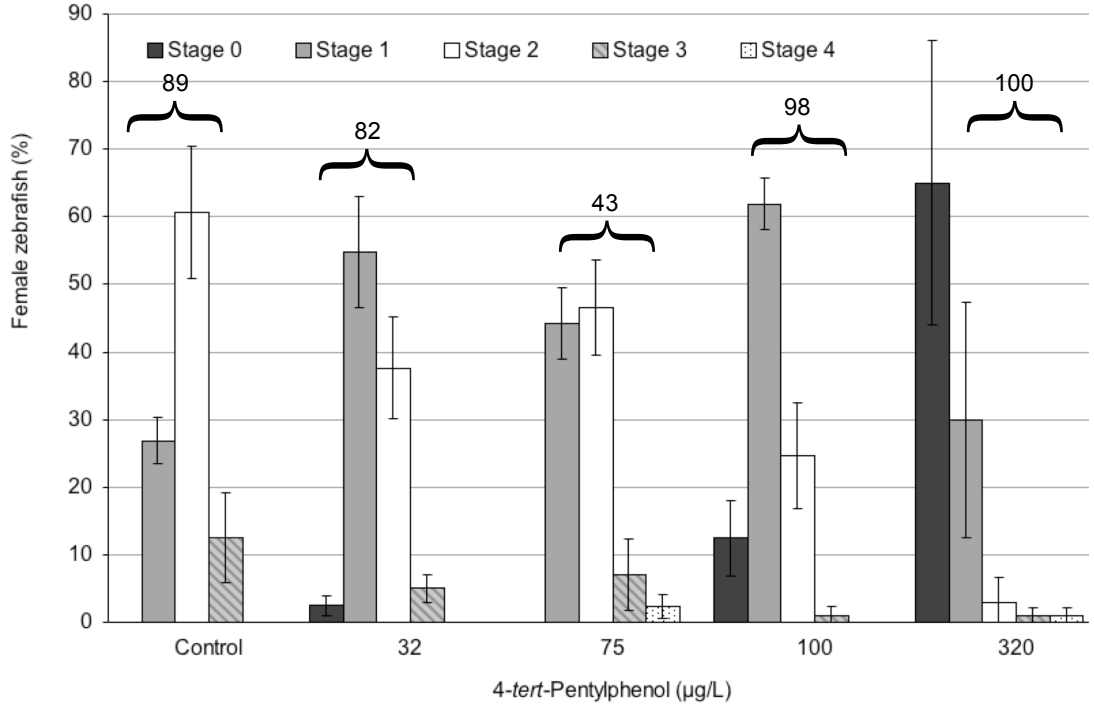


Fig. 4.4.1: Stages of maturity (%) of female zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. n = 4 (n =2 at 75 µg/L). Data at the top of the columns indicate the total number of female fish in each exposure group.

4.4.2 Staging of male zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol

Male zebrafish (*Danio rerio*) were categorized into stages of maturity 0 - 2 (Fig. 4.4.2). Fish from the control tanks were composed of 46.4 % stage 1 and 53.6 % stage 2 fish. The number of male fish belonging to stage 2 was higher at the 75 µg/L 4-*tert*-pentylphenol exposure (81.1 %) and lower (5.8 %) at 320 µg/L. Immature males of stage 0 only appeared at 32, 100 and 320 µg/L. A high percentage was found in 320 µg/L with 52.9 %, compared to only 4.5 % at 32 µg/L and 0 % in the control.

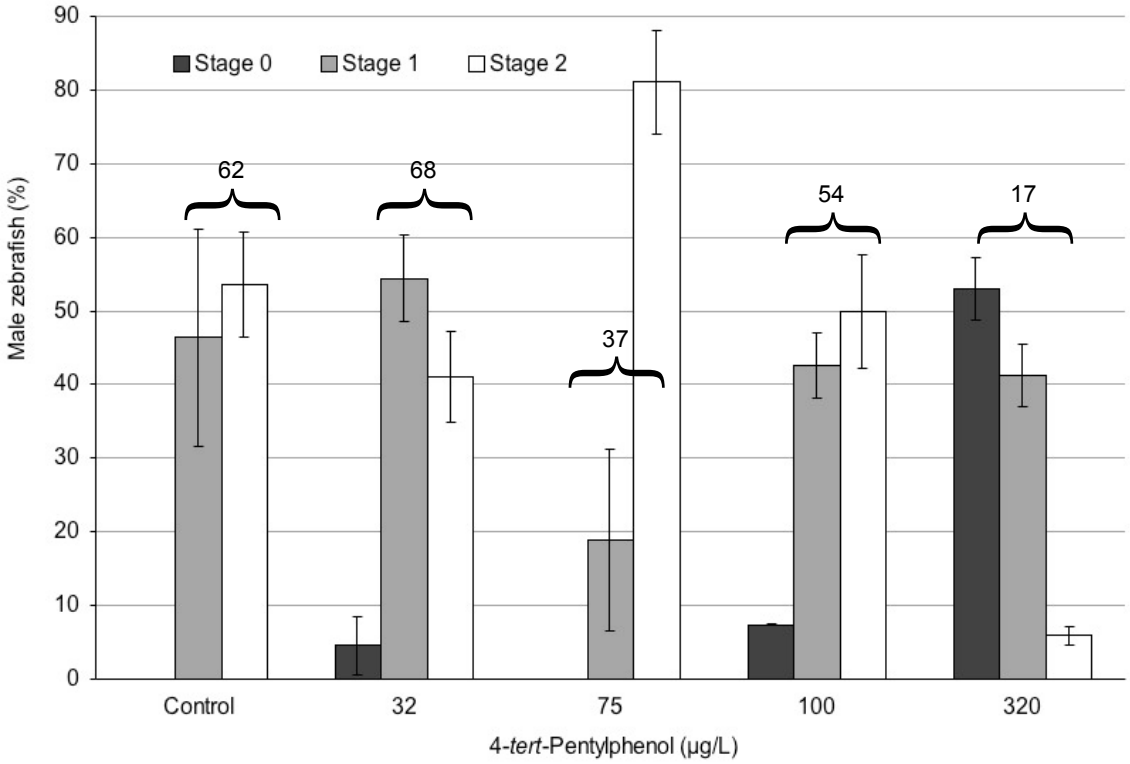


Fig. 4.4.2: Stages of maturity (%) of male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. n = 4 (n =2 at 75 µg/L). Data at the top of the columns indicate the total number of male fish in each exposure group.

4.4.3 Overall maturity index of zebrafish (*Danio rerio*) exposed to 4–*tert*-pentylphenol

The stages of maturity of female and male fish exposed to 4-*tert*-pentylphenol was combined and described with the overall maturity index (MI; described in chapter 2.4.4). This method is an amplification of the regular staging system from the OECD guideline. Each stage of maturity was attributed to a certain value, which increases with maturity of the fish. The values of each replicate were summed up, divided by the number of fish, and the mean value for the exposure group was calculated (Fig. 4.4.3). The results show a decrease of maturity for female and male zebrafish with increasing concentration of 4-*tert*-pentylphenol. Female fish had the highest maturity index at the control (MI 2.85). The values decreased significantly at 32 mg/L (MI 2.45), 100 µg/L (MI 2.14) and 320 µg/L (MI 1.49). No significant decrease could be determined at 75 mg/L (MI 2.64). The maturity index of male zebrafish varied from MI 2.86 to MI 2.39 at the control and from 32 to 100 µg/L 4-*tert*-pentylphenol, but a significant decrease could be determined at the highest concentration (320 µg/L) with a maturity index of only MI 1.5.

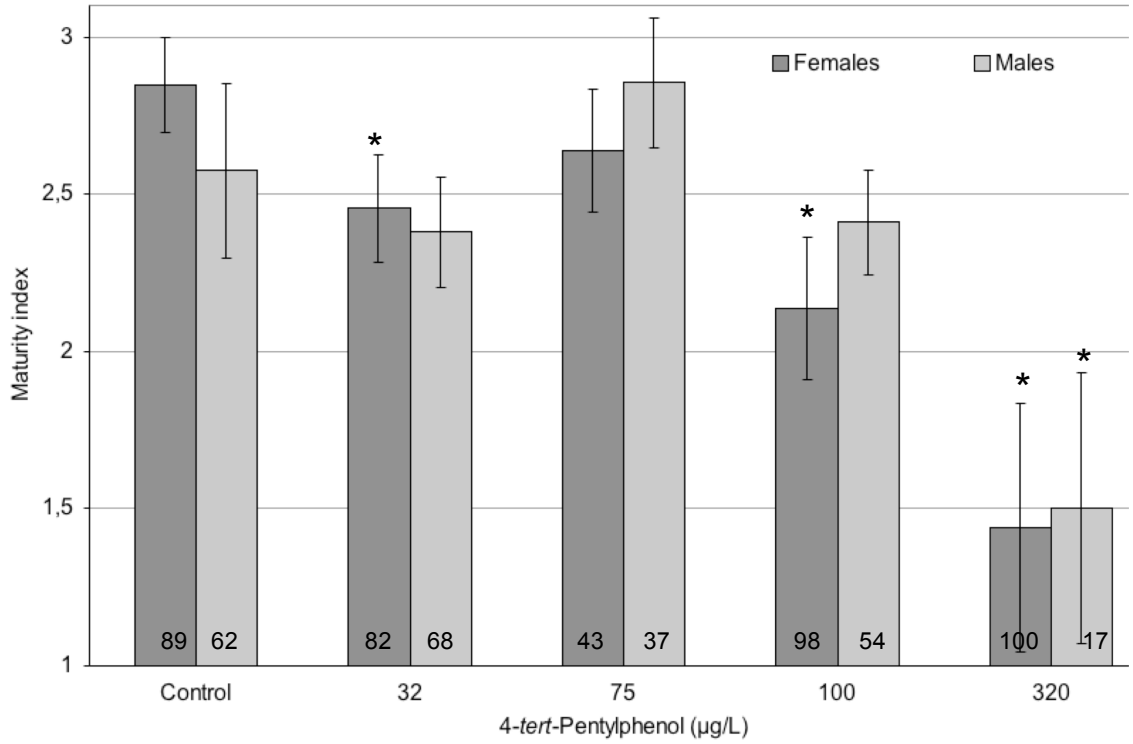


Fig. 4.4.3: Maturity index of female and male zebrafish (*Danio rerio*) 60 days post-hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L). Data at the base of the columns indicate the total number of female and male fish in each exposure group.

4.5 Histopathology of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

Only a marginal number of histopathological effects (minimal fibrosis and atresia) were found in female and male zebrafish from tanks of different concentrations of 4-*tert*-pentylphenol (details not shown). Since these effects did occur in both controls and exposure groups, no correlation between effects and concentration of 4-*tert*-pentylphenol could be determined.

4.6 Length and weight of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

4.6.1 Length and weight as correlated to gender and concentration

Length and weight of males and females were compared in correlation to the concentration of 4-*tert*-pentylphenol (Figs. 4.6.1a&b). The mean length of female fish ranged between 29.1 mm (control) and 27.3 mm (75 µg/L). Male fish had a mean length between 28.6 mm (320

µg/L) and 27.2 mm (75 µg/L). A significantly lower length was found at 75 µg/L 4-*tert*-pentylphenol (females and males) and 320 µg/L 4-*tert*-pentylphenol (females). With the exception of the 320 µg/L concentration, female fish were always longer than male fish. Similar variations were found in the range of mean weight. The mean weight of female fish ranged between 229.4 mg (control) and 194.8 mg (75 µg/L), and male fish weighed between 215.6 mg (320 µg/L) and 185.9 mg (75 µg/L). A significantly lower average weight was found for 75 µg/L 4-*tert*-pentylphenol (females and males). Female fish were always heavier than male fish, except for the concentration of 320 µg/L 4-*tert*-pentylphenol.

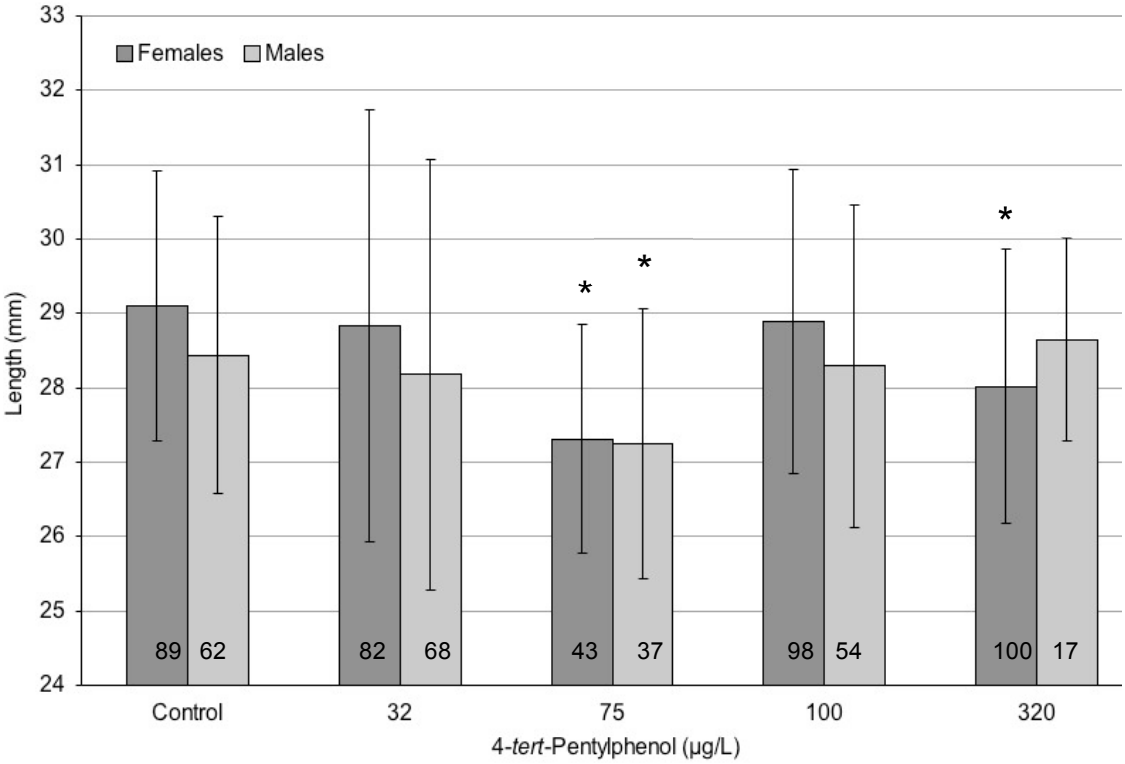


Fig. 4.6.1a: Length (mm) of female and male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L). Data at the base of the columns indicate the total number of female and male fish in each exposure group.

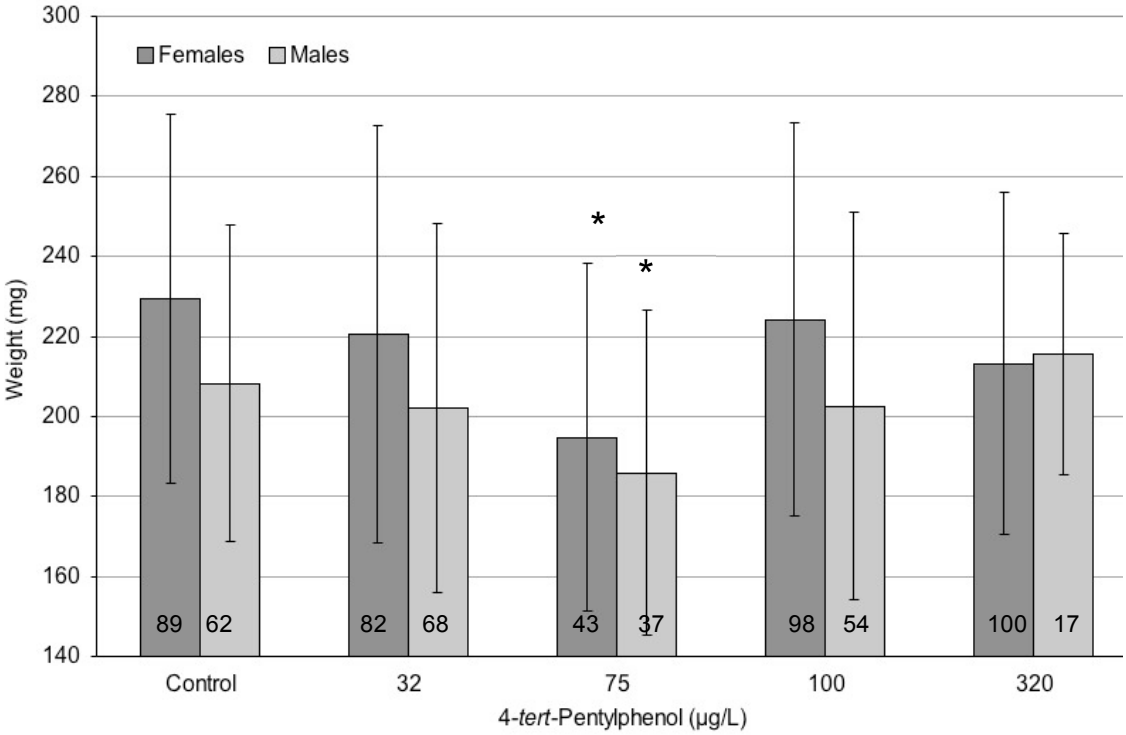


Fig. 4.6.1b: Weight (mg) of female and male zebrafish (*Danio rerio*) 60 days post hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L). Data at the base of the columns indicate the total number of female and male fish in each exposure group.

4.6.2 Length and weight as correlated to stage of maturity

In order to assess a possible correlation between the size (length and weight) of the fish and their stage of maturity, these parameters were correlated to each other (Fig. 4.6.2a & b). Fish from exposure groups and controls were mixed up in this approach, since the same correlations between growth and stage of maturity were found at all replicates independent from exposure to 4-*tert*-pentylphenol (details not shown). Only female zebrafish showed a significant increase of length and weight according to their stage of maturity. Male zebrafish even showed a light reduction of growth with increasing stage of maturity. Female zebrafish belonging to the immature stage 0 had an average length of 27.6 mm and weighed 198.6 mg. Both length and weight were higher in mature females of stage 4 with mean values of 29 mm (5 % more) and 266 mg (25 % more). Immature male fish belonging to stage 0 had an average length of 28.4 mm and weighed 211.4 mg. More mature fish from stage 2 had an average length of 28.1 mm (1 % less) and weighed 203.5 mg (3.7 % less).

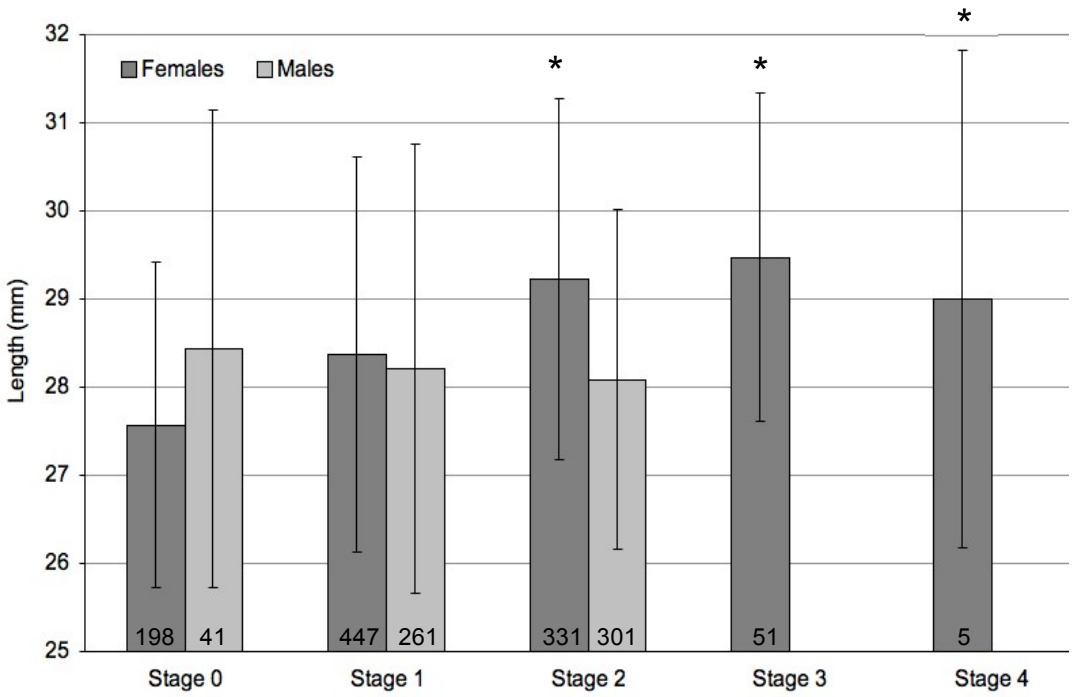


Fig. 4.6.2a: Length (mm) of female and male zebrafish (*Danio rerio*) 60 days post-hatch correlated to their stage of maturity. Asterisks: Values differ significantly from stage 0 (Anova on ranks; * $p \leq 0.05$). Data at the base of the columns indicate the total number of female and male fish in each group.

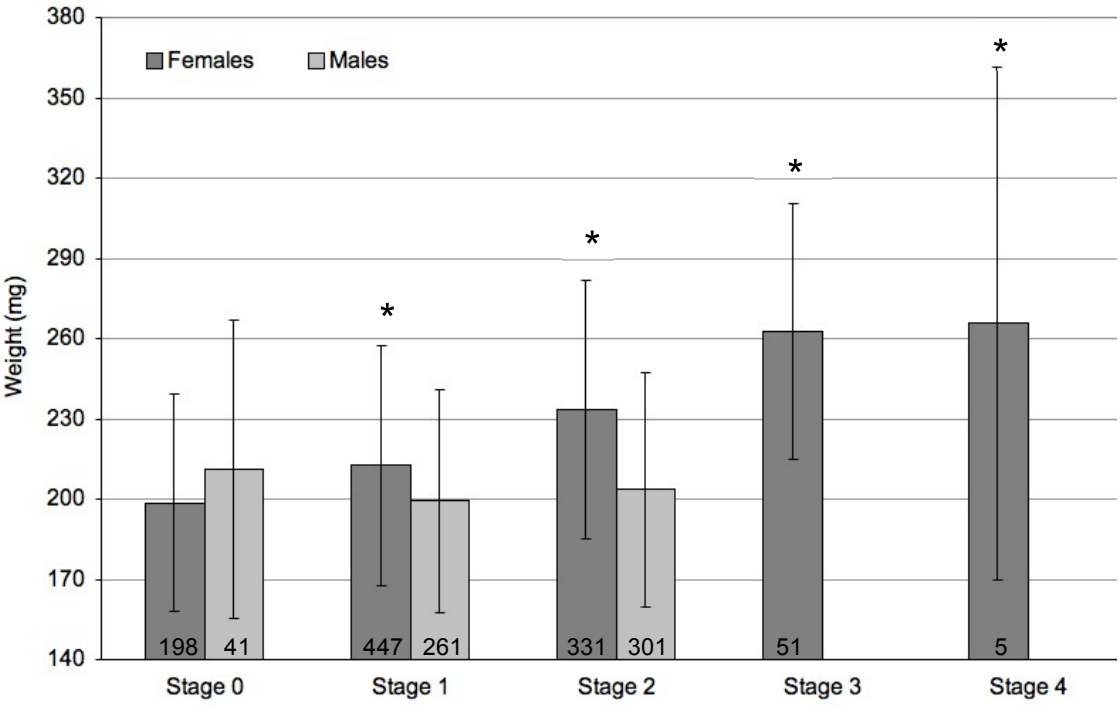


Fig. 4.6.2b: Weight (mg) of female and male zebrafish (*Danio rerio*) 60 days post-hatch correlated to their stage of maturity. Asterisks: Values differ significantly from stage 0 (Anova on ranks; * $p \leq 0.05$). Data at the base of the columns indicate the total number of female and male fish in each group.

4.7 ELISA with zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

4.7.1 Vitellogenin synthesis in female zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol

The concentrations of vitellogenin (vtg; ng vtg/g fish) in head and tail of female zebrafish exposed to different concentrations of 4-*tert*-pentylphenol was measured by ELISA (Fig. 4.7.1). The average concentration of vitellogenin ranged approximately between 2 and 6 ng vtg/g fish. A significant difference compared to the control could only be found at a concentration of 320 µg/L 4-*tert*-pentylphenol, where the females produced less vitellogenin than at the control.

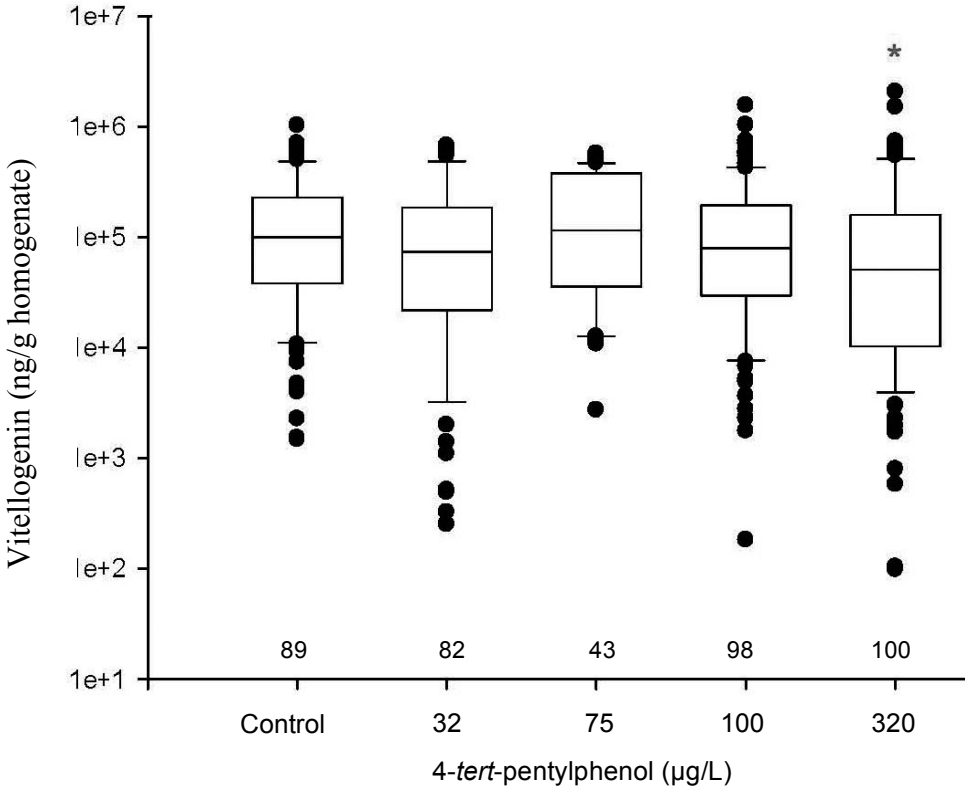


Fig. 4.7.1: Concentrations of vitellogenin (vtg; ng vtg/g fish) in head and tail of female zebrafish (*Danio rerio*) 60 days post-hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L). Data below the boxes indicate the total number of female fish in each exposure group.

4.7.2 Vitellogenin synthesis in male zebrafish (*Danio rerio*) exposed to 4-*tert*-pentylphenol

The concentrations of vitellogenin (vtg; ng vtg/g fish) in head and tail of male zebrafish exposed to different concentrations of 4-*tert*-pentylphenol were measured by ELISA (Fig. 4.7.2). The average concentration of vitellogenin ranged between 1 and 5 ng vtg/g fish, which is approximately 2 ng less than in females. A significant increase of the vitellogenin concentration in male zebrafish compared to the control was found at 100 and 320 µg/L 4-*tert*-pentylphenol.

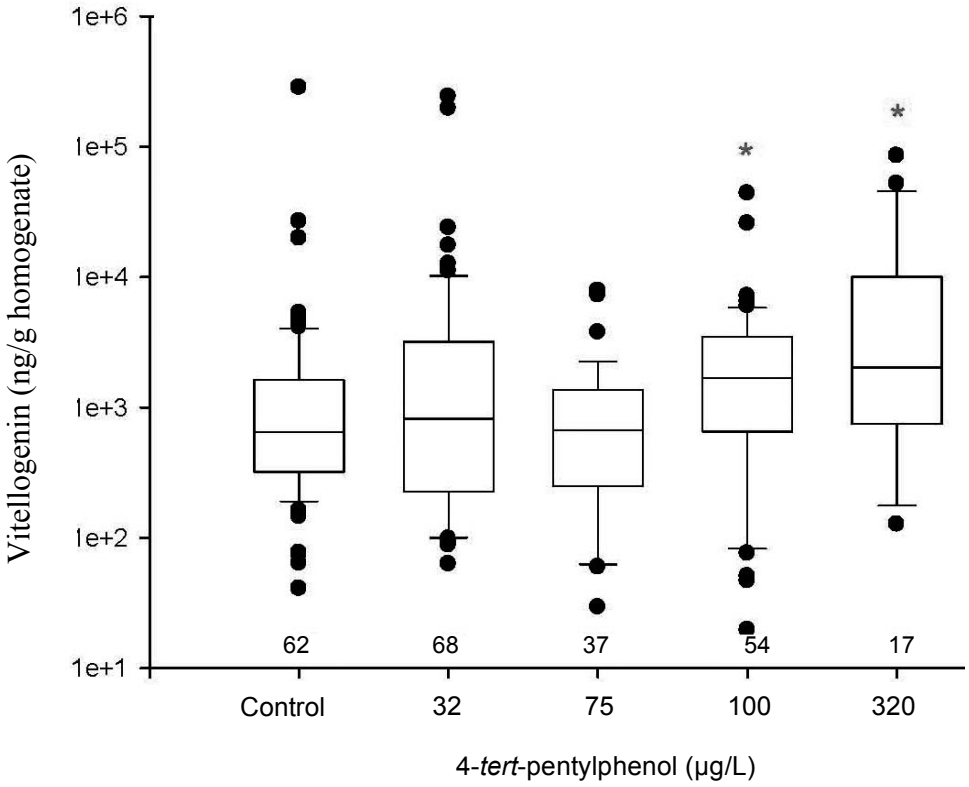


Fig. 4.7.2: Concentrations of vitellogenin (vtg; ng vtg/g fish) in head and tail of male zebrafish (*Danio rerio*) 60 days post-hatch (dph) exposed to different concentrations of 4-*tert*-pentylphenol. Asterisks: Values differ significantly from the negative control (Anova on ranks; * $p \leq 0.05$). n = 4 (n =2 at 75 µg/L). Data below the boxes indicate the total number of male fish in each exposure group.

4.8 Acute fish embryo toxicity assay with zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

4.8.1 Fish embryo assay 24 hours post-fertilization

Following endpoints were recorded after 24 hours of exposure to different concentrations of 4-*tert*-pentylphenol, dimethylsulfoxid (DMSO; solvent control), 3.4-dichloranniline (DCA; positive control) and artificial water (negative control): the number of coagulated eggs (lethal effects) and the number of normal and abnormal (sublethal effects) developed embryos (% of survived embryos). Four replicates were run with respectively 24 eggs per concentration (Fig. 4.8.1). 3.6 % of the zebrafish from the negative control (artificial water) were coagulated, 2.4 % at the positive controls (DCA) and 7.1 % at the solvent control (DMSO). The percentage of coagulated eggs increased constantly and significantly with increasing concentrations of 4-*tert*-pentylphenol. At the highest concentration, 5 mg/L, only 2.8 % of the embryos survived, which is 34 times less if compared to the negative control. Abnormally developed embryos were found at the positive control (11 %) and with increasing concentration of 4-*tert*-pentylphenol. 5.7 % of the embryos were abnormally developed at 3 mg/L 4-*tert*-pentylphenol and even 50 % at 5 mg/L. No additional core endpoints of the fish embryo assay were found. The LC_{50} after 24 hours of exposure was 3.5 mg/L. The EC_{50} for sublethal effects was 5 mg/L.

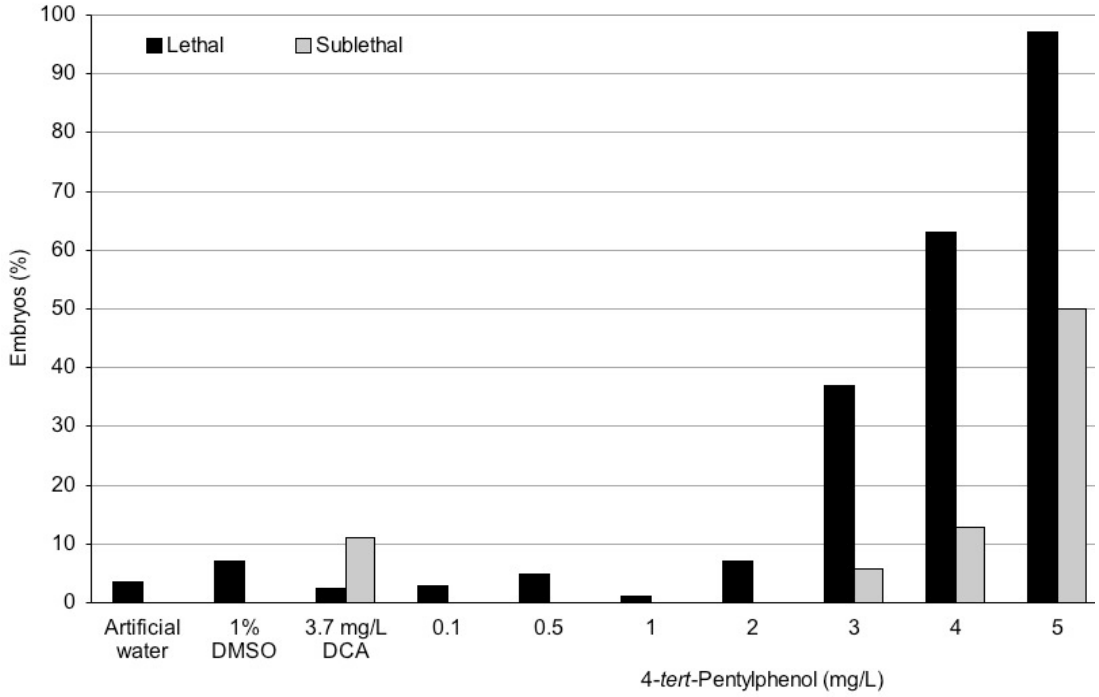


Fig. 4.8.1: Mortality and development of zebrafish (*Danio rerio*) embryos after 24 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

4.8.2 Fish embryo assay 48 hours post-fertilization

The same endpoints as after 24 hours post fertilization were recorded after 48 hours (Fig. 4.8.2). No additional eggs coagulated in the negative control. In the solvent control, the number of coagulated eggs increased from 7.1 % to 8.3 %. In the positive control, the number of coagulated eggs increased from 2.4 % to 6 % and the number of abnormally developed embryos increased from 11 % to 81 %.

Most abnormally developed embryos due to 4-*tert*-pentylphenol were found in the 5 mg/L exposure group, with 50 % of the surviving embryos. At a concentration of 2 mg/L 4-*tert*-pentylphenol, no abnormal embryos were found after 24 hours post-fertilization, but after 48 hours, 1.3 % were categorized as abnormal. The LC_{50} after 48 hours of exposure was 3.5 mg/L. The EC_{50} for sublethal effects was 5 mg/L.

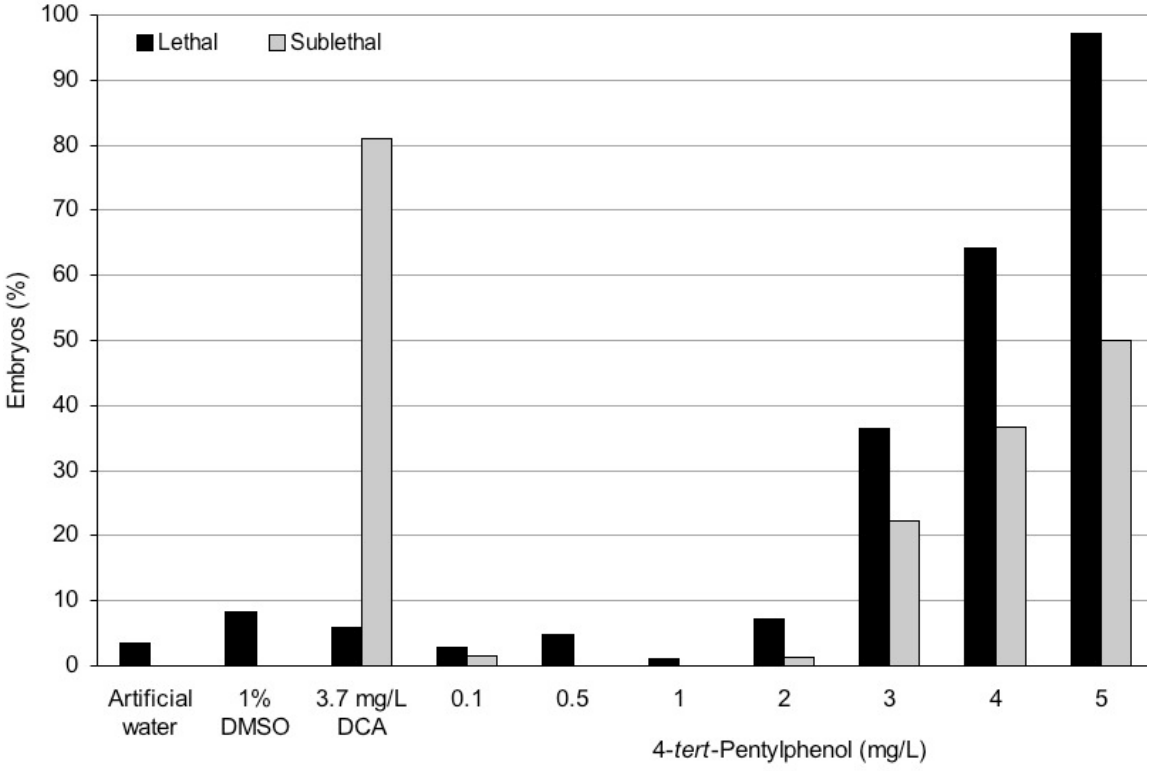


Fig. 4.8.2: Mortality and development of zebrafish (*Danio rerio*) embryos after 48 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

4.8.3 Fish embryo assay 72 hours post-fertilization

After 72 hours of exposure, the success in hatching was determined in addition to the endpoints recorded after 24 and 48 hours post-fertilization (Fig. 4.8.3a). 72.8 % of the embryos in the negative control had hatched, whereas less than a half (26.6 %) had hatched at the 4 mg/L 4-*tert*-pentylphenol concentration. Lethal and sublethal effects did not increase significantly if compared to 48 hours post-fertilization. Spinal distortions of hatched embryos correlated to the concentration of 4-*tert*-pentylphenol (Fig. 4.8.3b). Embryos from negative, positive and solvent controls did not show any spinal distortions. The percentage of spinal distortions increased with the concentration of 4-*tert*-pentylphenol: at 0.1 mg/L, all hatched embryos were normally developed, but at 0.5 mg/L 1.3 % had spinal distortions. The percentage increased extremely (1mg/L: 32.4 %, 2 mg/L: 87.5 %) to 100 % at concentrations of 3, 4 and 5 mg/L 4-*tert*-pentylphenol. The LC_{50} after 72 hours of exposure was 3.5 mg/L. The EC_{50} for sublethal effects was 5 mg/L. The EC_{50} for delay of hatch and spinal distortions was 1.4 mg/L.

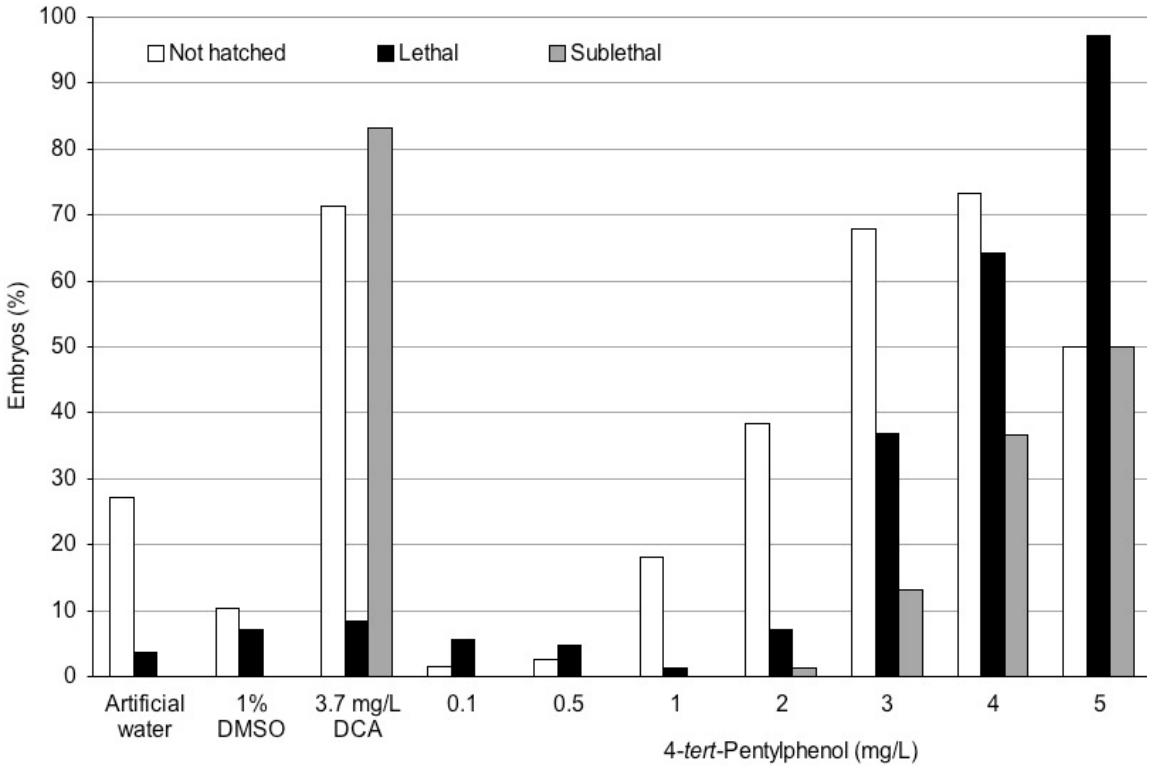


Fig. 4.8.3a: Mortality, hatch and development of zebrafish (*Danio rerio*) embryos after 72 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

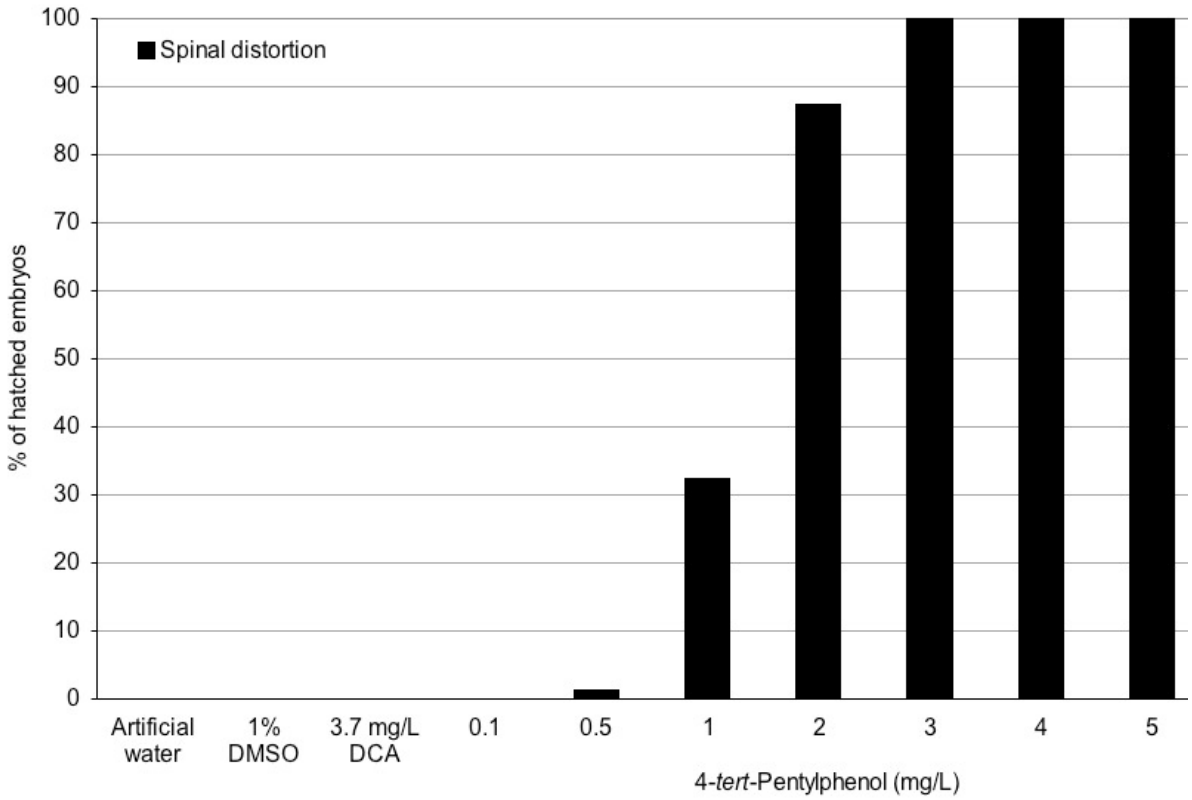


Fig. 4.8.3b: Spinal distortions of hatched zebrafish (*Danio rerio*) embryos after 72 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

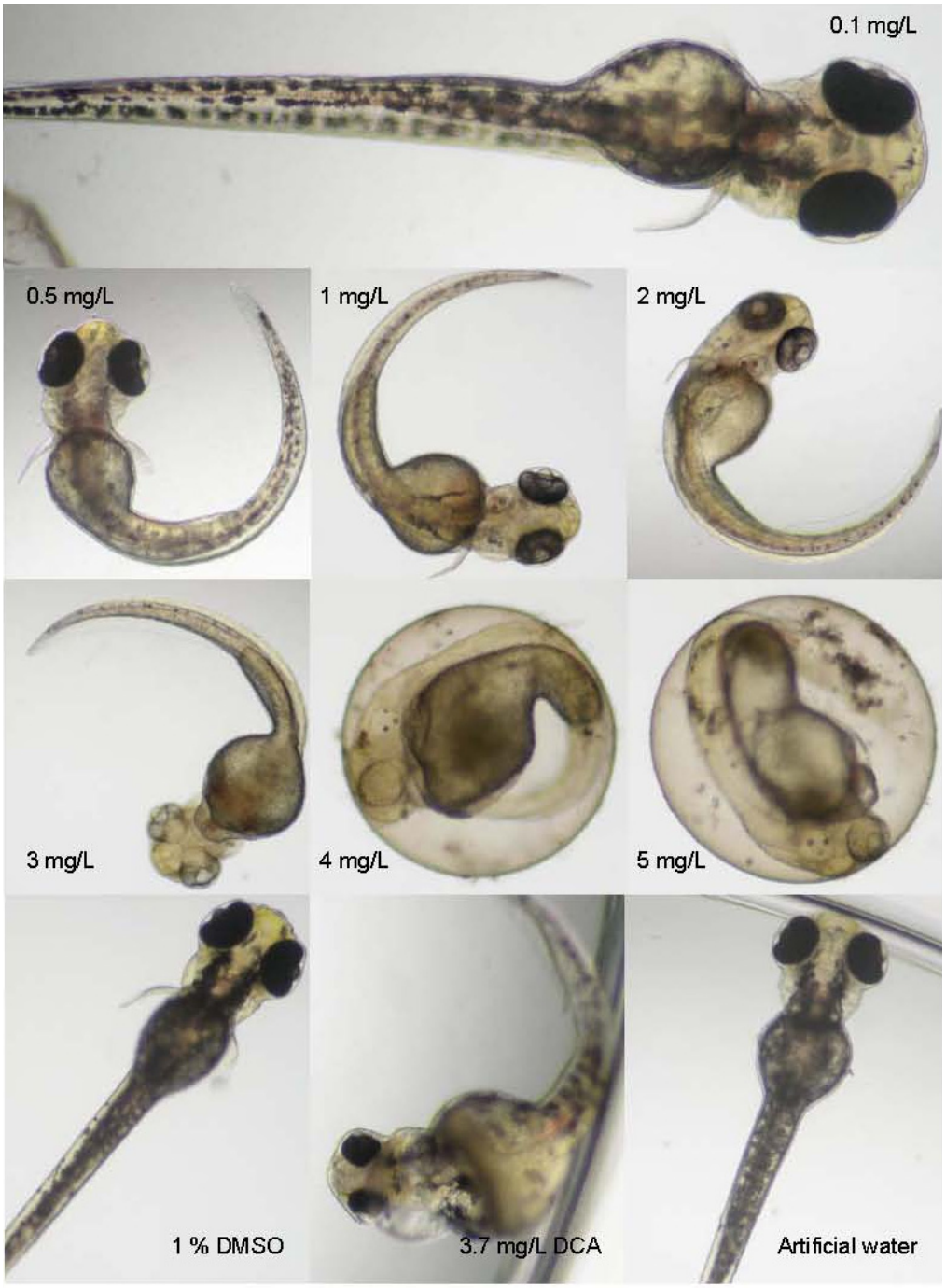


Fig. 4.8.3c: Zebrafish (*Danio rerio*) embryos 72 hours post fertilization. Embryos exposed to 4-*tert*-Pentylphenol had spinal distortions and retardation of hatch and pigmentation at high concentrations. Embryos from the water and solvent controls did not show any abnormalties. Embryos from the positive control had several developmental abnormalties, like edema.

4.8.4 Fish embryo assay 96 hours post-fertilization

Success in hatching, lethal and sublethal effects were determined after 96 hours of exposure (Fig. 4.8.4a). Lethal and sublethal effects only increased in the positive control (14.3 % lethal, 97.2 % sublethal). The percentage of hatched embryos increased at all concentrations (100 % at solvent control and 0.5 mg/L 4-*tert*-pentylphenol) and the percentage of hatched embryos with spinal distortions decreased at higher concentrations of 4-*tert*-pentylphenol (Fig. 4.8.4b).

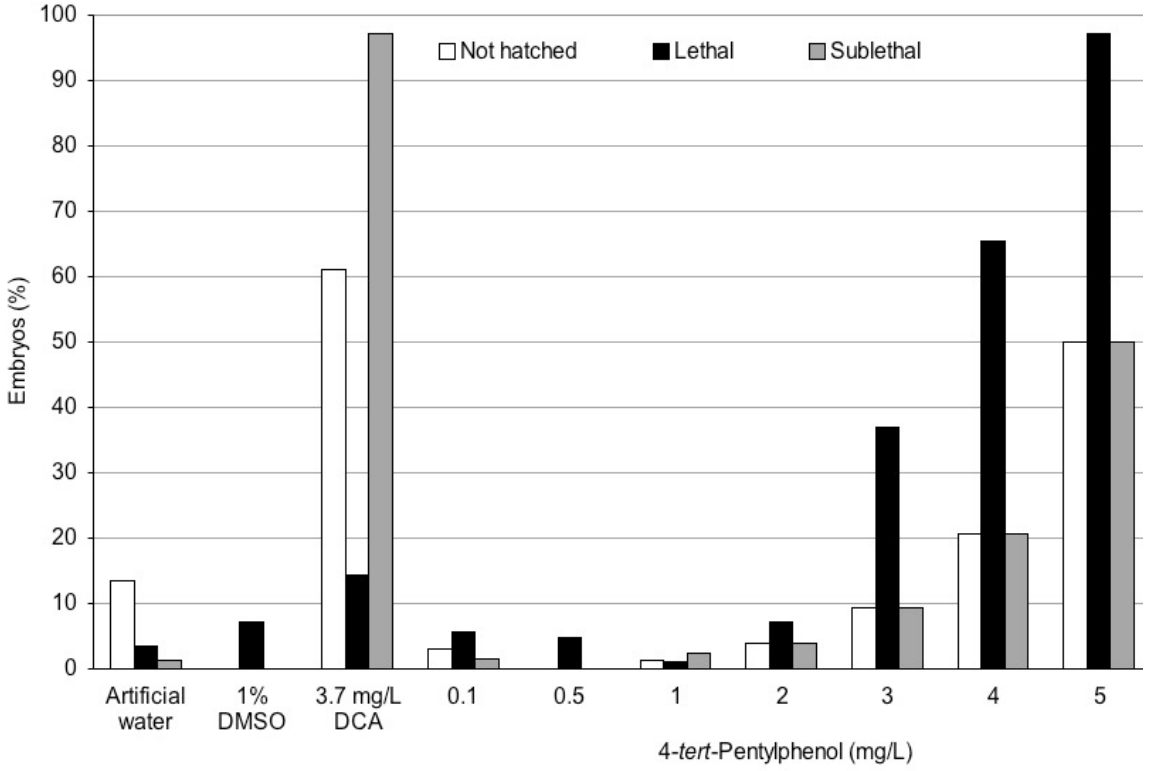


Fig. 4.8.4a: Mortality, hatch and development of zebrafish (*Danio rerio*) embryos after 96 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

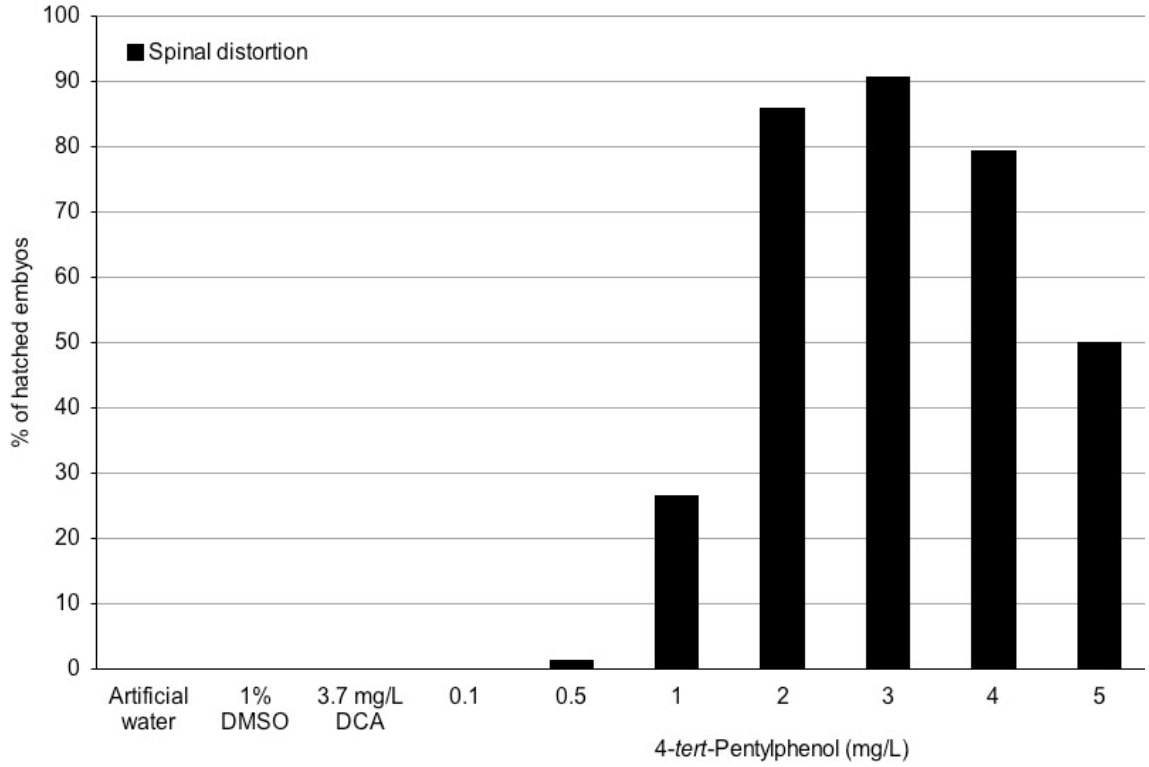


Fig. 4.8.4b: Spinal distortions of hatched zebrafish (*Danio rerio*) embryos after 96 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

4.9 Pigmentation of zebrafish (Danio rerio) eyes exposed to 4-tert-pentylphenol

The degree of pigmentation of the embryos eyes was detected after 48, 72 and 96 hours post-fertilization (Fig. 4.9a - c). The relative brightness was scaled between 0 (white) and 100 (black). The mean value at the negative control after 48 hours was 46.3 (Fig. 4.9a). This value decreased by 15 % with increasing concentrations of 4-*tert*-pentylphenol finally reaching a value of 38.5 at 5 mg/L. The same tendency could be determined after 72 hours (Fig. 4.9b): the highest degree of pigmentation was reached in the solvent control (81.5) and the lowest at 4 mg/L 4-*tert*-pentylphenol (55.4), which is 30 % less. The range of values became smaller after 96 hours of exposure, but still showed a correlation with the concentration of 4-*tert*-pentylphenol (Fig. 4.9c): 74.4 at artificial water and 63.4 (15 % less) at 4 mg/L 4-*tert*-pentylphenol. Fig. 4.9d shows the average values for 48, 72 and 96 hours of exposure: The mean value of the embryos from 4 mg/L 4-*tert*-pentylphenol (53) was 18 % lower than that of the negative control (65.1).

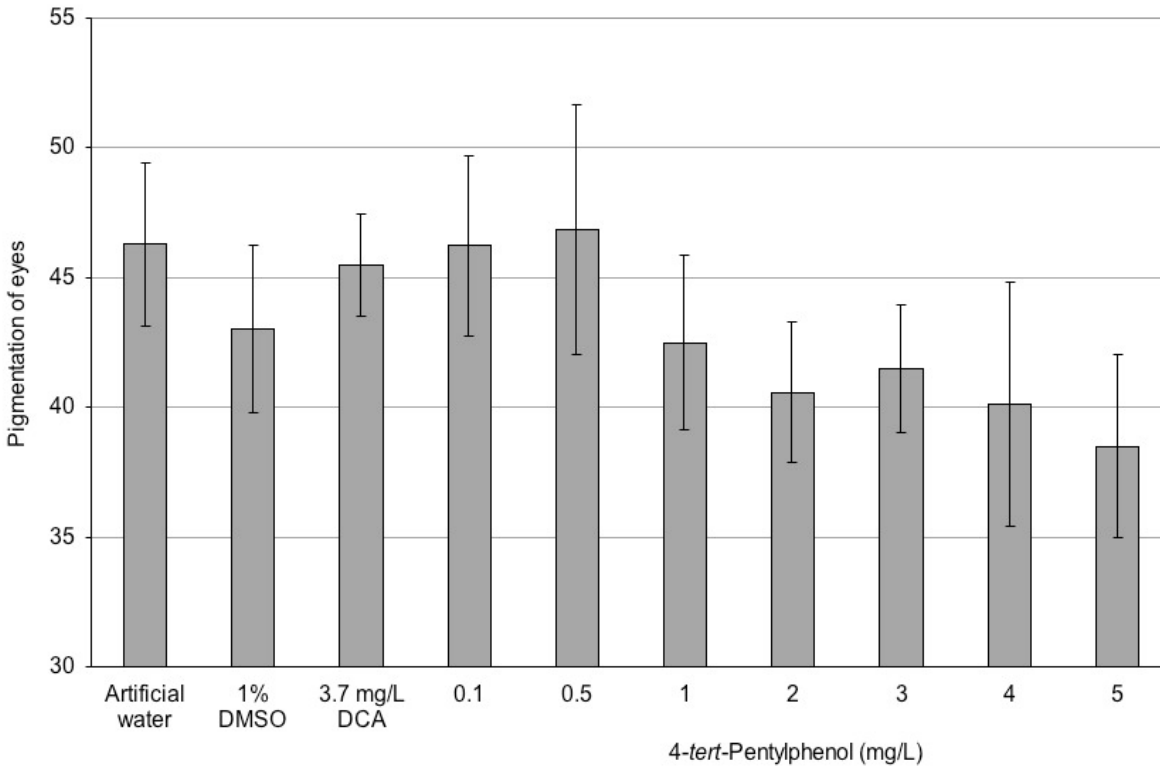


Fig. 4.9a: Pigmentation of zebrafish eyes after 48 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

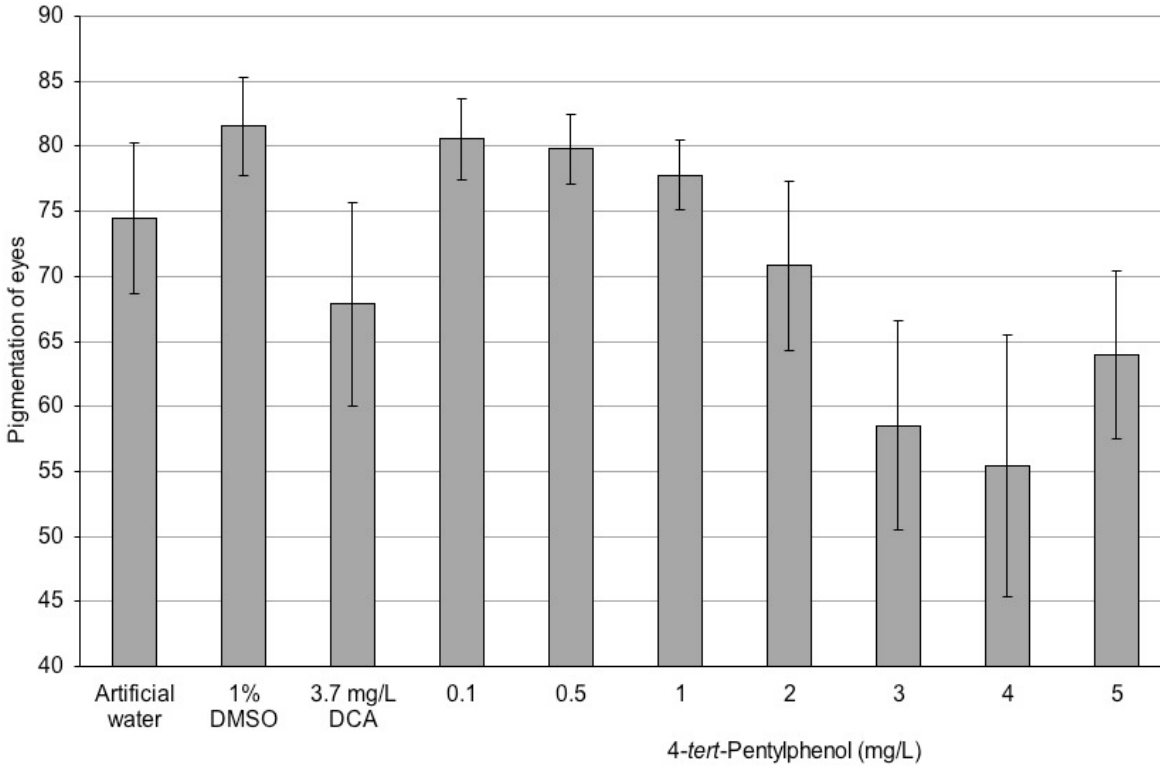


Fig. 4.9b: Pigmentation of zebrafish eyes after 72 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

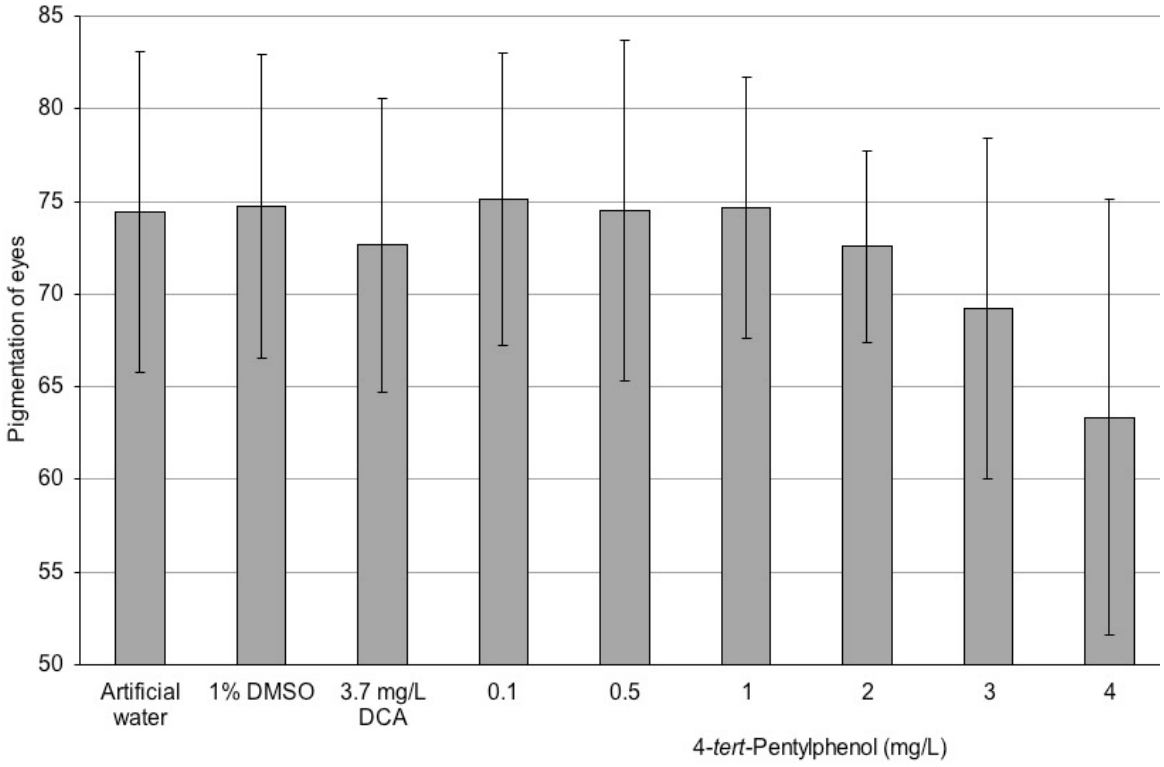


Fig. 4.9c: Pigmentation of zebrafish eyes after 96 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

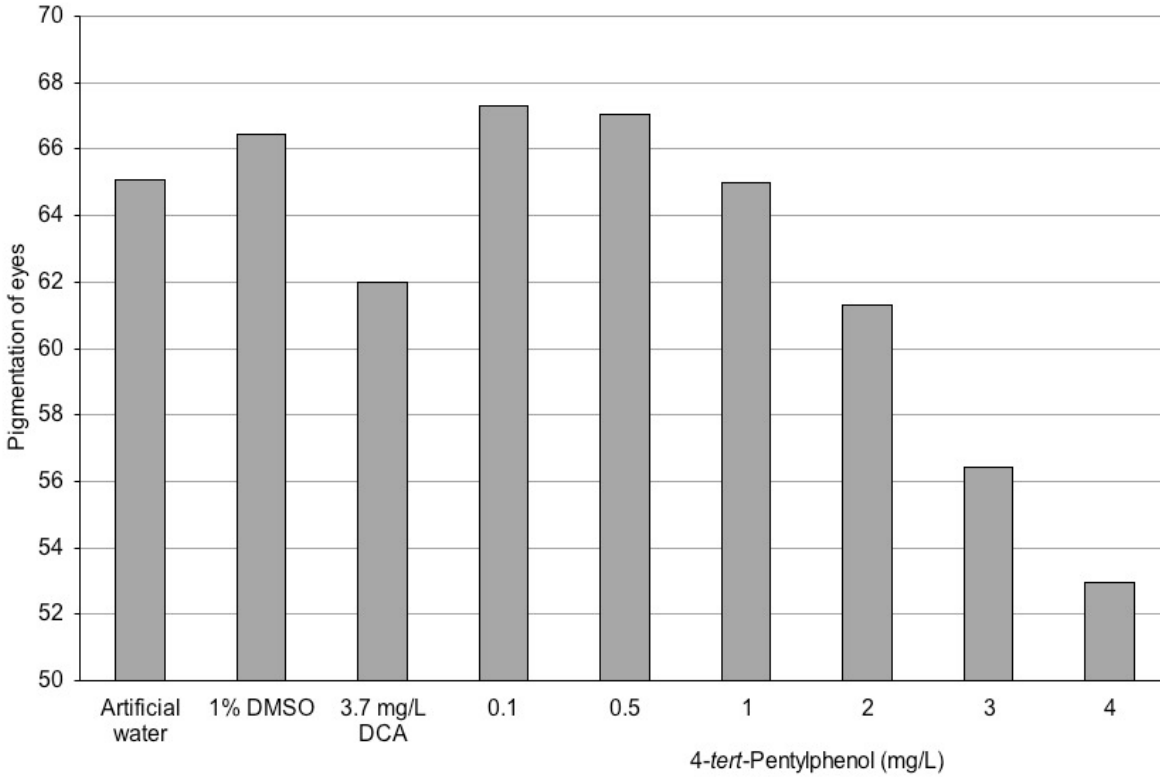


Fig. 4.9d: Average pigmentation of zebrafish eyes after 48, 72 and 96 hours of exposure to 4-*tert*-pentylphenol; n = 4; 24 eggs per replicate.

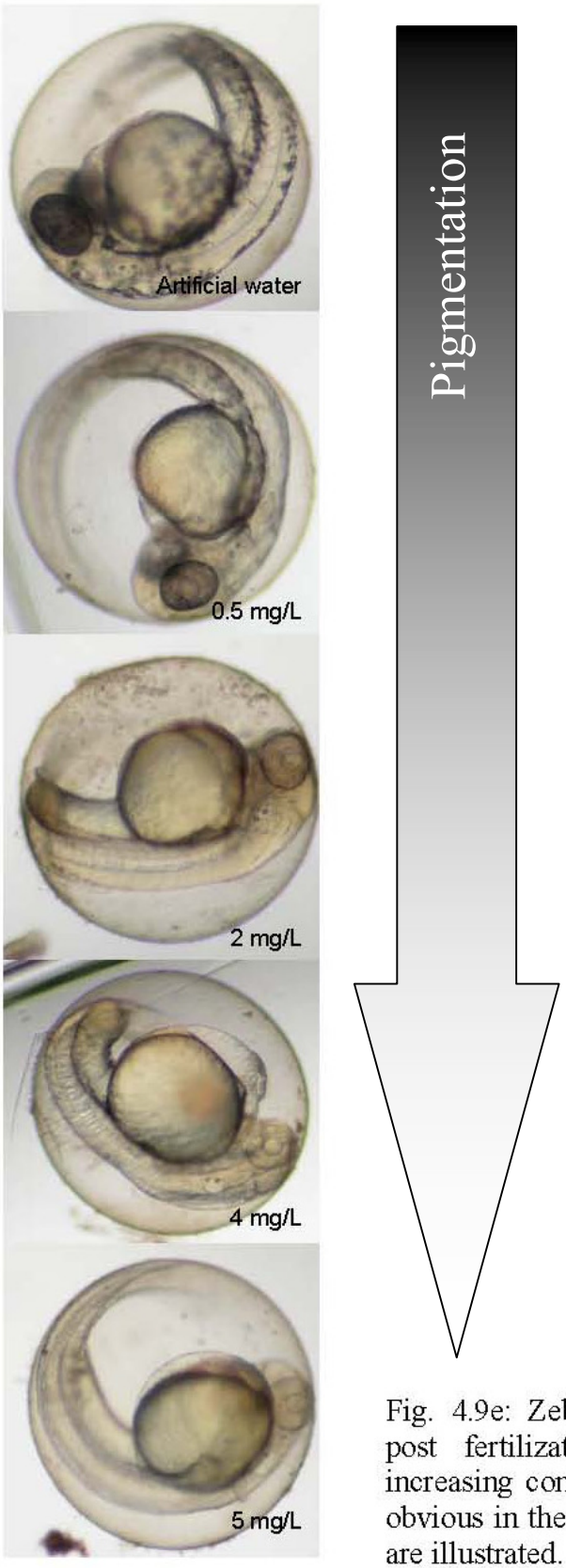


Fig. 4.9e: Zebrafish (*Danio rerio*) embryos 48 hours post fertilization. The loss of pigmentation with increasing concentration of 4-*tert*-Pentylphenol is most obvious in the eyes of the fish. Not all exposure groups are illustrated.

5. Discussion prochloraz

5.1 Sexual differentiation and vitellogenin synthesis in zebrafish (Danio rerio) within the scope of a FSDT with prochloraz

Sexual differentiation and vitellogenin synthesis in zebrafish are directly correlated to the hormonal status of the fish (Holbech 2001, 2006). Therefore, these two endpoints will be discussed together. 60 days post-hatch, the sex ratio of all zebrafish (*Danio rerio*) was determined by means of histological investigations (Fig. 3.3). A clear dose-dependent masculinization could be determined after prochloraz exposure combined with a significant reduction of female fish at all concentrations. The percentage of testis-ova increased significantly at concentrations of 150 and 300 µg/L prochloraz. The results from the present study thus correlate well with those from other studies (Ankley 2005, Blystone 2007, Kinnberg 2007, Vinggaard 2005) and prove the aromatase-inhibiting effects of prochloraz by inhibition of the estrogen synthesis responsible for the masculinization. Inhibition of the aromatase enzyme activity during a critical developmental period can cause genetically female fish to develop as phenotypic males (Kitano 2000; Kroon & Liley, 2000). In the frame of another FSDT, Kinnberg et al. (2007) also exposed zebrafish to prochloraz (16 - 202 µg/L) and also observed significant masculinization at the highest concentrations. The percentage of females decreased with increasing concentration of prochloraz in parallel to an increase in the percentage of undifferentiated and intersex fish.

These results are in accordance with the common opinion that exposure to EDCs in the transition stage of sexual development in zebrafish can cause a shift in sex ratio (Maack 2003). As the balance between estrogens and androgens is essential for sexual differentiation in fish (Baroiller 1999), the shift in sex ratio is probably due to the aromatase-inhibiting effect of prochloraz and therefore an endocrine effect. The lack of estrogen in the critical window of gonadal differentiation causes a masculinization (Maack 2003), since the surplus of testosterone supports the development of testes. This hormonal misbalance probably causes the increase of intersex/testis-ova, as well. One possible explanation for this phenomenon is that intersex fish might be retarded in their development. Their sexual differentiation is still in the juvenile hermaphroditic stage, where ovaries are transformed into testes. Another explanation might be that the deficit of estrogens and the surplus of androgens cause a masculinization of gonads, which were genetically determined to be ovaries, but are transformed to testes now.

The shift in sex ratio is directly correlated to the changes in vitellogenin synthesis, since both dependent on hormone levels. All zebrafish exposed to prochloraz (except those at 75 µg/L) showed a significant dose-dependent decrease of vitellogenin concentrations compared to the controls. This effect is consistent with the aromatase-inhibiting features of prochloraz, since it causes reduced estrogen levels. Especially in early stages of sexual differentiation, as in the present study, the occurrence of vitellogenin might be due to the transformation of ovaries to testes in the maturing male zebrafish. This might explain why males from the control group had vitellogenin concentrations of 3 ng/g homogenate in their head and tail.

Other studies also documented that aromatase inhibitors reduce vitellogenin synthesis in fish. Kinnberg et al. (2007) found a significant decrease in vitellogenin concentrations in female fish exposed to 202 µg/L prochloraz in a FSDT. However, they observed elevated concentrations of vitellogenin in male fish exposed to low concentrations of prochloraz and a decrease at higher concentrations. This discrepancy to the present study might be due to the fact that zebrafish in the FSDT of Kinnberg and colleagues were more mature compared to the control group. Immature males could have higher levels of vitellogenin as remnants from the ovaries they had during their sexual development. Studies with short periods of exposure support this thesis: Haverkate (2005) exposed zebrafish 21 days post-hatch to prochloraz and found a slight tendency of vitellogenin reduction in males. Transformation from ovaries to testes (juvenile hermaphroditism) starts approx. 23-25 days post hatch (Takahashi 1977) and might explain the high levels of vitellogenin in immature males. In an OECD validation ring test of the 21 - day fish screening assay with adult fish (OECD 2006), vitellogenin levels in female zebrafish decreased dose-dependently after exposure to prochloraz and no significant effects were observed in male zebrafish (20 - 300 µg/L prochloraz). This is not surprising, since the gonads were completely differentiated in the adult males.

All these results are consistent with those of the present study. Prochloraz is correctly classified as endocrine disruptor and its presence in environment (e.g. agriculture) makes it a potential risk for organisms. Even though the FSDT is not sufficient to show an impact on reproduction, we may conclude that organisms of wildlife might be affected by prochloraz in their reproductive processes. Masculinization will definitely take influence on the population structure and may eventually result in extinction. Likewise, reduction of vitellogenin synthesis in females might cause problems in egg production and, thus, in reproductive success.

5.2 Maturation and growth of zebrafish (Danio rerio) exposed to prochloraz

Since only two females remained at the highest exposure groups (300 and 600 µg/L), their development was excluded from interpretation. Likewise, statistical analysis for the 10 remaining fish in the 150 µg/L treatment group was also critical. Therefore, these results should be classified as a tendency rather than as effects.

The 3 remaining groups showed that prochloraz causes a slight developmental retardation of female gonads, if compared to the control. The percentage of females at late vitellogenic stage (stage 3) decreased strongly at all concentrations of prochloraz, whereas the percentage of immature females (stage 1) increased. In male zebrafish, a slight underdevelopment due to prochloraz could also be determined. Immature male fish (stage 0) were only found at high concentrations of prochloraz. The percentage of stage 1 increased slightly and the number of more mature males from stage 2 decreased. The same tendencies were revealed by the assessment of the maturity index. This approach allows to sum up the stages of maturity from all fish of one exposure group, which gives an overview about the rate of maturation. The use of maturity indexes as fixed values for each stage facilitates statistical analyses.

It should be noted that replicates of one exposure group were combined for reasons of simplification, since no significant differences among the replicates could be determined. A statistical significantly reduced maturation stage could only be determined in zebrafish at the lowest concentration of prochloraz (37.5 µg/L). All other exposure groups did not differ significantly from the control, however a slight tendency towards underdevelopment could be seen (mean values were all lower than in controls but standard deviations were high). These results show that similar tendencies of underdevelopment are caused by prochloraz in both sexes. Kinnberg et al. (2007) revealed basically similar effects in a FSDT with zebrafish exposed to prochloraz (16 - 202 µg/L). They observed significant underdevelopment of ovaries after exposure to 16 and 202 µg/L prochloraz, but males were more mature than in the control groups. Since the concentrations were higher in the present study, it cannot be excluded that the results differ from each other.

Prochloraz did not only affect maturation of the zebrafish, but also their growth. Results concerning growth and maturation are discussed together because there is a clear relation between both (see chapter 7).

Female and male zebrafish exposed to the highest concentration (600 µg/L) of prochloraz showed significant decreases of length and weight compared to the control, but growth was slightly increased at low concentrations. This might be explained by the effect of hormesis,

which is a dose-response phenomenon characterized by low-dose stimulation, high-dose inhibition and resulting in either a J-shaped or an inverted U-shaped dose-response curve. Kinnberg and colleagues (2007) did not find any affection of growth, which again can be explained by the lower concentrations they used. Haverkate (2005) exposed zebrafish to prochloraz (16 - 202 µg/L) for 21 days post-hatch and did not report any effects on growth of males, but a slight reduction of length in females. The results are difficult to compare, since the period of exposure was significantly shorter in this study and the concentrations were lower.

It is probable that a combination of endocrine and toxic effects of prochloraz caused the reduction of growth and maturation in the present study. Endocrine effects might have caused the gonadal underdevelopment of females: aromatase inhibition by prochloraz causes inhibition of estrogen synthesis (Hinfray 2006), which is needed for sexual maturation of females. This explanation however, does not readily explain the effects in males: the surplus of testosterone should support the maturation of testes.

There are several negative and positive feedback mechanisms within the hypothalamic–pituitary–gonadal axis that could be affected by xenoestrogens. For example, exogenous estrogens can inhibit gonadotropin-releasing hormone synthesis in the hypothalamus or gonadotropin synthesis in the pituitary gland (Gray 1997). Gonadotropins do not act directly, but exert their action through gonadal biosynthesis of steroid hormones, which, in turn, mediate various events of gametogenesis (Nagahama, 1993). Toxic effects are also probable as an explanation for the underdevelopment of zebrafish, since toxic stress is known to inhibit growth in several species (Hoar 1979). Toxic effects of prochloraz, e.g. delayed hatch (indication for delayed development), are also revealed in the acute fish embryo toxicity assay (see below for discussion).

The endpoints maturity and growth are indicative of the negative effects of prochloraz on the development of zebrafish. Even though the FSDT is not sufficient to show effects on reproduction and the population level, it can be a good starting point for further considerations. It may be assumed that underdeveloped fish suffer from disadvantages in wildlife. Reduced maturation certainly causes problems in reproduction, which can, e.g. *via* a shift in sex ratio and reduced vitellogenin synthesis, reduce population fitness. Reduced growth is likely to be a further disadvantage, with respect to the interaction with predators and congeners.

5.3 Histopathology of zebrafish (Danio rerio) exposed to prochloraz

The histopathological alterations found in female zebrafish exposed to prochloraz occurred in the control and the exposure groups. Therefore, no correlation to the exposure with prochloraz could be revealed. These results are consistent with those from the FSDT conducted by Kinnberg et al. (2007), who did not detect any effects in females and males after exposure to prochloraz.

Ankley et al. (2005) only found histopathological effects in male fathead minnows (*Pimephales promelas*) after 21 days of exposure to prochloraz (increased number of spermatogonia). In contrast, the present study revealed two significant effects were found in male zebrafish exposed to prochloraz: fibrosis and increased number and size of Sertoli cells. The main function of Sertoli cells is to support the developing sperm cells through the stages of spermatogenesis by secretion of different substances. This might be the reason for their increase in zebrafish exposed to prochloraz. The aromatase-inibition by prochloraz causes elevated levels of testosterone, which is responsible for maturation of the testes. The surplus of testosterone could therefore induce increased activation of the Sertoli cells, as was evident by hypertrophy and hyperplasia.

Another function of Sertoli cells is to control the transfer of nutrients, hormones and other chemicals into the testicular ducts. Since prochloraz is toxic, it might induce high activity of Sertoli cells as a protective process and, therefore, result in hypertrophy and hyperplasia. This toxic effect might also explain the dose-dependent occurrence of fibrosis. The development of connective tissue is normally interpreted as a result of inflammatory processes, and can be caused by chemicals or pathogens. Missing tissue is then replaced by collagen fibers. It is well possible that the toxic stress caused by prochloraz induced inflammation and.

5.4 Toxic effects of prochloraz

Beside the endocrine effects of prochloraz, two endpoints were studied in order to assess its toxic effects: After 30 days of exposure, the mortality of zebrafish in the FSDT was determined. In addition, an acute fish embryo toxicity assay was conducted. Both revealed significant toxic potentials of prochloraz, but due to the exposure design, in different concentration ranges. A significantly higher percentage of dead fish in the FSDT compared to the control was found at the highest concentration (600 µg/L prochloraz), where almost 50% of the fish died. Clear dose-dependant toxic effects were determined in the fish embryo assay with respect to hatch (EC_{50} delay of hatch 72 h: 1 mg/L) and lethality (LC_{50} 24 and 48 h: 2.9 mg/L). These results correlate very well with those from other studies: acute toxicity tests in fish gave a 96 h toxicity LC50 of prochloraz in rainbow trout (*Oncorhynchus mykiss*), harlequin fish (*Harlequin rasbora*) and bluegill sunfish (*Lepomis macrochirus*) in the range of 1.5 - 2.9 mg/L (Larsen 2004). However, when exposing juvenile rainbow trouts for up to 21 days to concentrations of 7.9 and 65.9 µg/L prochloraz, no mortality was observed (LeGac 2001). This difference is easily explainable by the lower concentrations used in the latter study compared to the present one.

As for the endocrine disruptive features of prochloraz, the toxic properties are also relevant for the situation in the environment: Elevated concentrations in aquatic systems are likely to affect fish populations.

6. Discussion 4-*tert*-pentylphenol

6.1 Sex ratio of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

Exposure of zebrafish for 60 days post-hatch to 4-*tert*-pentylphenol caused a clear-cut feminization as well as decreased maturity in both sexes (Fig. 4.3). At the highest concentration, only 10.63 % of the fish were categorized as males, which is nearly 4-fold lower than in the control. Several studies revealed that exposure to alkylphenols as pseudoestrogenic compounds in the sensitive stage of gonadal differentiation in fish causes a bias towards females in different species: Tanaka et al. (2002) exposed *Rivulus marmoratus* (Osteichthyes, Cyprinodontiformes), a self-fertilizing, hermaphroditic species, to 150 and 300 µg/L nonylphenol and reported feminization and underdevelopment of gonads. Yokota et al. (2005) as well as Seki et al. (2003) showed the same results after exposing medaka (*Orizyas latipes*) to 4-*tert*-pentylphenol in a concentration range between 121 and 931 µg/L.

Holbech et al. (2006) performed an FSDT with zebrafish exposed to three natural estrogens: estrone, 17β-estradiol, and estriol. The sex ratio was significantly shifted towards females from 49 ng/L estrone, 54 ng/L 17β-estradiol and 22 µg/L estriol, respectively. These studies, as well as the present one, reveal the estrogenic effect of alkylphenols (including 4-*tert*-pentylphenol) and, of course, natural estrogens. Zebrafish (*Danio rerio*) are protogynic, which means, that all fish first develop ovaries, from which approximately 50% will be transformed to testes later. The transformation starts approx. 25 days post-hatch. Maack et al. (2003) called this stage of sexual differentiation the “transition stage”. Endocrine disruption by xenoestrogenic compounds in this stage causes agonistic effects on the estrogen receptors in the fish and therefore inhibits the transformation of ovaries to testes. As 4-*tert*-pentylphenol acts as a pseudoestrogen, the observed feminization might be related to this agonistic effect in the transition stage. On the other hand, it may be discussed whether the increase of undifferentiated fish is an endocrine or a toxic effect. Reasons for the underdevelopment are discussed in 6.4.

A dose-dependent correlation for the development of intersex/testis-ova could not be determined in the present study. This observation is consistent with those from several other studies, which could not relate the occurrence of testis-ova in zebrafish to exposure to both natural estrogens and alkylphenols (Hill & Janz 2003, Oern 2003, Brion 2004, Weber 2003). However, after exposing medaka to different alkylphenols, testis-ova were induced (Seki 2003). Since the gonadal development of zebrafish differs from that of medaka (Oern 2006),

interspecies comparisons regarding the histopathology of gonads are difficult, and obvious differences are necessarily not contradictory.

Since 4-*tert*-pentylphenol causes feminization and underdevelopment, it poses a potential risk for wildlife organisms. Therefore, a classification of this substance as an EDC is mandatory. Even though the FSDT is not sufficient to show an impact on reproduction, the conclusion can be drawn that wildlife might be well affected by 4-*tert*-pentylphenol in its reproductive success. A population, mainly consisting of females and underdeveloped individuals is likely to have difficulties in reproductive behavior.

6.2 Maturation and growth of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

Male and female zebrafish (*Danio rerio*) were considerably underdeveloped at high concentrations of 4-*tert*-pentylphenol (concerning maturity and growth). A very high percentage of female and male zebrafish belonging to the stage of maturity 0 (immature gonads) was found at the highest concentration (320 µg/L). Additionally, the number of undifferentiated fish increased significantly at the highest concentration. These observations are supported by analysis of the maturity index (see 4.4.3). This method enables to summarize the stage of maturity of all fish from one exposure group in a single number. All female exposure groups, except at 75 µg/L, showed a significant decrease of the maturity indices compared to the control group. Male fish were significantly underdeveloped at the highest concentration (320 µg/L). This approach corroborates the results from the sex ratio, which showed that 4-*tert*-pentylphenol causes a distinct underdevelopment.

The delayed development in female and male zebrafish after exposure to 4-*tert*-pentylphenol might be related to the estrogenic characteristics of 4-*tert*-pentylphenol or its toxicity. Since male zebrafish need high levels of testosterone for maturation (Hoar 1969), the estrogenic activity of 4-*tert*-pentylphenol might be responsible for the observed underdevelopment of male fish. On the other hand, females need estrogens for maturation, which is a contradictory on a first glance.

Several studies were published which revealed similar effects of natural estrogens or alkylphenols: Fenske et al. (2005) showed that exposure to ethinylestradiol caused 20 % immature ovaries in zebrafish after 118 days of exposure. They also documented a suppression of gametogenesis in a life-cycle assay with ethinylestradiol. Hill & Janz (2003) reported delayed sexual maturation of zebrafish after exposure to ethinylestradiol at 60 days post-hatch. Similar results were achieved by Weber et al. (2003), who found a suppression of

gametogenesis in zebrafish dependent on the concentration of ethinylestradiol and nonylphenol they were exposed to. Estrogenic compounds may also influence the growth of gonads: Van den Belt et al. (2001) showed that ethinylestradiol and octylphenol reduced both the growth of testes and of ovaries in zebrafish.

An explanation, which is probably most relevant at high doses, are the direct cytotoxic (Kinnberg 2000; Kime 1999) and toxic (EPA 2007) effects of alkylphenols. However, the endocrine disruptive action of 4-*tert*-pentylphenol seems to be more likely to explain the inhibited sexual maturation. The disruption of one component of the hormonal network can disturb diverse mechanisms. For example, the agonistic effects of 4-*tert*-pentylphenol on the estrogen receptor in the organism may down-regulate the pituitary, which is also responsible for the regulation of several other hormones with manifold effects (e.g. sexual maturation and growth). The pituitary releases gonadotropins, which do not act directly, but exert their action through gonadal biosynthesis of steroid hormones, which, in turn, mediate various events of gametogenesis (Nagahama, 1993).

Apart from gonadotropins, several other hormones such as thyroxin, triiodothyronin, and growth hormone have also been implicated in the regulation of the oocyte growth including vitellogenesis (Gomez 1999; Pavlidis 2000). Tokumoto et al. (2005) suggested that different EDCs can act as agonists or antagonists in the induction of oocyte maturation in fish. Oocyte maturation in fish is triggered by the maturation-inducing hormone (MIH), which acts on receptors located on the oocyte membrane and induces the activation of maturation-promoting factor (MPF) in the oocyte cytoplasm. Similar processes exist in males. Disturbance of the estrogen balance by EDCs could, therefore, induce the underdevelopment observed in zebrafish exposed to 4-*tert*-pentylphenol.

Similar effects might be responsible for the growth inhibition found at different concentrations of 4-*tert*-pentylphenol. No clear dose-dependent effects on growth could be determined in the FSDT with zebrafish, but a significant decrease of length was found at concentrations of 75 µg/L (females and males) and 320 µg/L (females), and a significant decrease of weight occured at 75 µg/L (females and males). Research on alkylphenols has produced different results concerning the effects on growth. Some studies could not determine any effects of natural estrogens or alkylphenols on growth (Haverkate 2005, Gimeno 1998), others found only slight effects (Hill 2003). Only Seki et al. (2003), who studied the chronic effects of 4-*tert*-pentylphenol on the reproductive status of medaka (*Orizyas latipes*) over two generations under continuous exposure, observed significantly reduced growth after 60 days post-hatch at 931 µg/L. The comparability of these studies is problematic, since growth of fish

depends on several exogenous factors like nutrition, temperature, light and species. Comparison of different species from different laboratories (with different rearing conditions) cannot allow a general conclusion concerning the effects on growth of any chemical. Nevertheless, most studies prove at least weak effects of 4-*tert*-pentylphenol on growth.

A combination of toxic and endocrine effects is the most likely an explanation for the general underdevelopment of zebrafish in the present study. Toxic stress and disruption of hormonal processes are very likely to inhibit growth of body and gonads. These inhibitory effects of 4-*tert*-pentylphenol imply potential risk for wildlife organisms exposed to alkylphenols (e.g. in industrial areas). Especially the delay in maturation could affect individuals in their reproductive behavior, as well as reduced body size.

6.3 Vitellogenin synthesis in zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

4-*tert*-pentylphenol had gender-specific effects on vitellogenin synthesis in zebrafish: Females produced significantly less vitellogenin than controls at a concentration of 320 µg/L. A significant increase of vitellogenin concentration in male zebrafish compared to controls was found at 100 and 320 µg/L 4-*tert*-pentylphenol.

Since estrogens are known to induce the synthesis of vitellogenin (Lech 1996, White 1994, Holbech 2006), the effect on the females does, on first sight, not accord to the estrogenic properties of 4-*tert*-pentylphenol. Several other studies confirm this mode of action and therefore observed elevated vitellogenin levels in females and males (Haverkate 2005, OECD 2006). However, Seki et al. (2003) who exposed medaka for 101 days post-hatch to 4-*tert*-pentylphenol (51.1- 931 µg/L), also reported no elevated vitellogenin levels in females at concentrations lower than 224 µg/L, and significantly increased vitellogenin levels in males at 320 and 1000 µg/L 4-*tert*-pentylphenol.

In the present study and the experiment described by Seki et al. (2003), the vitellogenin induction did not occur, because females were significantly underdeveloped in all exposure groups (see chapter 4.4). Vitellogenin synthesis is directly correlated to oocyte maturation and yolk production. Thus, the low levels of vitellogenin are likely to be correlated to the immaturity of the females. Male zebrafish were also underdeveloped, but this might be the reason for the high vitellogenin levels measured. Immature males could have had higher levels of vitellogenin as remnants from the ovaries they had during their sexual development.

6.4 Histopathology of zebrafish (Danio rerio) exposed to 4-tert-pentylphenol

Only a marginal number of histopathological effects (minimal fibrosis and atresia) were found in female and male zebrafish after exposure to of 4-*tert*-pentylphenol (data not shown). Since these effects occured in both control and exposure groups, no correlation between effects and concentration of 4-*tert*-pentylphenol could be determined.

Other studies also revealed that 4-*tert*-pentylphenol (and nonylphenol) causes no severe histopathological abnormalities in zebrafish (Weber 2003, OECD 2006).

6.5 Toxic effects of 4-tert-pentylphenol

After 30 days of exposure, the overall mortality of all zebrafish (*Danio rerio*) in the FSDT was determined (Fig. 4.2). No correlation to exposure of 4-*tert*-pentylphenol could be determined, as the data showed high variation. This is in line with other studies showing that 4-*tert*-pentylphenol does not cause mortality at concentrations up to 1000 µg/L in zebrafish (Haverkate 2005), medaka and common carp (Gimeno 1998). Only Seki et al. (2003) published contradictory results, which reveal an LC_{50} of 693 µg/L for medaka exposed to 4-*tert*-pentylphenol 60 days post-hatch.

Although standard deviations of the present data for mortality between the replicates were high, the mean percentage of the highest concentration represents the maximum mortality within the whole test. This shows that 4-*tert*-pentylphenol had a tendency to increase mortality, even though it was statistically not significant.

This is confirmed by the results found in the acute fish embryo toxicity assay: Clear dose-dependent effects were determined concerning hatch, lethality (LC_{50} = 3.5 mg/L) and sublethality. These results are compatible with those from a report by the EPA concerning toxic effects of 4-*tert*-pentylphenol (EPA 2007), which gives an EC_{50} (96 h) for acute fish toxicity of 1.6 mg/L. The values are roughly in the same range, which is a confirmation for the sensitivity of the fish embryo assay compared to the acute fish assay.

The toxic effects of 4-*tert*-pentylphenol pose a potential risk for wildlife organisms, since it is widely spread in industrial use, together with other alkylphenols.

6.6 Effects of 4-tert-pentylphenol on pigmentation in zebrafish (Danio rerio) embryos

A clear dose-dependent decrease of pigmentation in embryos of zebrafish (*Danio rerio*) could be determined at 48, 72 and 96 hours post-fertilization. The pigmentation of their eyes was measured, which resulted in an approximately linearly descending curve. Thus, 4-*tert*-pentylphenol did disturb the normal development of pigmentation. In zebrafish, pigmentation is derived from two different sources: the pigment cells of the dermis and epidermis derive from the neural crest, and the pigment cells of the retina originate from the optic cup (Lister 2002). 4-*tert*-pentylphenol probably affected both: A central target of chemicals that interfere with pigmentation (e.g. 1-phenyl-2-thiourea (PTU), hydroquinone) is tyrosinase, (Camp 2001), which catalyses the oxidation of phenols (e.g. tyrosin) to melanin. A vague speculation could be that agonistic/competitive effects of 4-*tert*-pentylphenol disturbed tyrosinase activity. Maybe tyrosinase metabolized 4-*tert*-pentylphenol instead of tyrosine, since the two compounds are very similar in molecular structure and probably chemical features (Fig. 6.7). Consequently, less tyrosine was oxidized, and, therefore, less melanin was produced.

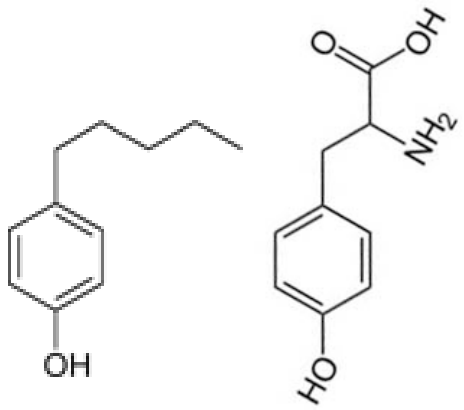


Fig. 6.7: 4-*tert*-pentylphenol (left) and tyrosine (right) have very similar molecular structures.

Research on this correlation is very scant, but there are studies that revealed that some xenoestrogene EDCs could also interact with the thyroid, as there is 'cross-talk' between the oestrogen and thyroid receptors (Waring 2005). In the latter study, agonistic effects of bisphenol A on thyroid hormone action were examined. Bisphenol A and triiodothyronine were found to be unexpectedly resembling in chemical structures. The same correlation was found between PCBs and thyroid hormones (Rickenbacher 1986). Triiodothyronine and thyroxine are thyroid hormones in the colloid of the thyroid and are also derived from tyrosine. Harvey & Williams (2002) also found out that plasticizers such as nonylphenol, bisphenol-A and butylbenzylphthalate are able to disrupt thyroxine binding to the transport protein transthyretin.

Of course, other (toxic) mechanisms caused by 4-*tert*-pentylphenol could also be the reason for the inhibition of pigmentation. Melanization of the outer retina is believed to be involved in responding to oxidative stress and in other aspects of metabolism (Schraermeyer & Heimann 1999). Further research is needed, which could be very helpful for scientific investigations that use transparent zebrafish. Inhibition of pigmentation by higher concentrations of chemical compounds may cause problems due to high mortality, reduced hatching frequency and teratogenesis. 4-*tert*-pentylphenol was proven to be toxic at high concentrations, but potentially it is less critical than chemicals currently used (PTU, hydroquinone). For example, PTU causes delayed hatching, retardation and malformation of embryos or larvae with increasing doses (Elsalini & Rohr 2003).

Another useful aspect of the present results is the method developed to quantify the reduction of pigmentation in the zebrafishs eyes. The embryos were photographed, and the brightness of their eyes was scaled between 0 (white) and 100 (black) with Adobe Photoshop (see 2.6.3). No technical method has yet been established to quantify the pigmentation of zebrafish embryos. The use of numerical systems based on optical inspection might be sufficient in some cases, but a more objective method should help to improve the quality of results. Various fields of research could profit from this simple and cost-effective method, for example investigations on pigmental disruption and painting compounds.

7. Correlation between maturation and growth in zebrafish (*Danio rerio*)

In the present study, a clear correlation between maturation and growth in zebrafish of both sexes could be determined. However, these results are in contrast to those of Maack and colleagues (2003), who could not reveal such an unequivocal relation between body size of zebrafish and their gonadal differentiation status. No other comparable data concerning zebrafish could be found, since examinations of growth in zebrafish have never been correlated to stages of maturity. In the present study, growth and maturity correlated in all exposure groups without any concentration-dependency of prochloraz and 4-*tert*-pentylphenol. No significant difference between fish was found, when comparing individuals of the same maturation grade throughout the different concentrations. Since mechanisms of growth and endocrine regulation are different, the results for female and male zebrafish are discussed separately.

In both sexes, body growth is, among others, under control of growth hormones. Growth hormones were found to interact with the reproductive axis (Zou 1997), e.g. modulate gonadal steroidogenesis (Nagahama 1993) in addition to its effects on somatic growth (Peter 1990) and osmoregulation (Sakamoto 2006). These observations indicate the existence of a pituitary GH–gonadal feedback system in teleost fish (Zou 1997). Central messengers of this system are sex steroids like testosterone and estradiol. Mature males produce androgens (testosterone) in their testes, which have growth-promoting effects (Hoar 1979). This explains why mature males are bigger than immature. Their mature testis is able to produce more testosterone, and, therefore, more GH is released.

To some extent, the same relation between growth and maturity can be found in females. Estrogens can also have anabolic effects, but may suppress secretion of growth hormones. The mechanisms behind this effect are vague, but some simple physical relation could at least explain why mature females are bigger than immature: late vitellogenic follicles are much bigger than immature ones, especially due to the high amount of yolk produced. It is evident that huge eggs require more space than small ones.

8. Evaluation of the FSDT

Tab. 8: Overview of the LOECs and NOECs from the FSDTs with prochloraz and 4-*tert*-pentylphenol.

Endpoint	**Prochloraz** (0, 37.5, 75, 150, 300, 600 µg/L)		**4-*tert*-pentylphenol** (0, 32, 75, 100, 320 µg/L)			
			LOEC		**NOEC**	
	LOEC	**NOEC**	*females*	*males*	*females*	*males*
Mortality	600	300	-	-	320	320
Sex ratio	37.5	-	320	320	100	100
Maturity	37.5	-	32	320	-	100
Growth	600	300	75	75	32	32
Vitellogenin	37.5	-	320	100	100	75
Histopathology	37.5	-	-	-	-	-

The present study is part of an evaluation study for the "fish sexual development test" FSDT (an extension of the existing OECD TG 210, fish early life stage toxicity test) as a draft proposal for an OECD guideline. The FSDT, in which changes in vitellogenin concentration and sex ratio are the main endpoints, has, among others, been proposed as a test guideline for the detection of EDCs affecting fish (Petersen 2001). The main idea is to establish an *in vivo* test for EDCs that is relatively easy, cheap and more rapid than full life cycle tests (FLCT).

One of the central questions is, if the FLCT could in some cases be replaced by the FSDT. Therefore, it is essential to evaluate the different endpoints examined in the FSDT and to compare them to those in a FLCT. Are they sufficient and sensitive enough for the detection of the endocrine potential of a chemical? Are the endpoints recorded of comparable sensitivity in both test systems?

One of the most striking and evident endpoints is the assessment of the sex ratio. The exposure duration of 60 days post-hatch was chosen in the FSDT with zebrafish (*Danio rerio*) since their sexual differentiation is accomplished at this point of time (even though they are not yet sexually mature). Endocrine disruption during the course of transformation from ovary to testes is known to cause a shift in sex ratio (Fenske 2005, Hill 2003, Holbech 2006, Kinnberg 2007, Maack 2004, Mc Allister 2003, Orn 2006). Thus, the assessment of a shift in sex ratio is definitely a useful biomarker for the detection of endocrine effects.

One of the main critiques for the FSDT is its insufficiency to show effects of EDCs on reproduction, as FLCTs can do. This is a disadvantage concerning a detailed assessment of an EDC, but a forecast concerning reproductive problems is nevertheless possible: an unequivocal feminization or masculinization of a population is likely to cause reproductive difficulties. The evaluation of other endpoints in the FSDT can give similar forecasts: The examination of the stages of maturity is a very important aspect that provides detailed information about the current state of reproduction in the fish. It is certain that an underdeveloped or even immature fish will have reproductive problems in wildlife. However, this observation cannot completely replace the detailed data concerning reproduction of a FLCT, but the results are of high predictive quality.

Both EDCs used in the present study caused sexual underdevelopment in zebrafish. This fact became more obvious by determining the maturity index (see chapter 2.4.4) than only by classification into stages of maturity (method of the OECD guideline). This evaluation method seems to be useful, since the results of the maturity index allow to obtain a general overview about the developmental status of an entire exposure group. The evaluation of mean values might, of course, generalize conclusions in this case, but on the other hand helps to detect dose-dependent effects. The results from this analysis proved to be the most sensitive endpoint (together with vitellogenin induction and sex ratio). It even facilitated the documentation of the clear correlation between maturation and growth in zebrafish. This finding corroborated the necessity of assessment of the endpoint growth. The present study showed that growth is a sensitive parameter that can detect dose-dependent effects. However, the effects on growth might also be related rather to toxic than to endocrine mechanisms. Therefore, effects on growth need to be considered with care.

The second major endpoint examined in the FSDT is vitellogenin induction. It is indispensable for the evaluation of endocrine potential of a compound, since it gives essential information about the hormonal condition of a fish. The measurement *via* ELISA opens up the possibility to detect even slight hormonal disruptions. Especially vitellogenin induction in male fish provides essential data for the assessment of estrogenic compounds. Several studies including the present one have revealed that vitellogenin induction is a useful and sensitive biomarker for assessment of the endocrine potential of EDCs (Rose 2002, Orn 2003, Andersen 2006, Holbech 2006). Vitellogenin induction after exposure to prochloraz and 4-*tert*-pentylphenol turned out to be as sensitive as the assessment of the sex ratio (see Tab. 8).

All the endpoints mentioned and examined in the present study are of great importance for the assessment of the potential risk of existing and new chemicals. Their high sensitivity towards

exposure of EDCs to zebrafish could be revealed in the FSDT. The detection of histopathological alterations was the only ambivalent endpoint: the rating of severity and interpretation of the results can be subjective and demand routine. An OECD validation ring test of the 21 day fish screening assay with adult fish (OECD 2006) revealed that inter-laboratory results concerning histopathological alterations can differ within a wide range. One idea for the improvement of histological analyzes is to replace the HE-staining by a multi-color-staining. This could bring some striking advantages: by staining different tissues in different colors, histopathological alterations could be much easier to identify, and can, therefore, easier be distinguished from each other. This could facilitate the identification of fibrosis, inflammation or proteinaceous fluid in the gonads.

In summary, the results show that the FSDT has strong potentials to replace FLCTs, especially for the collection of data concerning EDCs, as currently required for REACH. The most important argument is that the FSDT with zebrafish covers its phase of sexual differentiation (as FLCTs do), which is supposed to be sufficient to detect endocrine disruption. Segner et al. (2003) as well as Fenske et al. (2005) compared the sensitivity of zebrafish in partial-life-cycle tests and FLCTs with different EDCs. They both pointed out the need to cover the period of final gonad differentiation of zebrafish for the detection of EDCs. Under this condition, they found more advantages for the FSDT than for FLCTs.

Therefore, the main advantages of the FLTC can be summarized as follows:

1) Even though reproductive and population-relevant effects cannot be detected by the FSDT, it has at least important predictive power for FLCTs. FLCTs could then reveal reproductive and long-term effects of EDCs mediated by bioaccumulation or adaptation.

2) The main advantage of a FSDT compared to a FLCT is its practicability: it is short, cheap and easy to perform.

Summing up, the present study provides strong evidence that the FSDT is a sensitive test for estrogenic and aromatase-inbiting exposure, and its validation as an OECD test guideline should continue. Compared to tests with adult fish, the FSDT is just as sensitive to estrogens and more sensitive to androgens (Holbech 2006). The FSDT has proven to be effective in detecting compounds with estrogenic (Orn 2003; Holbech 2006), androgenic (Orn 2003; Holbech 2006), anti-estrogenic (Andersen 2004) and aromatase inhibiting (Andersen 2004) effects.

9. Summary

So-called “endocrine disrupting chemicals” (EDCs) are ubiquitous in the environment, especially in aquatic systems close to industrial and agricultural areas. Disturbances of the hormonal balance between estrogens and androgens are one central target of EDCs. Especially during sexual differentiation of organisms, the consequences of such a misbalance may easily lead to population-relevant effects. Thus research into EDCs has been massively intensified over the last decades. Since, at international levels, there is high interest to establish validated test systems for the evaluation of endocrine effects, the OECD has initiated a progran to develop test guidelines for the detection of EDCs. Within this scope, the present work forms part of a validation exercise for the so-called “fish sexual development test” (FSDT), an extension of the existing OECD test guideline 210, the fish early life-stage toxicity test as an OECD guideline. The main idea is to establish an *in vivo* test for EDCs, which is relatively straightforward, cheap and on the one hand more rapid than full life-cycle tests, but, on the other hand, more sensible than the currently discussed 21-day-screening test. In the present study, the two EDCs prochloraz and 4-*tert*-pentylphenol were selected as exemplary substances to be tested in the FSDT. Prochloraz is a fungicide that inhibits the enzyme aromatase and therefore the synthesis of steroids. 4-*tert*-pentylphenol belongs to the group of alkylphenols, which are known to interact with the estrogen receptor, and, therefore, are classified as pseudoestrogens. Zebrafish (*Danio rerio*) were exposed to these chemicals in flow-through systems for 60 days post-hatch, a period in which zebrafish finalize their sexual differentiation. Within this sensitive phase the sexual development can easily be disturbed by EDCs. After exposure, the zebrafish were measured and weighed, the gonads were examined by means of histological analysis, and vitellogenin concentrations were measured in heads and tails *via* ELISA. Both chemicals caused a shift in sex ratio (prochloraz: masculinization; 4-*tert*-pentylphenol: feminization), underdevelopment of the gonads, inhibition of growth and altered vitellogenin production. For the two substances examined, the sex ratio and the evaluation of maturity stages were both the most sensitive and the most relevant endpoints. Thus, even though reproductive and population-relevant effects could not be documented by the FSDTs, the test clearly had at least predictive power for what could have been seen in full life-cycle tests. Therefore, the present study corrobates the conclusion that the FSDT is an adequate test system for the evaluation of EDCs and that its further validation as an OECD test guideline is definitely required.

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