Markers of endocrine disruption in fish

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detail of the Fish Fountain at the Fish market in Leiden, The Netherlands (*Rinus Bogers*)

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For all the women in my life,
especially Lia, my other half

One of you can be content,
however, happiness can be shared
only when there are at least two of you.

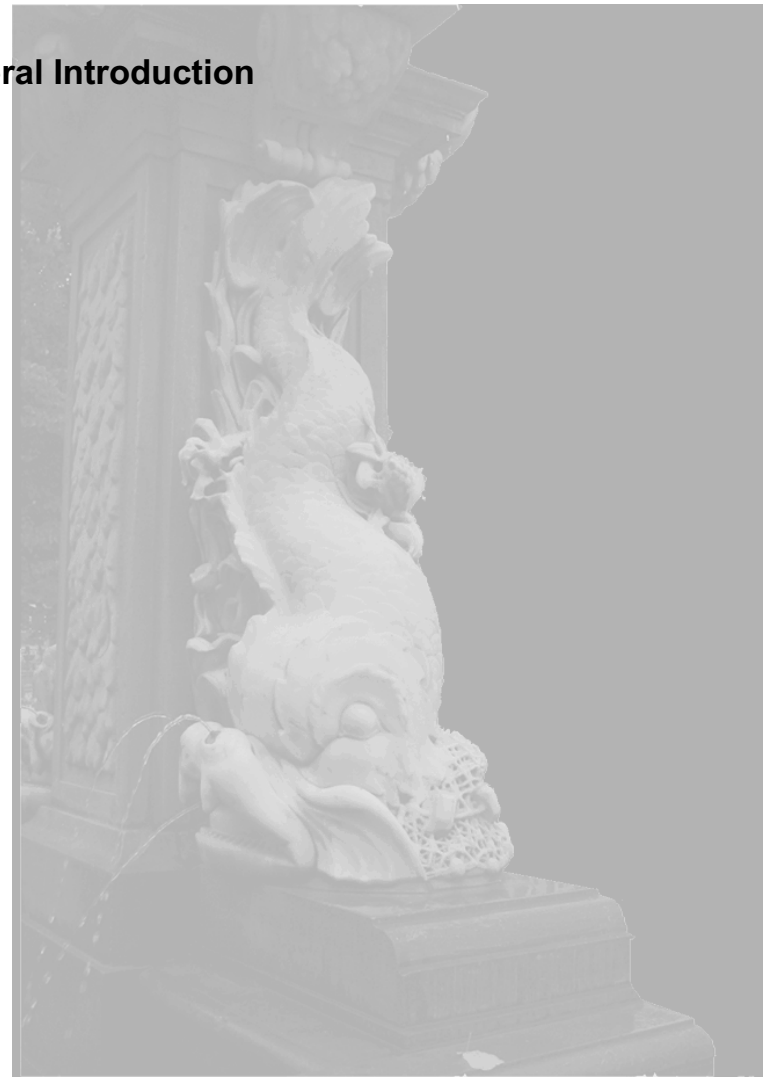
Alleen kun je tevreden zijn,
echter, geluk deel je alleen
als je minimaal met tweeën bent.

Rinus Bogers

Contents

Chapter 1

General Introduction



History of a battle between fact and fiction

In the early 1990's, an interdisciplinary group of scientists in the United States started a discussion about chemicals with a potential to specifically induce adverse effects on the endocrine systems of both human and wildlife. The result, the "Wingspread Consensus Statement", triggered further debate in both the USA and Europe [1]. The general public was confronted with this issue by television through programs like the 1993 BBC Horizon documentary with the dramatic title “Assault on the Male” and the well known 1996 publication “Our Stolen Future” by Colburn, Dumanoski and Myers. This mobilization of public awareness culminated in urging the regulatory bodies to take their responsibility regarding the risk of synthetic chemicals and environmental contaminants acting as endocrine disrupting compounds (EDCs[1]). The concern focused especially on the potential for adverse effects on the reproductive systems of human and wild life populations around the world.

EDCs are suspected to interact with the endocrine systems and as such disturb hormone homeostasis. Most of them are lipophilic and have a potential for chemical transformations. Although different in structure and size, an unknown number of exogenous chemicals may interact with the endocrine system. One group of mayor concern consists of those EDCs that disturb the action of sex hormones. Such EDCs can be natural like phyto- and myco-estrogens, or anthropogenic such as certain pharmaceuticals, pesticides and other industrial chemicals.

The hypothesis was put forward by the early publications that synthetic estrogens (xeno-estrogens) and other EDCs dispersed in the environment may well be associated with a global decrease in human sperm counts, increase of testicular dysgenesis syndrome (TDS), decrease of male reproductive capacity and/or increased incidence of breast and testicular cancer. Later studies performed in several countries tended to support this hypothesis [2, 3, 4]. However, these studies also showed a high variation in the frequency of male reproductive tract disorders among regions and in time, which challenged the hypothesis of an integral and global effect of environmental chemicals on human male reproductive capacity [5, 6].

Although this thesis focuses on man-made EDCs, it is interesting to mention that human and animal exposure to endocrine disrupters is mainly expected from phyto-estrogens, i.e. isoflavones present in soybeans, chickpeas, and soy-based products, coumestans present in herbs, forest crops and vegetables, and lignans present in oil seeds, such as flaxseed, cereal bran and legumes [7]. When consumed as part of a regular diet, phyto-estrogens are considered safe and even thought to be beneficial as possible cancer preventatives or advantageous in treatment and/or prevention of menopause and osteoporosis [7, 8]. On the other hand, various civilizations made or still make use of certain herbs, carrots or other plants for contraceptive purposes [9, 10] Further, phyto-estrogens have since long proven to significantly affect the fertility and reproductive capacity of herbivore animals both domestic and wild, especially in seasons when or areas where the diversity of available plants is limited [11, 12, 13].

[1] Note that these substances are addressed to as endocrine active chemicals (EACs) in Chapters 3 and 4. To remain in conformity with the original publications, this term is maintained in these chapters.

The first accounts of endocrine disrupting effects in wildlife suggesting the presence of environmentally relevant doses of man-made chemicals, are about effects on the sexual development of mollusks and gastropods related to exposure to organotins (e.g. tributyltin and triphenyltin) [14, 15]. Another classic study related the cause of a 90% decline in the birth-rate of alligators and disturbed sexual development of young male alligators living in Lake Apopka in Florida to an accidental spill of DDT from a chemical plant [16].

Soon after the publication of “Our Stolen Future”, most of the "evidence" brought forward by the authors roused scientific criticism. Serious doubts were expressed against the hypothesis that industrial-derived EDCs would be a real important factor in causing future effects at the population level of both human and wildlife, considering the accumulating experimental evidence of their relatively low endocrine potency compared to naturally occurring phyto-estrogens in food and the environment [17, 18, 19, 20]. In the years that followed, several studies confirmed alkylphenols to be potentially pseudo-estrogenic in fish [21, 22, 23, 24, 25]. Further, estrogenic effects were detected in fish exposed to surface waters receiving effluents from sewage treatment works (STW) or industrial plants [22, 25]. Depending on the site, various types of estrogenic compounds were detected including natural estrogens like ß-estradiol (E2) and estrone (E1), synthetic estrogens like ethinylestradiol (EE2) and industrial chemicals like alkylphenols [22, 27]. Incidentally also indications of androgenic responses were found in fish exposed to effluents of industrial plants [28].

Still, some parties argued that environmental concentrations of most industrial EDC’s were too low to induce any significant effect on aquatic organisms based on studies published at the end of the nineties [27, 29]. However, there is increasing evidence that certain organisms and life stages are more sensitive to exposure to these EDCs than expected. Considering the lack of ecologically relevant exposure data the possibility of subtle endocrine effects on populations in the environment can therefore not be excluded. Especially persistent EDCs in the environment pose continuous exposures, and the possibility of additivity [30] or bioconcentration [31], especially in aquatic organisms. In a recent 7-year, whole-lake experiment in Canada, Kidd et al. [32] found that chronic exposure of fathead minnow to low EE2 concentrations (5 - 6 ngl^{-1}) led to feminisation of males and, ultimately, a near extinction of this species from the lake. Further, an example for the complexity of environmental contamination with EDCs is the report of Parrot et al. [33] on changes in growth and secondary sex characteristics of fathead minnows exposed to bleached sulphite mill (BSM) effluent. They found that male fathead minnow exposed to dilutions of this effluent developed female secondary sexual characteristics, while their female counterparts developed male characteristics. The threshold for the most sensitive effects was <10% BSM effluent. Finally, accumulation in sediment or in the food chain may also increase the potential of relevant effects of EDCs in wildlife.

The concern regarding the presence of EDCs in the aquatic environment led to nation-wide surveys in different Western countries. A nation-wide survey in the UK [24] showed that essentially all STW effluents were estrogenic to fish. Analysis of some estrogenic effluents showed that most of the estrogenic activity in STW effluents did not come from man-made non-steroidal EDCs such as alkylphenols, but instead was contributed by natural estrogens, such as E2, E1 or the man-

made steroid EE2. Further studies on wild populations of freshwater fish have shown that intersex fish are present in almost all rivers of Great Britain. In fact, in some of the rivers, which receive large inputs of effluent from STWs, all of the male fish were intersex to varying degrees. As the rivers containing the most severely affected fish also received relatively large inputs of industrial effluent, a possible contribution from industrial chemicals to the observed effects could not be excluded. This could also be the case in flounders caught in estuaries around the UK showing signs of exposure to estrogenic chemicals, which receive large amounts of industrial and domestic effluent [24].

A nation-wide survey in the Netherlands [25] came up with similar findings although the Dutch STWs appeared to be more effective in removing natural and synthetic estrogens from the untreated wastewater than the British ones. The EDCs identified were bisphenol-A, nonylphenol, nonylphenolethoxylates and phthalates. In contrast to the findings in the UK, hardly any estrogenic effects, except for incidental cases of vitellogenin induction, were found in male flounder caught on most Dutch offshore locations, coastal waters and open estuaries. Freshwater fish from some locations showed estrogenic effects, though, e.g. bream from a small river (Dommel) receiving sewage treatment plant (STP).

International strategies

Near the end of the past millennium, the European Community Strategy on Endocrine Disrupters (ECOM, 1999) identified actions for the short, medium and long-term. The short-term actions led to a list of substances for further evaluation in the context of existing legislation to control the environmental risk. The medium-term actions focussed on the identification and assessment of endocrine disrupters, as well as on further fundamental research with respect to the mechanisms involved in endocrine disruption by chemicals. The long-term actions concerned the development of further legislative actions to protect both human health and the environment. The European activities in this field are to be viewed as part of the international co-operation with the USA and Japan on research of the environmental risks of endocrine disrupters. This co-operation exists within the WHO on health issues and the OECD on screening and testing issues.

The USA has planned to screen a very large number of chemicals (probably 15,000) through a tier of assays for endocrine disrupting activity. In this program, termed the Endocrine Disrupter Screening programme (EDSP), the Environmental Protection Agency (EPA) focussed on the development, standardisation and validation of screens and tests. The core elements of the tiered approach include initial sorting, priority setting, tier one screening, and tier two testing. Tier two testing followed tier one testing in 2004. A combination of *in vivo* and *in vitro* screens will be utilized in Tier 1, and Tier 2 will involve *in vivo* testing methods using two-generation reproductive studies. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays. The difficulties encountered mainly relate to data interpretation, to relating results to the development of policies for future research on EDCs, and to the subsequent development of legislation to protect human health and the environment.

Processes related to sexual development in fish

Already a lot of scientific work has been performed to establish relevant screening tests for the assessment of the sex hormone disrupting potency of man-made and natural chemicals (34). The relevance of such work demands consideration of the fact that the whole process of reproduction, the main target for this assessment, has so many different steps that there are numerous events in this process, which may be the specific target of EDCs. In this context the warning of professor Alan Heath in his book 'Water Pollution and Fish Physiology' (35) should be considered: "In carrying out studies on the effects of pollutants on reproduction, care must always be taken to avoid attributing mechanisms of toxic action to particular processes, unless other processes have also been examined."

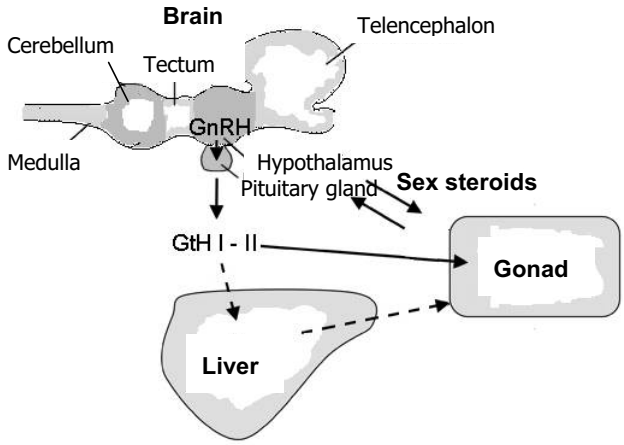


Figure 1.1 Schematic presentation of the hypothalamus-pituitary-gonadal axis in fish.

The complex physiology of reproduction in fish, as in other vertebrate species, consists of two major parts, i.e. sexual and gonadal development of males and females before spawning and fertilisation followed by embryonic and larval development after spawning. Different organs are involved in the sexual development and maturation of fish. For more detailed discussion of the various processes involved with reproduction the reader is advised to consult one of the review books on fish physiology and endocrinology (35, 36, 37). As in mammals, the basic organ in fish regulating sexual development and maturation is the hypothalamus (Figure 1.1), which acts on the second organ involved, the pituitary gland, by producing the gonadotropin releasing hormones (GnRH). In turn the pituitary then starts to release gonadotropins (GtH I and II), which stimulate the development of the spermatozoids or oocytes in the gonads (gameto-genesis). This neuro-endocrine system is generally referred to as the hypothalamus-pituitary-gonadal axis. Teleost fish differ from other vertebrates in that a well-developed portal blood supply between the hypothalamus and the adenohypophysis of the pituitary gland does not exist. Instead the adenohypophysis is directly innervated with neurosecretory fibers originating in the hypothalamus. The pituitary gland also produces growth hormone and thyrotrophin, the latter controls the thyroid

gland as a thyroid-stimulating hormone (TSH). Although the thyroid hormones are related to regulation of growth and development, they probably also play a role in regulating reproductive processes.

Normally the gonads in fishes are paired structures lying adjacent to the air bladder or dorsal kidney, but there are many exceptions like the single ovary in medaka. Another feature in most fishes is that the ovaries differentiate earlier than the testis. As an example the gonadal differentiation in juvenile fatheads and medaka is distinct from zebrafish in that male gonads do not differentiate at the same moment in time when female gonads develop, whereas in zebrafish all juveniles develop ovarial tissue until 25 days post hatch (dph), after which testes formation in males starts.

In female fish the gametogenesis (Figure 1.2) starts with the development of an ovarian follicle, basically a central cavity surrounded by *follicular* and *thecal* layers of cells, which secrete the sex steroid hormones, and of yolk, all activated by the GtH I and II hormones. Estrogenic steroids like E2 stimulate the production of vitellogenin (VTG) by the liver, which is transported by the blood to the ovary to be taken up into the developing oocytes. The blood levels of steroids not only affect vitellogenesis in the liver, but also reach the hypothalamus affecting the release of GnRH, and as a consequence the release of GtH I and II by the pituitary gland. This process of feedback can be positive, e.g. in immature fish to stimulate gonadal development, or negative, e.g. in mature fish to prevent overstimulation of oocyte ripening.

In male fish, the early parent cells in the developing testis develop to spermatogonia, which develop to sperm cells through different stages. Important roles are played by Sertoli and Leydig cells, which support and regulate the spermatogonia in their maturation e.g. by producing sex steroids, in this case androgens like testosterone (T) and 11-ketotestosterone (11-KT). It should not be neglected that steroids like T, and also E2 can be found in both male and female fish. In female fish T is a precursor of E2 and converts to E2 in the process of aromatisation of T. In male fish the activity of this process is much lower, but still present and may be involved in regulation of T levels.

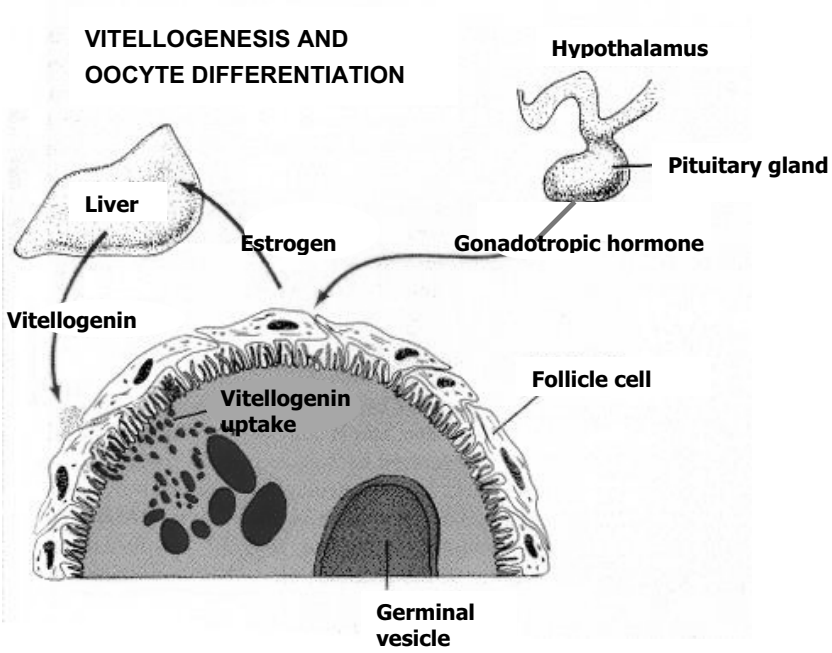


Figure 1.2 The various organs involved in oocyte differentiation and vitellogenesis in female fish (courtesy of Prof. William Wasserman, Dept. of Biology, Loyola University of Chicago).

Regulation of hormone synthesis and secretion within the hypothalamus-pituitary-gonadal axis and thyroid system occurs from feedback control exerted by the secreted hormones themselves or by the steroids that are induced by hypothalamic-pituitary hormones. In fish, an example of feedback loops within the neuroendocrine system is the effect of sex steroids on gonadotropin release. Gonadotropins (the tropic hormones) stimulate the synthesis and release of sex steroids (the target hormones) by the gonads, and these sex steroids in turn affect the release of tropic hormones by the pituitary. Both positive and negative feedback can occur to either stimulate or inhibit release of the tropic hormone in synchronous spawning fish. This is probably also true for asynchronous spawning fishes such as those typically used in regulatory testing, although published data are lacking.

Sex steroids are derivatives of cholesterol and possess a four-ring structure with variations in substituting groups on the rings. Specific addition of methyl or ethyl substituent provides the basic structural skeleton for the estrogens, androgens, and progestrogens (Figure 1.3). Estrogens like E2 and E1 are C18 steroids and are primarily synthesized in the ovary. Androgens are C19 steroids with the 11-oxygenated derivatives (e.g. 11-KT) being the most important. The testis is the primary site of androgen synthesis, but since T and androstenedione are precursors for estrogen synthesis they are also formed in the ovaries. In male fish the androgen 11-KT is generally present at higher levels than T, while in females it is found at barely detectable levels. Unlike T, 11-KT cannot be aromatized to an estrogen.

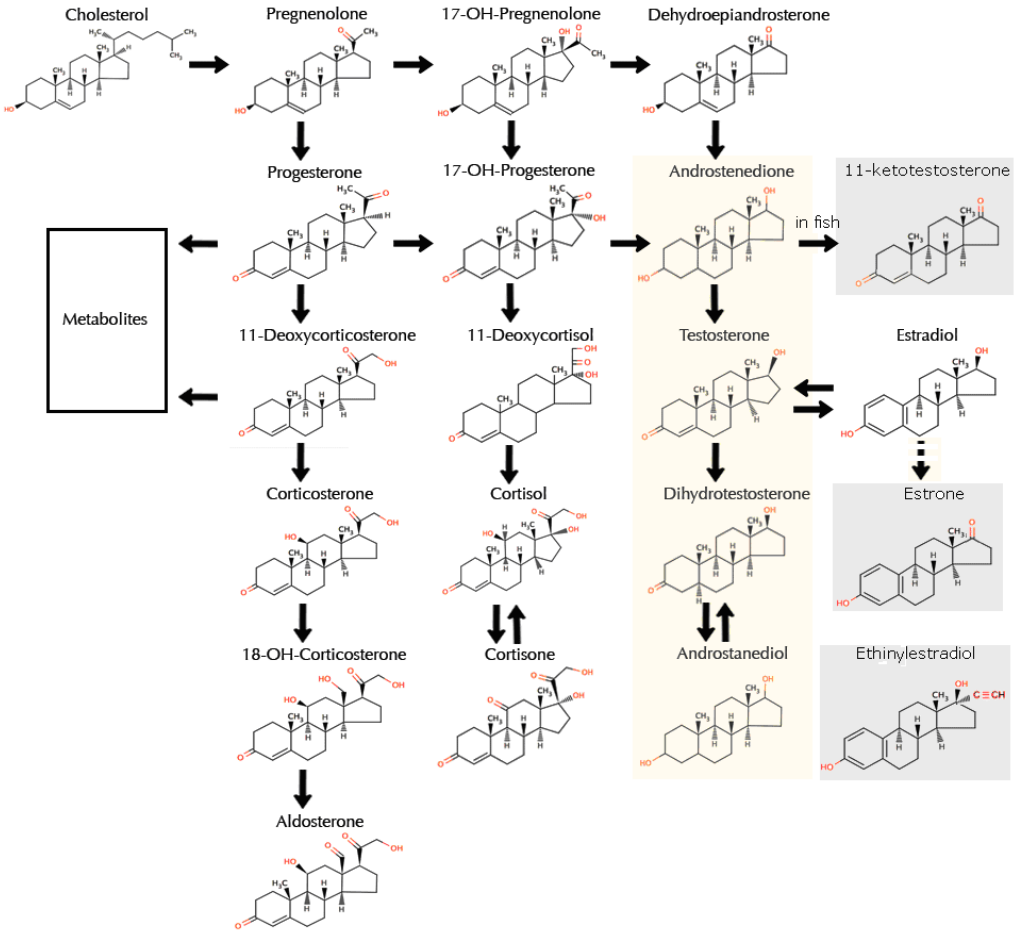


Figure 1.3 Examples of routes of synthesis of sex steroids in mammals with estrone (E1) and the synthetic ethinylestradiol (EE2) included for comparison. In fish (11β-hydroxy) androstenedione is converted to 11-Ketotestosterone (11-KT). This scheme was adapted based on a figure obtained from the Tulane/Xavier Center for Bioenvironmental Research, New Orleans, Louisiana, USA.

The testes of teleost fishes are distinct from other vertebrates like mammals with respect to the high capacity for glucuronide conjugation of androgens. These steroid conjugates may play a role as male sex pheromones [38, 39].

Steroids are not only produced in a natural way but also commercially by pharmaceutical industries with well-known examples being the steroids in the anticonceptive pill and anabolics.

Anticonceptive pills mostly contain one of the estrogens E2, EE2 or progestrogens such as mestranol, with EE2 being present in most prostagenic products.

The list of anabolic steroids commercially available is much longer including testosterone propionate (Testex), methyltestosterone (Metandren Oreton Methyl), fluoxymesterone (Halotestin), oxandrolone (Anavar), methyldihydrotestosterone (Dianabol) and many others.

As stated in former sections, nation wide surveys in different countries showed that detectable traces of estrogenic substances like EE2 and E1 were present in STW effluents and in surface waters receiving these effluents, while detectable traces of androgenic active substances were present in industrial effluents.

Sex reversal in fish

In aquaculture, total functional sex reversal of fish by exposing them to sex hormones has widely been applied, especially during their early life stages [40, 41]. Depending on the species, females or males grow faster, and faster growth gives higher output. In the case the roe is commercially important, females are preferred, whereas for ornamental fish, the breeding of males may be preferred. There is also evidence that sexual differentiation in certain fish species (expressed as sex ratios) can be influenced by environmental variables such as pH [42] or temperature [43]. As a consequence, natural populations of certain fish species may possess a proportion of phenotypically sex-reversed fish, resulting in altered sex ratios. However, these proportions appear to be much lower than those found in more polluted waters [44].

In fish, the actual genetic sex of individuals cannot easily be identified due to the lack of genetic markers or discernible chromosomes. Instead, the sex of fish is identified based on the gonad type, i.e. ovary or testis, or external sexual characteristics. The term gonad type reversal is used instead of sex reversal for the development of the opposite gonad type in an individual with a given genetic sex. Definition of sex based on gonad type is also applicable in the case of the development of testes with ovarian cells or ovaria with testicular structures. In such a case, this is identified as intersex, without implying a certain genetic sex.

Two patterns of gonad development can be distinguished in fish. The gonad develops directly into testis or ovary (fathead minnow, medaka) or first into an ovary-like gonad before genetic males develop testes (zebrafish). As in other gonochorist species, intersex can occur spontaneously in fish characterized by the presence of both ovarian and testicular tissue within the gonad. In natural populations at non-polluted locations, the occurrence of this phenomenon is relatively low and species dependent, with percentages of < 0.1 to 4 [45, 46].

Current methods to study endocrine disruption in fish

The various workshops almost unanimously agreed on defining a hazard identification strategy including initial screening of the intrinsic endocrine activity of chemicals based on Structure Activity Relationships (SARs) and *in vitro* tests. In most cases it is difficult to assess their effects solely on a structural basis, due to the diversity of chemical classes the EDCs belong to and their specific biological interactions (bioavailability, bioactivation, bioconcentration etc.). Presently

different *in vitro* assays are available with the estrogen receptor (ER)-binding assay and the reporter gene effect assays: a yeast estrogen screen (YES) and the ER-mediated chemically activated luciferase gene expression (ER-CALUX®) assay, as most prominent examples [47, 48] These standardized short-term *in vitro* assays are applied to identify EDCs as well as to determine the relative endocrine-disrupting potency of environmental matrices, e.g. effluents and surface waters [49, 50]. Further, these assays may be useful as tools to prioritize chemicals or environmental matrices for more extensive *in vivo* studies. *In vitro* bioassays for EDCs are designed to assess effects like competitive ligand binding or receptor mediated activation of gene transcription [49, 50]. However, these assays still do not account for interactions of EDCs with the various processes related to the hypothalamus-pituitary-gonadal axis, gonadal maturation, synthesis of sex steroids and *in vivo* processes like bioconcentration, bioactivation and metabolism. These assays are therefore mainly suggested as the initial step in an overall assessment that eventually includes verification of a potential effect *in vivo* prior to implicating a substance or environmental matrix as potentially endocrine disrupting. Hence, short and long term *in vivo* tests can be considered essential for further screening of potential EDCs.

As at the time the first workshops were held no appropriate short term *in vivo* tests for EDCs were available, various types of *in vivo* tests were developed in the years that followed. Evaluation of the numerous studies performed with endocrine active substances during the last decades shows application of many different experimental designs as well as differences in the choices made for the life-phase of exposure, endpoints detected or fish species used. Although this provides a wide perspective into the different ways for assessment of endocrine activity, it hampers a reliable comparison between the results of these experiments as needed for regulatory processes. At present these types of experiments include testing during reproduction, early development or young adult stages of fish [51, 52, 53]. After initial assessment and screening, sub-chronic and/or chronic *in vivo* tests are necessary to determine a possible relationship between concentration and endocrine effect and to obtain more specific detail on the modes of action in the living organism [54]. Further, species-related differences are often seen when examining sub-lethal effects on growth or larval development. Similarly, comparison of results of various experiments showed species-related differences in sensitivity towards endocrine activity of chemicals [52, 54, 55, 56].

Most importantly, the decision which design will be the most appropriate for the testing of potential EDCs in fish should be guided by the type and sensitivity of endpoints predictive of endocrine disruption at the level of sexual development. In addition, the design should consider the type of effect parameter most applicable for risk evaluation, i.e. to assess the hazard of effects of exposure to an EDC on sexual development and fecundity of fish. Evidently, differences between species determine the species-related exposure duration to allow comparison of phases in development. In all, tests with potential EDCs may include the testing of endocrine effects on separate sexes, fertility and spawning experiments with pair or group breeding fish, or early life stage studies including embryonic, larval and early juvenile development. For chronic tests, the experiment may include more than one life phase, and preferably also include spawning and F1

development. Examples of this kind of studies are the partial life cycle test, the two-generation test and the full life cycle test (61, 62).

Essentially, *in vivo* screening assays should combine a relatively short exposure period with an endpoint sensitive enough to detect significant endocrine related changes in fish. In principal, the *in vivo* screening assays for assessment of endocrine disruption are not designed to identify specific mechanisms of hormonal disruption. However, there certainly is an advantage in applying alternative test designs that also include endpoints, which identify the mechanism of action. The test concentrations should be sub-lethal and not exceeding water solubility limits.

In spite of the wide range of publications there is still a relative lack of information on the reproductive endocrinology of even the most frequently used test species. Although a great variety of endocrine parameters have been examined in numerous *in vivo* studies, only a small number of parameters have been widely applied, with VTG induction being the most commonly used endpoint in *in vivo* EDC research. Up to now, research has mainly focused on estrogens and estrogenic compounds, but the work on anti-estrogens, androgenic and anti-androgens is limited. Partly because the endpoints for the more intensively studied estrogenic effects are not all useable in tests with e.g. anti-estrogens or androgens, new endpoints have to be developed first. Examples of biological endpoints often studied are:

1. gross morphology, including secondary sexual characteristics;
2. the hepato- or gonado-somatic index (HSI or GSI);
3. liver VTG or VTG mRNA levels;
4. plasma concentrations of estrogens and androgens;
5. histopathology of excised gonads, and optionally also liver.

The first endpoint is rather species specific. For example, the parameters involved are not easy discernable in zebra-fish but very prominent in adult male fathead minnow (57, 58, 59) or medaka (60). The somatic indices HSI and GSI are used to decrease the individual variability observed in the absolute weights of body and the respective organ. However, also these indices can vary considerably between and within species and this variation may well be species or gender related (21, 59). As stated earlier, VTG induction is probably the most commonly applied endpoint in estrogenic research. VTG is generally quantified using an enzyme-linked Immunosorbent Assay. The principle of ELISA is based on the reaction between the protein (or steroid) and a specific antibody, one of which is covalently adsorbed to a solid surface (e.g., the wells of a microtiter plate). The antibody can be mono- or polyclonal and developed in one specific species or for a specific group of species (e.g. Cyprinidae). Other schemes for ELISA can be used to measure sex-steroid hormone levels in plasma or homogenates. Commercially developed ELISA kits for E2, T or 11-KT (e.g., Cayman Chemical) are available, but have not yet been applied extensively for measurement of E2 levels in fishes.

Aim and outline of the thesis

In vitro bioassays for detecting EDCs do not account for interactions of the EDCs with the various processes related to the hypothalamus-pituitary-gonadal axis, gonadal maturation, synthesis of sex steroids and *in vivo* processes like bioconcentration, bioactivation and

metabolism. Therefore, further screening of potential EDCs should include *in vivo* verification of potential effects. As discussed earlier, many different in vivo assays with different fish species as the test organism and a wide range of possible endpoints are being developed. This raises the need for more insight into the relative sensitivity and applicability of these different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals. Such insight, would add considerably to the definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs. Therefore, the aim of the present thesis was to investigate the relative sensitivity and applicability of different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals, in order to contribute to definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs

To this end, in the different experimental chapters of this thesis, different endpoints for testing EDCs in *in vivo* experiments with fish were investigated and compared, ultimately revealing their promises and pitfalls. Table 1.1 presents an overview of the different endpoints investigated in the the different chapters of this thesis.

The study described in Chapter 2 incorporated specific endpoints for estrogenic activity, namely vitellogenin (VTG) levels, as an extra parameter in an early life stage (ELS) test carried out as described in the guideline 210 of the Organisation for Economic Co-operation and Development (OECD) and traditionally used for toxicity screening of chemicals. The sensitivity of this widely used endpoint was compared to that of a newly developed endpoint based on the use of a transgenic zebrafish model expressing an estrogen receptor-mediated luciferase reporter gene. The transgenic zebrafish were exposed to EE2, and luciferase activity as well as VTG was measured.

Table 1.1 Summary of all endpoints (markers) examined in the various chapters of this thesis

Endpoints or markers	Chapter			
	2	3	4	5
Vitellogenin (VTG)	X	X	X	X
Vitellogenin messenger RNA (VTGmRNA)				X
Behaviour & appearance		X	X	
Secundary sexual characteristics (SSC)		X	X	X
Body weight-length-condition factor	X	X	X	X
Hepatic somatic weight (HIS)				X
Gonadal somatic weight (GSI)				X
Gonadal histology		X	X	X
Peritoneal attachment of gonads		X		
Luciferase induction in transgenic zebrafish	X			
Fecundity (egg & spawn production)			X	
Egg-survival	X	X	X	
Hatching	X	X	X	
Overall survival	X	X	X	
Sex-ratio		X		

In addition to extending the OECD test guideline 210 by adding an extra parameter like VTG levels, the Endocrine Modulators Study Group (EMSG) of the European Chemical Industry has proposed to develop an extended fish ELS test based on OECD test guideline 210 by combining it with a short-term fish pair-breeding reproduction study as a possible alternative for fish full life cycle testing. In this thesis, parameters quantifying effects on early life stages were investigated in Chapter 3 and those quantifying fish pair breeding success in Chapter 4. These parameters were used as endpoints for evaluating endocrine disrupting activity comparing their sensitivity to that of VTG as a possible endpoint. Chapter 3 describes the testing of the androgen methyldihydrotestosterone (MDHT) in an extended ELS test with fathead minnow supplemental to such a test with the weak estrogen 4-*tert*-pentylphenol (4TPP). Main endpoints studied were secondary sexual characteristics (SSC), plasma VTG induction and gonadal development.

The studies described in Chapter 4 were undertaken to further investigate the use of fathead minnow in pair-breeding reproduction studies. The results of two fish pair breeding reproduction studies with MDHT are presented and the reproductive performance of breeding pairs of fathead minnows was examined. Other endpoints examined were somatic growth, the prominence of nuptial tubercles (male related) and VTG induction (female related). EE2 was included as an estrogenic reference substance.

In additional studies, presented in Chapter 5, the usefulness of fathead minnow in the Non Spawning Male Fish Assay and possible endpoints in this fish model for testing endocrine disruption were investigated. Furthermore, VTG mRNA levels were examined as an additional endpoint and compared with VTG (protein) induction. The experimental setup also included the endpoint of nuptial tubercles development tested in Chapter 3 and 4, which appeared to be a sensitive sex-related characteristic of fathead minnow. Adult male fathead minnow were exposed for 14 or 28-days under flow-through conditions to undiluted filtered water samples from the rivers Meuse and Rhine in the Netherlands. Additional groups were exposed to EE2 as a reference and untreated drinking water as a negative control. Other major endpoints examined included hepato- and gonadosomatic indices (HSI and GSI) and gonadal histology.

In Chapter 6 the results obtained in the experiment of Chapter 2, 3, 4 and 5 were summarized and integrated comparing the endpoints studied and drawing conclusions on what would be the best endpoints to be considered in future work. Altogether the results obtained should provide insight in the relative sensitivity and applicability of different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals, thereby contributing to the definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs.

Chapter 2

Estrogenic endpoints in fish early life-stage tests: luciferase and vitellogenin induction in estrogen-responsive transgenic zebrafish

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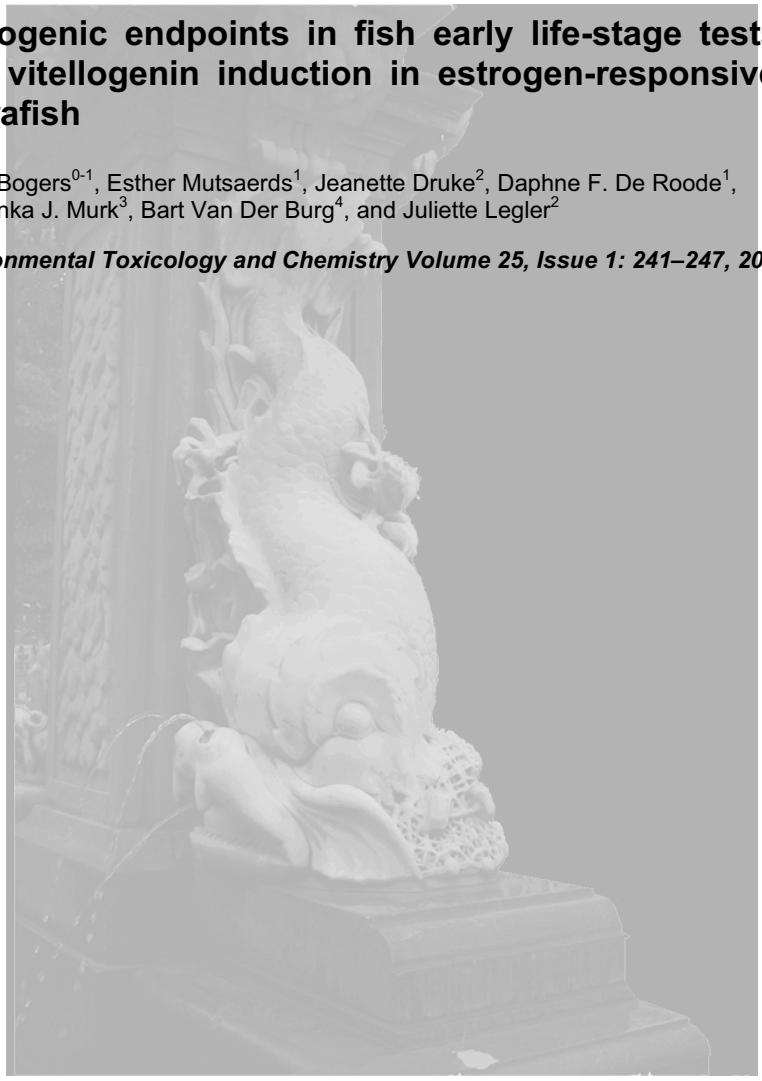

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Abstract

This study incorporated specific endpoints for estrogenic activity in the early life stage (ELS) test as described in the guideline 210 of the Organisation for Economic Co-operation and Development (OECD) and traditionally used for toxicity screening of chemicals. A transgenic zebrafish model expressing an estrogen receptor-mediated luciferase reporter gene was exposed to ethinylestradiol (EE2), and luciferase activity as well as vitellogenin (VTG) was measured. Concentrations of EE2 were tested at 1, 3 or 10 ng l^{-1} for 30 d from fertilization or during only the last 4 d with dimethylsulphoxide (DMSO) as pre-solvent (0.01 %). Exposure to EE2 induced no toxic effects. Mean body weights were significantly higher in groups exposed for 30 d in the presence of DMSO, but condition factors were not affected. Significant luciferase and VTG induction occurred at 10 ng EE2 l^{-1} following the 4 and 30-d exposure, while only VTG levels were affected at 3 ng EE2 l^{-1} in the 30-d exposure.[2] This study demonstrated the usefulness of incorporating estrogenic endpoints in the OECD ELS test, fitting the requirements for screening estrogenic activity of chemicals. Quantitative measurement of both VTG and luciferase activity proved to be rapid and sensitive. Additional value of using transgenic zebrafish lies in combining VTG measurement with the more mechanistic approach of luciferase induction in one experiment.

[2] In the original publication the sentence: "Significant luciferase and VTG induction occurred following 30-d exposure (3 and 10 ng EE2/L), while only VTG levels were affected in the 4-d exposure (10 ng EE2/L)." did not match with the respective results, and was therefore adjusted.

Introduction

Endocrine disrupting compounds (EDCs) present in the aquatic environment may interact with endocrine systems of aquatic organisms and as such disturb hormone homeostasis [64]. Of major concern are those compounds that disturb the action of sex hormones, such as estrogens. Estrogenic active compounds can be natural like phyto- and myco-estrogens [17, 18, 19] or anthropogenic such as certain pharmaceuticals, pesticides and industrial chemicals [21, 22, 24, 25, 65, 66]. Estrogenic effects have been detected in fish studies in surface waters receiving effluents from STWs or industrial plants [21, 25, 26, 67]. Depending on the site, various amounts of the estrogenic compounds have been detected including natural estrogens like E2 and E1, synthetic estrogens like EE2 and industrial chemicals like alkylphenols [25, 66, 68]. But also unidentified estrogenic compounds associated with sediment have been demonstrated [69]. Incidentally also indications of androgenic responses have been found in fish exposed to effluents of industrial plants [28].

The evidence of estrogenic effects in wildlife has fuelled international organizations responsible for test guideline development to give priority to the development of aquatic screening tests with relevant test species. Existing guidelines for tests with the early-life stages (ELS) of fish are intended to define the lethal and sub-lethal effects of chemicals on the stages and the species tested [70]. This information can be used to estimate the chronic lethal and sub-lethal effects of chemicals on other fish species. Originally, ELS tests were not intended to measure specific effects of hormone disrupting chemicals [70]. In recent years, however, the measurement of vitellogenin, a female yolk precursor protein, has been widely incorporated in ELS tests to provide an indication of estrogenic effects [71, 72, 73]. VTG is an estrogen-dependent yolk protein precursor synthesized in the liver of female fish and transported by the blood stream to the female gonads where it is incorporated in the developing oocytes. Vitellogenin induction following exposure of male fish to estrogens has been measured in various species, and studies have shown that the synthetic estrogen EE2 is more potent in fish than the natural estrogen E2 [55, 74, 75, 77, 78]. As a result, VTG induction is the most commonly applied endpoint in research of estrogenic effects in fish with EE2 as the most useful reference substance. Vitellogenin is generally quantified using an Enzyme-Linked Immunosorbent Assay (ELISA).

An alternative method for detecting estrogen activity in fish has recently been developed, which uses transgenic zebrafish. These estrogen responsive transgenic zebrafish were developed by stably introducing a reporter gene containing a minimal promoter sequence (TATA box) with three estrogen response elements linked to luciferase into the genome [63, 79]. Exposure of these transgenic zebrafish to estrogenic chemicals results in binding to endogenous estrogen receptors (ER) and the subsequent transactivation of the estrogen receptors, which induces luciferase gene transcription. Luciferase is then easily measured in tissue lysates. The *in vivo* transgenic zebrafish reporter gene assay demonstrated a concentration-response-related luciferase induction when fish are exposed to estrogens, e.g. E2, E1 and EE2 [79]. In these studies, juvenile transgenic zebrafish were tested undergoing gonad differentiation (four to five weeks old). Short-term (96 h) exposure was sufficient to measure the estrogenic activity of a number of compounds [27]. In comparison to

the traditional long term tests, the transgenic zebrafish model has potential to offer a more rapid and specific means for screening substances with a suspected estrogenic mode of action [63, 79].

The objective of the present study was to test luciferase activity as a useful endpoint for effects of exposure of fish to estrogenic compounds in a standard early life stage test using transgenic zebrafish and compare it with vitellogenin induction. Eggs sampled from transgenic zebrafish were exposed during embryonic and larval phases to the highly potent estrogen EE2. The transgenic fish were exposed to EE2 during two exposure periods: from egg-stage to 30 d according to standard ELS protocols; and for four days only at the end of the 30d test period (days 26 to 30). This 4d period is at the beginning of the sensitive period of gonad differentiation and the experiment was simultaneously performed by the Institute for Environmental Studies (IVM) in Amsterdam, The Netherlands. This inter-laboratory comparison included two different methods of administration of EE2: continuous flow dosing (at NOTOX, 's-Hertogenbosch, The Netherlands) and static renewal of the test medium (at IVM).

Results indicate that combining vitellogenin and luciferase induction as endpoints in a fish early-life-stage test is particularly useful for the short-term screening of chemicals for possible estrogenic activity.

Materials and methods

Test substances

The 17β-estradiol (E2: $C_{18}H_{24}O_2$) was purchased from Sigma Aldrich Chemistry, Zwijndrecht, The Netherlands and purity was >99.9%. Also 17α-Ethinylestradiol (EE2) was obtained from Sigma with a purity > 98%.

Test organism

Zebrafish (*Danio rerio*, prev. *Brachydanio rerio*, Hamilton-Buchanan) is a teleost belonging to the family of *Cyprinidea*. Fertilized eggs arrived within two to four hours at our laboratory. Wild type zebrafish originated from a commercial supplier (Ornamental Fish Hatchery Atlanta, Hellevoetsluis, The Netherlands). Transgenic zebrafish [63] were cultured at the IVM. The transgenic brood stock was held in tap water at 26± 2°C. Age of the brood stock was between one and two years. The brood stock received dry feed (Tertra-min Pro) 2 times a day and frozen brine shrimp (*Artemia salinas*) once daily. Fish were set out at a ratio male : female of 3:2. Nets were placed in the spawning tank to prevent the fertilized eggs from being eaten by the parent fish. Males and females were put together in spawning tanks prior to spawning and lights were switched off at 21.00 h; spawning started the following day after lights had been switched on at 08.00 h. Directly after spawning, eggs were collected for dispatch to NOTOX.

Early life stage test with transgenic zebrafish

The experiment started with 45 fresh and healthy fertilized eggs of the zebrafish, between four to six hours old, per test group and for each of the controls. Due to the low solubility of EE2 in water, stock solutions were made up in dimethylsulphoxide (DMSO). The final concentration of the solvent in the test media was < 0.1 ml l^{-1} (< 0.1 g l^{-1}). The final target EE2 concentrations were 1 ng

l^{-1} (0.0034 nM), 3 ng l^{-1} (0.01 nM) and 10 ng l^{-1} (0.034 nM). The fertilized eggs were randomly distributed and divided equally over three petri dishes per test group. After all larvae had hatched and were free-swimming, they were transferred to stainless steel vessels in a flow through system, each provided with a steel mesh that separated the exposure compartment from an outlet pipe. The flow through systems contained computer-controlled dispensers, which dosed the stocks at short intervals into a mixing vessel. The dilution water entered the mixing vessels simultaneously and was mixed with the dosed volumes of the stocks by continuous stirring. The final test solutions entered the test vessels via a distribution vessel. Sufficient volumes for one day of the nominal test concentration EE2 were prepared in dilution water. The flow rate during the first 26 d of the second experiment was 400 ml per h.

The dilution water for feeding the 12 vessels for the 96-h exposure was supplied via a dilution water cell with a multi-way capillary flow tube system. The maximum flow rate was 3 liters per h (50 ml per min). On day 26 the dosing of EE2 in DMSO and DMSO alone was introduced via the multi-way valve. The flow rate was increased to a maximum of 300 ml per h to approach the flow rate of the continuously dosed groups (400 ml per h). The whole system was checked daily. The test vessels were protected against unwanted disturbance by performing the test in a climate room with no entry for unauthorized persons.

During the first half of the experiment the photoperiod was 24h per day due to an error in the illumination programming of the climate room. However, the light intensity was limited, i.e. between 400 and 940 lux. During the second half of the test period the photo-period was set at 16 h per day. Dilution water was tap water with a hardness ranging between 180 and 220 mg l^{-1} expressed as $CaCO_3$ with pH of 7.8 ± 0.2 after aeration. The tap water used during the embryonic stages was sterilized by filtering through a 0.2 μm filter. Temperature of the test solutions was 24.8 ± 0.8 °C, oxygen concentration was > 60% air saturation value throughout the test and pH varied from 7.6 to 8.7 with an average of 8.0. Larvae and juveniles were fed *ad libitum* with *Brachionus rubens* and live Artemia nauplii (24 h old), respectively. At the end of the 30d study period, all fish including those exposed only for days 26-30 were euthanized by exposure to buffered ethyl 3-aminobenzoate methanesulfonate salt (MS222). Thereafter the fish were instantly frozen in liquid nitrogen directly after measuring weight and length and stored at -80 °C.

In addition to the experiment using continous flow dosing of EE2, a parallel experiment was performed at the IVM laboratory with zebrafish of the same batch and age (26 dpf), using a previously published method [29]. In this static renewal set-up, zebrafish (*n*=5) were held in duplicate beaker glasses containing 250 ml water to which EE2 dissolved in DMSO had been added (0.01%). Half of the test solution was renewed daily for four consecutive days. Fish were sacrificed as described above.

Luciferase analysis

For measurement of luciferase activity, the frozen zebrafish larvae were kept in eppendorf vials on ice. An aliquot of 250μl triton-lysis buffer containing 1 mM fresh dithiothreitol (DTT) was added to each eppendorf vial. Then fish were homogenized with a micropestle. Subsequently the eppendorf vials were vortexed and then centrifuged for 15 min at 4 °C, 13.000 rpm. Luciferase was measured in a luminometer (LUCYII, Anthos) with automatic injection of luciferin substrate.

Relative Light Units (RLUs) were measured in 75 µl triton-lysis (blank) and in duplicate 75 µl samples of the zebrafish lysates.

Vitellogenin analysis

The early life stage test according to the OECD guideline 204 [70] was already a standardized test at our facility which, however, does not include VTG analysis as one of the endpoints. Introduction in our laboratory of VTG analysis as endpoint in a fish early life stage test started with a preliminary experiment with wild-type zebrafish exposed to E2. The ELS test started with 30 fresh and healthy fertilized eggs of the wild-type zebrafish, 3.5 h old, per concentration. Stock solutions of E2 were made up in tertiary-butyl alcohol (TBA). The final concentration of the solvent in the test media was < 0.1 ml l^{-1}(< 0.1 g l^{-1}). The final target E2 concentrations were 8.25 ng l^{-1} (0.03 nM), 82.5 ng l^{-1} (0.3 nM) and 825 ng l^{-1} (3 nM). The wild-type zebrafish were exposed in a flow-through system for 42 d. Vitellogenin was analysed in whole body homogenates of 10 fish per treatment. Whole body homogenates were prepared by homogenizing the whole bodies of individual fish in ice-cold ELISA assay buffer and centrifuged at 3000 g (4200 rpm) for 10 min. At least three successive dilutions of the supernatants were included in the ELISA as duplicate or triplicate samples.

In the ELS test with transgenic zebrafish exposed to EE2, whole body homogenates of three fish of each replicate were used for VTG measurements. Homogenates were prepared by homogenizing the whole bodies of individual fish in ice-cold ELISA assay buffer with 0.2 units Aprotinin per ml in a 1:20 ratio wet weight:buffer volume using a potter. The homogenates were centrifuged at 20000g for 30 min (temperature 4 °C). The supernatant was stored at –80°C until further analyses.

The supernatants were used to measure the vitellogenin content using the Zebra fish Enzyme Immuno Assay (EIA) kit of Biosence, Bergen, Norway (Biosence product number V01008401). The ELISA protocol is based on a competition for the Zebrafish-VTG antibody between VTG coated on the wells of the ELISA-plate and free VTG in solution.

Analysis of actual EE2 concentrations

The analysis of actual EE2 concentrations is described in detail elsewhere (M. Lamoree, IVM, Free University, Amsterdam, The Netherlands). Briefly, one to two liter water samples were concentrated using solid phase extraction (SPE) and were subsequently cleaned up and derivatized by silylation. Analysis of the extracts was performed by gas chromatography combined with ion trap detection (GC-ITD-MS). Deuterated EE2 was added as internal standard for quantification. The procedure recovery was 95% (±23%).The limit of detection ranged from 1 to 2.5 ng l^{-1}, depending on the volume of the sample and amount of impurities in the sample.

Data handling and statistics

For each concentration the relevant data were tested for normality and for homogeneity of variance. An effect was considered to be significant (at α=0.05) based on statistical analysis of the data obtained for the treated groups compared with those obtained in the negative and/or solvent

control. For this, analysis of variance (ANOVA) combined with a mean comparison test (Dunnet or Bonferroni *t*-test) and the Tukey multi-comparison test was applied [28].

Results

Actual EE2 concentrations

The results of chemical analysis on EE2 are shown in Table 2.1. On day 21 no reliable data were obtained due to disturbing peaks in the chromatograms. The lowest level of 1 ng l^{-1} was below the detection level and thus no reliable concentrations were measured. In two of the four samples taken at 3 ng l^{-1}, the concentration was detectable and at a level of 80% after 28 d of exposure. Though the average EE2 level measured in samples taken from 10 ng l^{-1} group was about 80% of the nominal concentration, a rather high variation from 5.4 to 9.9 ng l^{-1} was found during the 30d period.

Table 2.1 Concentrations of ethinylestradiol (EE2) measured in samples taken during the early life stage test with transgenic zebrafish. Time weighed averages are shown in the last column.

Nominal concentration EE2 (ng l^{-1})	Measured concentration EE2 (ng l^{-1})			
	Day 7	Day 14	Day 28	Average
0 (solvent)	< LOD (2.5)	< LOD (2.5)	< LOD (1.0)	< LOD
1	< LOD (1.3)	< LOD (1.3)	< LOD (1.0)	< LOD
3	2.9	< LOD (2.5)	2.4	2.6
10	7.4	9.9	5.4	7.8

LOD = Limit of detection (Actual values between parentheses).

Survival and development of embryos and larvae

During the first 24 h after the start of the exposure of fertilized eggs, an average of 30% of the embryos died in the various treatments (Table 2.2). No apparent effects on either embryonic or larval development were observed which could be related to EE2 exposure. Survival of the transgenic larvae was in agreement with the validity criterion normally applied in Early Life Stage tests, i.e. no less than 70% of the larvae should survive the test period.

On the average about 10% of the larvae were albinos and completely lacked pigmentation (Table 2.2). These larvae were not included in further testing and discarded after euthanization since these were expected not to survive the additional exposure period of 25 d (based on earlier experience). Incidentally, embryos developed to larvae with a bent spine and these died shortly after hatching. This was not treatment related and also observed in several of the 15 replicates receiving tap water only.

There was no significant difference between the mean half time for hatching in the controls receiving tap water only (15 vessels in total), and the means in the EE2 treated groups, although there was a slight delay in the latter groups when compared to the solvent control (Table 2.2).

Table 2.2 Data on egg survival after 24 h, hatching time (*t*50= time of 50% hatching), percentage albinos, survival, fish length and body weights during the early life stage test with transgenic zebrafish exposed to ethinylestradiol (EE2).

Concentration EE2 (ng l^{-1})	24h Survival (%)	t50 hatch (h)	Albinos (%)	26d survival (%)	30d survival (%)	30d length (mm)	30d weight (g)
0 (blank) [a]	71	71	2	77	70	9.92	14.39
0 (solvent)	67	67	8	91	91	10.91 [b]	19.43 [b]
1	83	74	13	91	91	10.46	20.13 [b]
3	59	74	14	100	100	10.57	18.62
10	65	75	15	96	96	11.00 [c]	21.01 [b]

[a] Tap water only

[b] significantly different from negative control two-sided α = 0.05; if the means of the EE2 treated groups are compared to the mean of the solvent-control they were not statistically different.

The juvenile fish were weighed and measured at the end of the 30d period. Evaluation of the mean body weights per replicate showed no significant relationship with the number of larvae per vessel but there was a positive relationship with the presence of the solvent DMSO during 30d of exposure (Table 2.2, Figure 2.1). The mean body weights of the fish exposed to the vehicle DMSO for 30 d were statistically higher than the blank control (Figure 2.1), and higher than the mean body weights of the animals exposed to DMSO for four days only. There was no relationship between the presence of solvent and the number of surviving larvae per vessel. The number of larvae present in a vessel can influence growth, as fish tend to become bigger when the volume of water per individual increases. However, when condition factor (100 x weight divided by length^3) was compared, there was no significant difference between the 30d treatments and the controls (Figure 2.1).

Luciferase and VTG induction

Significantly higher levels of luciferase were found in transgenic zebrafish exposed to the nominal concentration of 10 ng l^{-1} EE2 in both the 30d and 4d exposure groups, while at the lower levels of EE2, luciferase induction was hardly above background values (Figure 2.2). There was no significant difference between luciferase responses in homogenates of fish exposed for 30 d and those exposed during the last four days. No luciferase activity was observed in the negative and solvent controls, indicating negligible endogenous estrogen levels.

In a simultaneously performed test at the IVM laboratory with eggs from the same batch as used for the present study, a similar 4d exposure resulted in comparable induction of luciferase at 10 ng l^{-1} EE2 (Table 2.3). However, in this test, fish were exposed via a static renewal set-up as opposed to the continous flow set-up at the NOTOX laboratory.

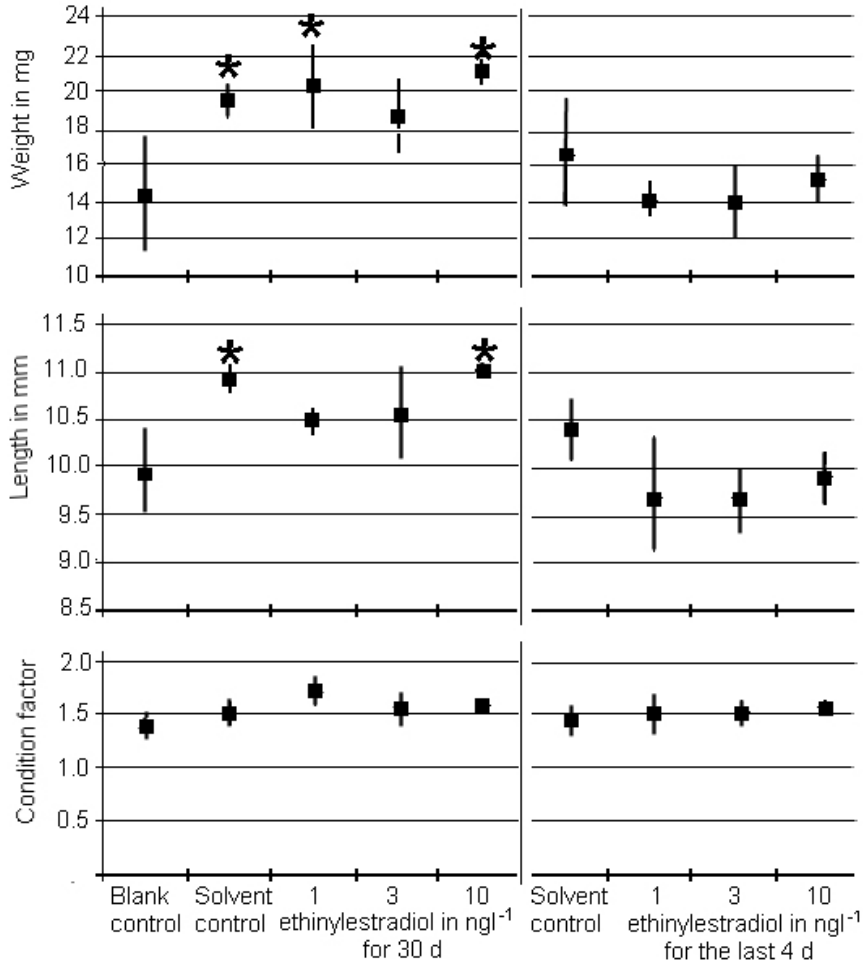

Figure 2.1 Effect of ethinylestradiol (EE2) on mean body weight (mg), body length (mm) and condition factor of transgenic zebrafish exposed from 0-30 d or 26-30 d; one asterix = significantly different from negative control, two-sided $\alpha = 0.05$.

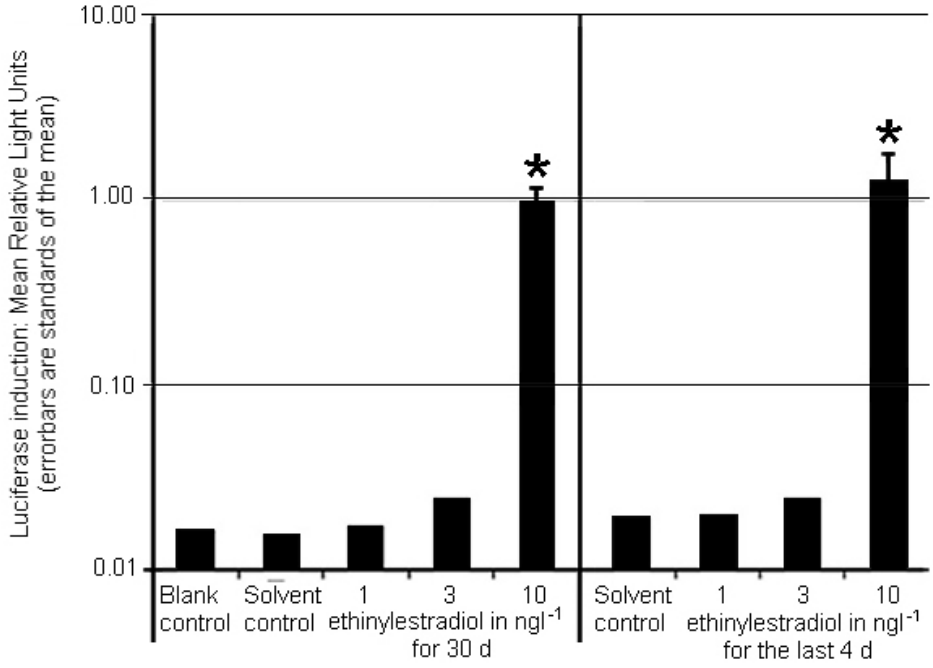

Figure 2.2 Luciferase activity (relative light units (RLU)) in transgenic fish exposed to ethinylestradiol (EE2) from 0-30 d (left series of bars) and 26-30 d (right series of bars). Both bars at 10 ng l^{-1} were significantly higher than the controls (α=0.05).

Table 2.3 Comparison of induction factors of luciferase in transgenic fish exposed to ethinylestradiol (EE2) for days 26 to 30 with continuous flow dosing at the NOTOX laboratory and static renewal dosing at the IVM laboratory.

Nominal concentration EE2 (ng l^{-1})	NOTOX Continuous Flow dosing		IVM Static renewal dosing	
	Induction factor (SEM)		Induction factor (SEM)	
0 (solvent)	1.0		1.0	
1	1.0	(0.1)	1.1	(0.1)
3	1.3	(0.1)	1.6	(0.1)
10	67.3	(19.7)	90.9	(31.5)
E2: 2750	Not tested		622	(220)

NOTOX = NOTOX Safety & Environmental Research, 's-Hertogenbosch, The Netherlands.
IVM = Institute for Environmental Studies, Amsterdam, The Netherlands.
SEM = Standard error of the mean.
E2 = Estradiol.

Prior to commencing the experiments with EE2, VTG analysis was introduced in our laboratory with a preliminary experiment in which VTG was measured in wild type zebrafish exposed to E2 for 42 d. Levels of VTG had increased 9.10^4 –fold in zebrafish exposed to the E2 concentration of 825 ng l^{-1} compared to 0.1 μg/g in the control groups , which were four orders of magnitude higher than the levels measured at 82.5 ng l^{-1} (Figure 2.3).

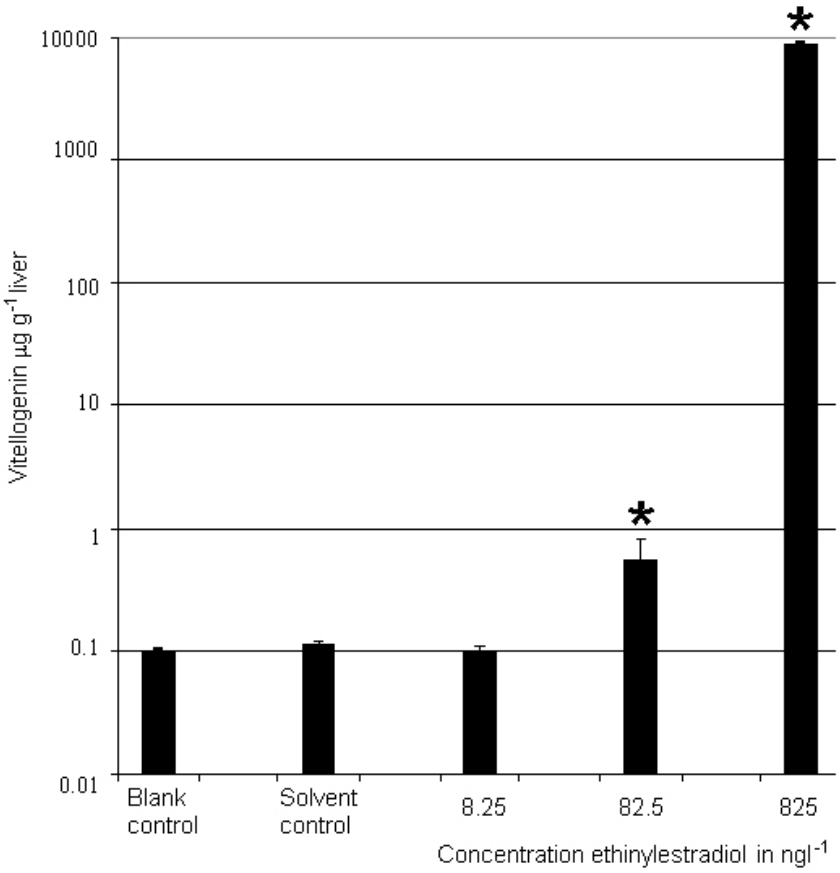

Figure 2.3 Vitellogenin concentrations (±standard error of the mean) in whole body homogenates of wild type zebrafish exposed to 17 β-estradiol (E2) for 42 d (one asterix: significant, α=0.05).

Compared to DMSO-controls, VTG were elevated 5- to almost 10000-fold in the homogenates of fish exposed to 3 and 10 ng l^{-1} EE2 for 30 d, respectively (Table 2.4, Figure 2.4). Furthermore, the mean VTG level measured in the homogenates of fish exposed to 10 ng l^{-1} for 30 d was less than 2 orders of magnitude higher than the mean level measured in the homogenates of fish exposed during the last four days of the 30d period (Table 2.4).

Table 2.4 Comparison of induction factors of VTG in transgenic fish exposed to ethinylestradiol (EE2) for 30 or 4 days

Concentration EE2 (ng l^{-1})	30 days		4 days	
	Induction factor	(Sem)	Induction factor	(Sem)
0+DMSO	1.0		1.0	
1	0.8	(0.0)	1.2	(0.2)
3	5.2	(0.4)	3.7	(1.1)
10	8307	(2351)	106	(17)

Discussion

The main purpose of this study was to both incorporate and compare endpoints for estrogenic exposure in experiments based on the standardized Early Life Stage test [70] with transgenic zebrafish. Exposure of transgenic zebrafish to non-toxic concentrations of EE2 induced an estrogenic related response at EE2 levels known to be potentially estrogenic in fish [54, 74, 81]. Luciferase activity was induced at the nominal EE2 concentration of 10 ng l^{-1}. Interestingly, exposure duration had no effect on luciferase activity in contrast to the increasing effect it had on the extent of vitellogenin induction. This indicates that, in short-term exposures during the critical life stage of gonad differentiation, measurement of the activation of endogenous ERs may be predictive of estrogenic effects in case of longer-term exposure.

Previous studies have also demonstrated that a 96-h exposure to estradiol and nonylphenol in transgenic zebrafish at the ages of 4-5.5 weeks correlates well with longer-term (three week) exposure [82]. Also, there were no inter-laboratory differences found in luciferase induction between the studies performed at IVM and our laboratory, in spite of the fact that IVM used a static renewal set-up opposed to the flow-through design used at NOTOX. Hence, for short-term screening of estrogens, a quick and simple dosing set-up could be sufficient provided that the substance of concern is hydrolytically stable and test concentrations can be maintained throughout the test period.

Unlike luciferase, quantitative comparison of the VTG levels in the zebrafish larvae exposed to EE2 for 30 d showed significantly higher levels of VTG at both 3 and 10 ng l^{-1} compared to controls. Levels of VTG measured in the homogenates of fish exposed during the last four days showed that only those at 10 ng EE2 per liter were significantly higher.

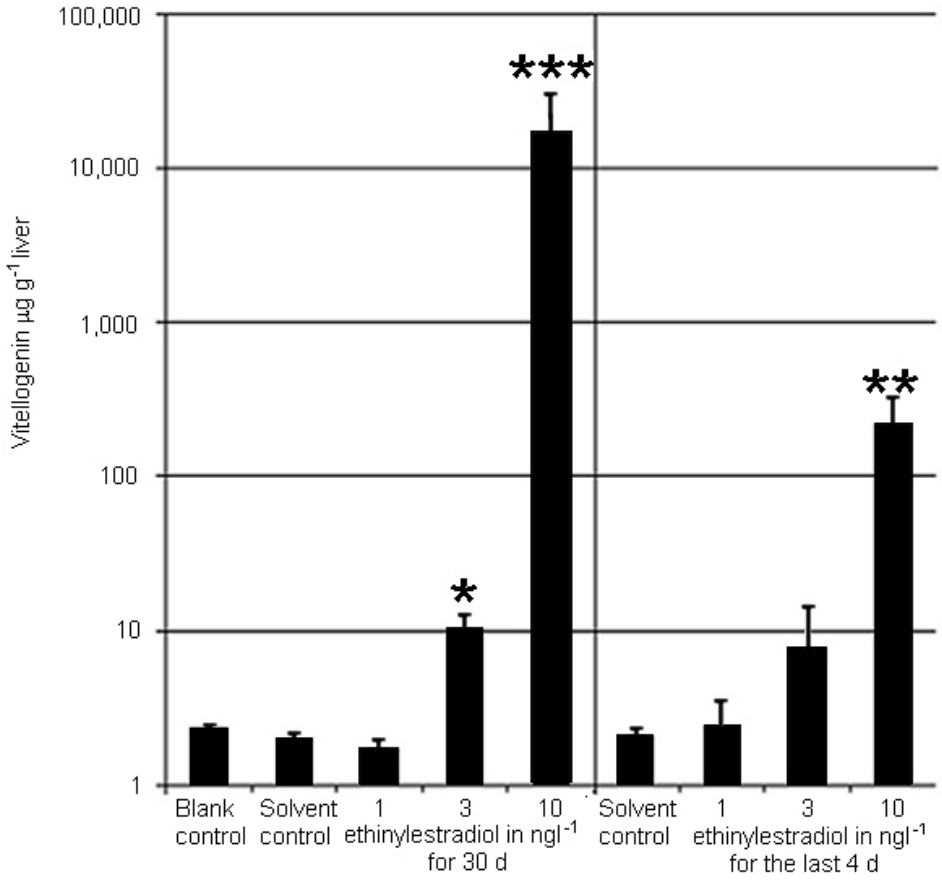

Figure 2.4 Vitellogenin concentrations (± standard error of the mean) in whole body homogenates of transgenic zebrafish exposed to 17 α-ethinylestradiol for 0-30 d or the last 4 d (26-30 d). One asterix: > Blank and solvent, two asterices: > 3 ng l^{-1}-30 d, three asterices > 10 ng l^{-1}- 4 d, α=0.05).

Previous results with adult zebrafish males exposed for eight days showed a lowest effect concentration at 3.0 ng EE2 per liter [74]. It is apparent that longer exposure periods result in the accumulation of vitellogenin, which is far more stable than luciferase with a half-life of merely two hours in live mammalian cells [83]. Schultz *et al* [84] found an elimination half-life of about two days (42–49 h) for VTG in rainbow trout exposed to EE2. The pattern of VTG induction was similar for all doses of EE2, with a 12h lag-time before a sharp increase from basal levels to maximum levels within seven to nine days. Plasma levels of intra-arterially injected purified VTG in rainbow trout declined tri-exponentially with an elimination half-life of 43.7h. Further comparison of the VTG levels at 10 ng EE2 per liter between the 30d and 4d exposure indicates that larval sensitivity for vitellogenin induction starts earlier than 26 d post fertilization or approximately 21 d post hatching.

As VTG tends to accumulate, this period could be in the range of one week earlier. Further, in our study relatively low or no exposure to estrogenic compounds give a relatively low background response of VTG production and this makes exposure during the early life stage a very sensitive method for detecting estrogenic active compounds.

The preliminary experiment with wild type zebrafish exposed to E2 also showed that measurement of VTG in the early life stage test is indicative for the estrogenic activity of test substances. The VTG response of the wild type fish was significantly higher at 82.5 ng E2 per liter compared to the mean response at 8.25 ng E2 per liter and the controls, although the absolute mean was only a factor of 2.5 higher. This indicated that the method of VTG measurement applied was relatively sensitive compared to other reports, in which VTG induction was measured only at a concentration of 275 ng E2 per liter in juvenile zebrafish exposed for 42 d [85]. However, in a comparable study design with fathead minnow exposed to E2 for 30 d during the early life stages, VTG levels detected were one order of magnitude higher than the controls at 50 ng per liter and two orders of magnitude higher at 100 ng per liter [86]. The absolute VTG levels in the fathead minnow controls of this experiment were comparable with those found in the present experiment with zebrafish. These results indicate a species-specific sensitivity to estrogens. In a study with adult male zebrafish VTG levels were three orders of magnitude higher than in the controls in males exposed to 80 ng E2 per liter for not more than eight days [74]. Also in their study Wester et al. [85] found that VTG induction following estrogen exposure was more sensitive in adult males than in young juveniles during their larval development. Comparison of the effect concentrations shows that fish are more sensitive to the artificial steroid EE2 than to E2. This confirms earlier findings for zebrafish [74,75,79], but is also found in other fish species [55, 76, 77, 78, 78]. The fact that EE2 is manmade and far more stable in water, this steroid is preferred as a reference or positive control for estrogenic responses in this type of experiment [70].

Our results indicate that measurement of VTG is a more sensitive endpoint for estrogen exposure than luciferase. However, it should be taken into account that VTG induction may not be as specific an indicator for estrogenic activity as compared to the specific ER-mediated mechanism of action of chemicals measured with the luciferase response. As luciferase reporter gene activity is under control of a minimal promoter sequence, only chemicals acting directly through activation of the ER will be measured. Cross talk with other hormone pathways will not be detected by this endpoint, as may be the case with vitellogenin, e.g. VTG has been shown previously to be induced by androgenic chemicals, such as methyldihydrotestosterone [85] and 17-β-trenbolone [87]. In addition, any substances that affect the neuro-endocrine system, generally referred to as the hypothalamus-pituitary-gonadal axis, can affect VTG induction. These differences in specificity should be taken into account when testing with an unknown mechanism of action or environmental samples, which contain mixtures of chemicals.

Conclusions

This study demonstrated the usefulness of incorporating endpoints for estrogenic effects in a standard ELS test. The early life stage test is a relatively straightforward experimental design, and, as it is already standardized, can be easily adjusted to fit the requirements of a test that can be

used to screen chemicals for their estrogenic activity. Exposure of zebrafish for 30 d is sufficient to measure possible estrogenic potential of industrial chemicals with EE2 as a reference substance in a positive control group. Both the quantitative measurements of luciferase activity and VTG proved to be rapid and relatively sensitive as endocrine endpoints, which make them both particularly useful for rapid (96 h) *in vivo* pre-screening of chemicals, and to direct further detailed testing. Measurement of VTG is a more sensitive endpoint when used in experiments that include prolonged exposure like the early life stage tests. However, the advantage of the application of the transgenic zebrafish in early life stage tests is the combination of the sensitive endpoint of VTG measurement with the specific ER-mediated mechanism of action of chemicals measured with the luciferase response in one experiment.

Application of the early life stage test can point out substances that have endocrine disrupting potential with much less animals and within a substantially shorter time that full life cycle tests. This makes this type of experiment both animal friendly and cost-effective. Hence, added value can be expected from the application of this strain of transgenic zebrafish as test organisms in an early life stage test for the assessment of estrogenic activity of industrial chemicals in the context of classification and risk evaluation.

Acknowledgements

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Chapter 3

An extended fish early-life stage test with an androgenic chemical in the fathead minnow (*Pimephales promelas*)

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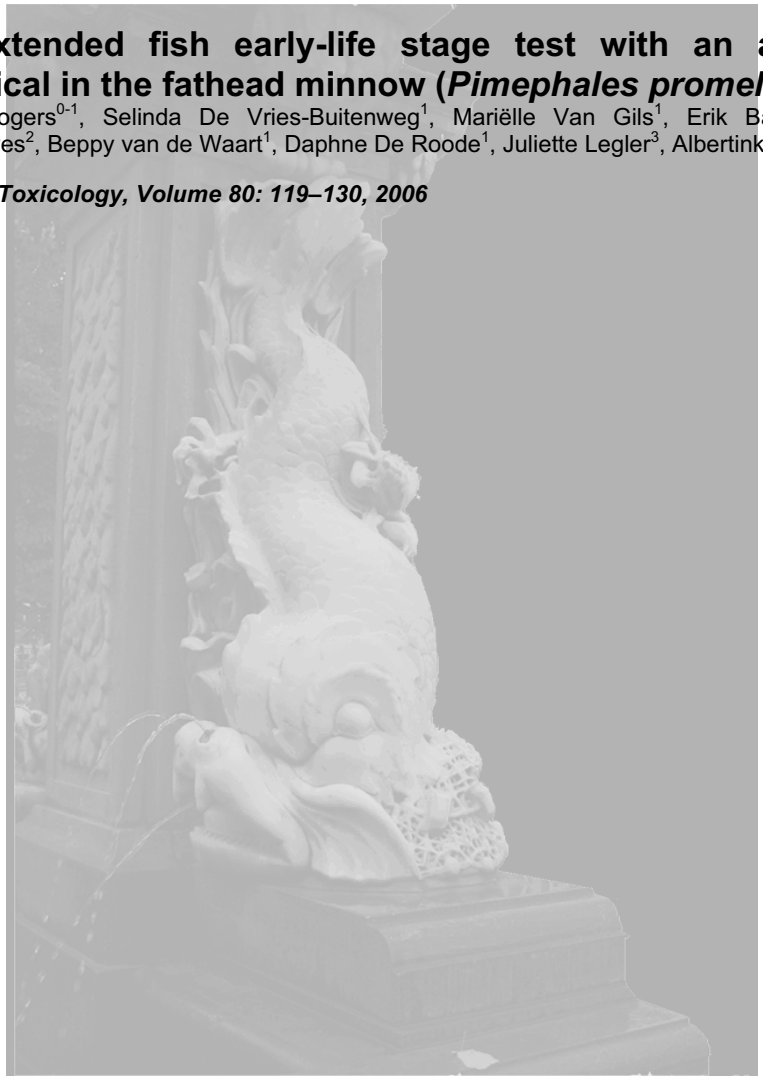

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Abstract

The Endocrine Modulators Study Group (EMSG) of the European Chemical Industry has proposed an extended fish early-life stage (ELS) test based on OECD test guideline 210 in combination with a fish pair-breeding reproduction study as a possible alternative for fish full life cycle testing. In this paper the androgen methyldihydrotestosterone (MDHT) was tested in an extended ELS test with fathead minnow supplementary to such a test with the weak estrogen 4-*tert*-pentylphenol (4TPP). Main endpoints were secondary sexual characteristics (SSC), plasma vitellogenin (VTG) induction and gonadal development. Early blastula embryos were exposed to 0, 0.10, 0.32 and 1.0 μgMDHTl^{-1} for up to 114 days post-hatch (dph). A batch of fish exposed to 1.0 μg l^{-1} was transferred to clean water after 30 or 63 dph for the remainder of the study. Ethinylestradiol (EE2) was included as estrogenic reference substance at 0.01 μg l^{-1}. Exposure to MDHT had no significant effect on hatching success or survival, but significantly increased the condition factor of fish exposed for 63 and 114 dph (up to 150% of the control). At 63 dph MDHT exposure induced appearance of tubercles on the snout (a male SSC) of more than 80% of fish. Compared to the controls, plasma VTG was not detectable or significantly lower in fish exposed to MDHT at 0.10 μg l^{-1}, but not significantly affected at higher MDHT concentrations. Both lower levels of MDHT significantly inhibited the development of female gonads as of 30 dph. Fish exposed to MDHT at 0.32 and 1.0 μg l^{-1} showed higher incidences of mixed sex gonads (10–25%) and smaller testes or dysplasia of gonadal tissue. Dysplasia was present in 80% of the fish continuously exposed to 1.0μg l^{-1} up to 114 dph, but reversible when fish were transferred to dilution water. Results indicate that suppression of ovarian development was the most sensitive endpoint for MDHT exposure after 30 dph. Other endpoints (e.g., growth and SSC) required exposure during at least up to 63 dph to yield a significant effect. Androgenic effects on VTG production required even longer exposure, i.e., until sufficient number of females had matured.

Introduction

As part of its Aquatic Research Programme to assess the effects of endocrine active chemicals (EACs), the Endocrine Modulators Study Group (EMSG) of the European Chemical Industry has proposed to extend the existing fish early life-stage test (OECD test guideline 210 [70]), to be used for environmental risk assessment purposes to confirm or refute the endocrine activity potential of suspected chemicals by assessing adverse effects. An earlier study on the development of chronic tests for EACs was conducted with a weak estrogen 4-*tert*- pentylphenol (4TPP) and published in this journal [88]. The proposal is based on the principle that the period of sexual differentiation, is a sensitive window of exposure to endocrine active chemicals [21, 40, 41]. This hypothesis has also potential for androgens such as testosterone [89] or methyltestosterone (MT) [90]. Environmentally induced androgenic effects in fish collected downstream of paper mills were already described in the early 1980s [91] and more recently in fish exposed to diluted effluents originating from plants and mills [33, 92, 93, 94] or cattle feedlot [95]. Androgenic activity in effluents was further confirmed by testing samples in androgen bioassays [96].

The purpose of the present study is to investigate whether the extended early life stage test, as described earlier [88], is also suitable for the assessment of androgenic effects in the fathead minnow. The synthetic steroid methyldihydrotestosterone (MDHT) is a non-aromatizable, methylated form of the natural endogenous androgen 5α-dihydrotestosterone [97, 98]. MDHT has a higher androgenic potency in fish than methyltestosterone or 11-ketotestosterone [99-103] and a slower degradation [97]. MDHT has also been chosen as a reference substance for androgen-related effects in a OECD draft proposal for a Fish Sexual Development Test [104]. Assessment of the effects of MDHT on gonad histology included the primary characteristics (histological sex, as defined by the germ cells present) and secondary characteristics (peritoneal attachment). MDHT was tested at sub-lethal concentrations [103] and confirmed in a range-finding study. The concentration levels were not related to concentrations of androgens measured in nature. Instead, the concentrations were tested at such levels that significant effects would occur on all endpoints examined and also the extent of reversibility of effects could be examined in fish transferred to clean water.

The extended early life stage test consisted of the exposure of fathead minnow to MDHT during the embryonic and larval stages with prolonged exposure until sexual maturation. Three time points of sampling were included to investigate the first time to detect a significant change in each endpoint. Fish from the highest MDHT concentration were returned to clean water in order to determine if any of the observed changes were reversible.

Materials and methods

Test species

Fathead minnow (*Pimephales promelas*, Teleostei, Cyprinidae) embryos were obtained from broodstock fish bred at the Ecotoxicology facilities of NOTOX B.V.. The broodstock were fed daily

a mixed diet of pelleted food (Bio-Optimal C80, Brande, Denmark) and adult brine shrimp. Male and female fish at a ratio of 1:1 were maintained under a 16-h light:8-h dark photoperiod to allow natural spawning. In the early morning eggs were collected from a substrate placed in each vessel and checked for successful fertilization and stage of development. All exposures were initiated with embryos at the early blastula stage. The embryonic stage of fathead minnow lasts for approximately four days, when larvae hatch. A larva is a recently hatched fish, which has physical characters other then those seen in adult. After the yolk-sac is completely absorbed larval fish commence with active feeding (swim-up fry). Post-larval fish develop to young adult fish in 90 to 120 days post hatch (dph).

Test substances

The androgen MDHT (17α-Methylandrostan-17β-ol-3-one, CAS 52-11-19) and the estrogen EE2 (Ethynyl estradiol, CAS 80-46-6) were at least of 98% purity (Sigma Chemical Co. Ltd. ,Dorset, England). Stock solutions of MDHT and EE2 were prepared in Trigol (Triethyleneglycol, CAS 112-27-6) at the lowest possible concentration, i.e. 0.00033 %.

Exposure design

Stock solutions were prepared in deionized water by adding 330 μl of primary stocks in Trigol per litre water, providing nominal concentrations of a factor of 100 higher than the final test concentrations. These stock solutions were used for dosing in the flow-through system at a rate of 10 ml per hour using computer controlled dispensers (Gilson, Velliers Le Bel, France). In mixing vessels, the dosed stocks were mixed with dilution water at a flow of 16.7 ml min^{-1} before it entered a 2-litre test vessel during the first 30 dph (ca. 10 renewals per vessel per day). During the remaining test period up to 114 dph, stocks were dosed at 60 ml per hour mixed with a dilution water flow of 100 ml min^{-1} before entering a 30-litre test vessel (5 complete renewals per vessel per day). All tubing used was made of glass or Teflon. The system was checked daily for any type of failure, flow rates and other technical features.

The dilution water was non-chlorinated tap water maintained between 24 and 27 °C. The hardness of the test water was < 200 mg l^{-1} measured as $CaCO_3$. The dissolved oxygen, temperature and pH values were measured in all vessels three times a week. The dissolved oxygen concentration remained above 70% of the air saturation value throughout the exposures. The pH was between 7.6 and 8.1. A 16:8 hour light:dark photoperiod with a staged change of light intensity (20 minutes) for dawn and dusk periods was provided.

Exposure protocol

The range of exposure concentrations for MDHT was based on a range finder study of 35-day duration at 10 μg MDHT l^{-1} and 0.01 μg EE2 l^{-1}. The nominal exposure concentrations selected for MDHT were 0.10, 0.32 and 1.0 μg l^{-1}. Two additional treatments were set up at the highest test concentration (1.0 μg l^{-1}), one where exposure was limited to the first 30 days (treatment 1A) and another to 60 days (treatment 1B). At the end of these exposure periods, fish were kept in dilution water until the end of the study (114 dph).. EE2 was used as potent estrogenic reference, and was

tested at 0.01 μg l^{-1} as in Panter et al. [88]. A schematic of the dosing regime is shown in Figure 3.1.

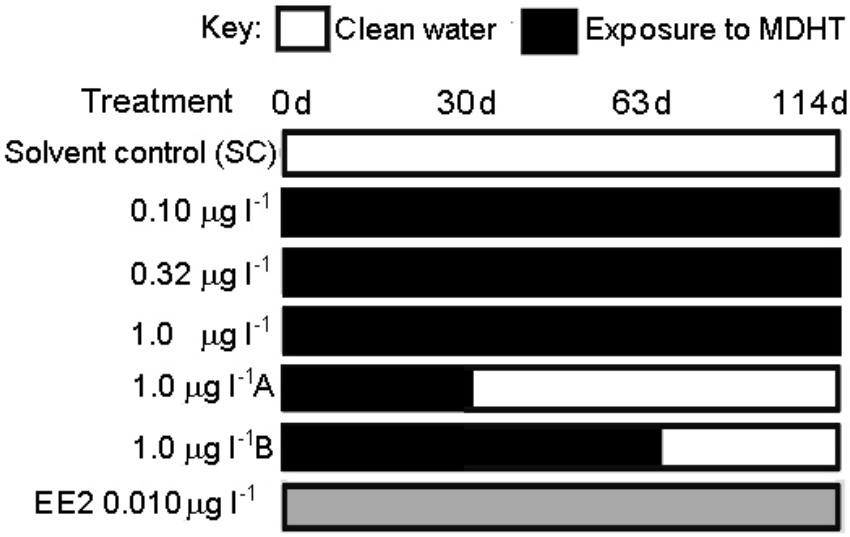

Figure 3.1 Schematic dosing regime. MDHT = methyldihydrotestosterone, EE2 = ethinylestradiol, dph = days post hatch.

No dilution water control was deemed necessary since the solvent control (Trigol) was used at a negligible level, i.e. 0.00033 % and no effects were observed at a Trigol concentration of 0.01% in a similar test with 4TPP [88]. This reduced the number of animals in the test, fulfilling the requirements of the Dutch Animal Control Act for reducing numbers of experimental animals without invalidating the integrity of the test.

At the start of the study early blastula embryos were obtained from the spawning of at least 10 pairs of fish. Batches of embryos were collected, pooled and then distributed randomly, in batches of 30 per petri-dish, with four replicate dishes per treatment. After 24 hours, all embryos were observed for mortality. Live embryos were transferred and kept for 4 days in sterile petri-dishes (diameter: 15 cm) that were gently shaken on a shaking device with daily renewal of the solutions. On day 4, all hatched larvae and any remaining embryos were transferred to stainless steel vessels of ca. 2 litres in a continuous flow-through system (four per treatment) and grown up until 30 dph. On this day, the remaining juvenile fish were transferred to 30-litre glass vessels in the flow-through system. Newly hatched larvae were fed rotifers (*Brachionus plicatilis*) and subsequently *Artemia* nauplii. Juveniles and adults were fed *ad libitum* with adult brine shrimp supplemented with pellet food (Bio-Optimal C80, Brande, Denmark). Fish were observed daily on working days for abnormal behaviour and appearance. When two fish (3 %) in the control group developed a deformation a vitamin solution was added to the test solutions daily during one week (Thiamine at 75 ppb, B12 at 1.0 ppb and Biotin at 0.75 ppb). Since they were added to the test

solutions for only one week these concentrations of vitamins were thought to not have affected the results.

Sampling of fish

At 30 dph, three fish were sampled randomly from each tank (thus, 12 per treatment of 0, 0.1, 0.32, 1.0 μg MDHT l^{-1} and the EE2 group), anaesthetized (with buffered MS222) and sacrificed according to Dutch Animal Control Act. Concentrations of MS222 used were adjusted to size of the fish. Fish were immediately wet weighed (to the nearest 0.1 mg) and measured (standard length to 0.5 mm). Whole fish were fixed in Bouin's solution and then stored in 70% industrial methylated spirit (IMS) for histological processing, described below.

At 63 and 114 dph, five fish were sampled randomly from each tank (i.e. 20 fish per treatment). After anaesthetization, blood was sampled by cardiac puncture using 1-ml syringes containing phosphate buffered saline (PBS) with heparin (0.16 mg ml^{-1} or 25 units ml^{-1}). The blood samples were collected in Eppendorf vials and subsequently centrifuged (7000 g at 4°C for 5 or 10 minutes). The supernatants were transferred to new Eppendorf vials and then stored at -20 or -80 °C dependent on the period needed for storage until actual VTG analysis. After blood sampling, fish were instantly killed by a cut through the spinal cord. Thereafter fish were immediately wet weighed (to the nearest 0.1 mg) and measured (total body length to 0.5 mm). Prior to fixation and storage fish were slit ventrally from the anus to the gills to allow the fixative to penetrate the body cavity to avoid lytical processes. At 114 dph, the remaining fish from each tank were sacrificed and measured, but no blood samples were taken. After sacrificing the fish, they were cut in three parts: head, body and tail. The head and the body were separately immersed in Bouin's solution for 24 hours and stored in 70%-ethanol. The head part was used for determination of nuptial tubercles and the body part for histological processing. The tail part was discarded.

Histological processing and analysis

Normally female 30-dph fathead minnow larvae are distinguished from males by the presence of early oocytes in already differentiated ovaries each with two peritoneal attachments. In contrast, male gonads have not yet developed, but are distinct in their single peritoneal attachment. In general male gonadal tissue has started to differentiate after 63 dph.

Fixed body parts were cut transversally into 2 mm slices for smaller fish and into 3 to 4 mm slices for larger fish. The slices were embedded in a paraffin wax and the resulting wax blocks were sectioned with the microtome at 3 μm thickness, mounted on glass slides and stained with haematoxylin and eosin. The resulting transverse whole-body sections were microscopically examined to determine, where possible, fish sex and stage of testicular or ovarian development. Fish gonadal sex defined histologically as 'male' when cells of spermatogenic lineage were present, or 'female' when cells of oogenetic lineage were present. When both male and female germ cells were recognized within a gonad, this was recorded as 'mixed'. The designation 'sexually undifferentiated' was given to fish with gonads containing germ cells of an, as yet, undetermined lineage. Gonadal staging was performed according to Leino et al. [104]. Morphology of the reproductive tract was assessed, i.e. male-type (single attachment to the coelomic wall),

female-type (double attachment to the coelomic wall) as described by van Aerle et al. [106] for the fathead minnow or "not assessable". Additional gonadal features were examined e.g. abnormal spermatogenesis visible by deviating distributions of staging in testes or missing stages, oocyte atresia, collapsed follicular structures, "cavitation of the gonad" or hypoplastic/vestigial. The histological analysis was also conducted on the liver and kidney to assess possible impact on cell/tissue structure in these organs.

Quantification of secondary sexual characteristics

After 90 to 120 dph fatheads become sexually mature and show distinct secondary sexual characteristics (SSCs). The SSC determined was the number and prominence (grading) of nuptial tubercles (a male characteristic), which has been shown to be responsive to steroid hormones [107]. Counting and grading of the nuptial tubercles was conducted at 63 and 114 dph, using a binocular microscope following Smith [57] where the grading relates to the progressive prominence of the tubercles.

Vitellogenin measurement

Blood plasma samples were assayed for VTG using an established heterologous carp enzyme-linked immunosorbent assay. Biosense Laboratories AS (Bergen, Norway) supplied all reagents in the form of kits including carp-VTG standards and primary antibody, which has been shown to be suitable for determination of VTG concentrations in the fathead minnow [72]. VTG measurement was conducted according to standard protocols.

Analytical chemistry

Water samples for chemical analysis were taken from the stocks and test solutions at the start of the experiment and at regular intervals of 1 or 2 weeks during the exposure including the EE2 group. The aqueous samples were concentrated using solid-phase extraction (SPE). Quantitative analysis was based on the areas of the MDHT and EE2 peaks in a GC-MS chromatogram divided by the area of an internal standard peak (β-Estradiol). Further details of the analytical methods are available upon request from the corresponding author.

Statistical analysis

The data for wet weight, standard length, condition factor (body weight/ (0.01*length^3), SSCs and plasma VTG concentrations (log transformed) were analysed by analysis of variance (ANOVA). Where assumptions of normality and homogeneity were met, ANOVA was followed by a Dunnett's test to compare the treatment means with respective controls. Where the assumptions were not met, data were analysed using a suitable non-parametric test (Steel's Many-one rank test, Wilcoxon Rank Sum test and/or Kruskal-Wallis test). Tests were performed using a two-sided α of 0.05 [80].

Male and female data were based on histological examination. Histopathological results were analysed based on arcsine transformation of the percentages of female gonads for gonadal sex ratios, and of the percentages of female reproductive tract (double attachment) for comparison between the proportions of male and female reproductive tracts (single and double attachments)

(ANOVA Dunnett's test). Further the weighed Cohen Kappa measure for agreement was applied to compare the relation between two different histological observations: first, the gonadal sex (male, female, undifferentiated) and second, the type of reproductive tract (single/male-like; double/female like). This measure is theoretically between -1 (negative agreement) and 1 (perfect agreement). An agreement of 0 indicate the absence of any correlation. Where possible gonadal staging and microscopical observations were tested for significance applying a family-wise approach.

The distribution of the ovarian and testicular stages, the distribution of seminiferous tubule expansion within the fish with testis, and the distribution of dysplastics were tested for a monotonous relation between the observation ad either the concentration level or the exposure period using the non-parametric Jonckheere-Terpstra test. The results are reported as means ± standard deviations or percentages.

Results

Analytical chemistry

Concentrations in the solvent control were below the limit of detection < 0.0021 μg l^{-1} for MDHT and < 0.0013 μg l^{-1} for EE2 (Table 3.1). The EE2 concentrations were in agreement with nominal (100% when corrected for analytical recovery). The time weighed average (TWA) concentrations for MDHT shown in Table 3.1 are based on the concentrations actually measured at regular intervals of 1 or 2 weeks. The actual concentrations of MDHT deviated by -30 to +9 % from nominal. For reasons of clarity the concentrations in the results are based on nominal concentrations.

Table 3.1 Time weighed average concentrations (TWA) of methyldihydrotesto-sterone (MDHT) and ethinylestradiol (EE2) measured in the test solutions during exposure of fathead minnow with respective standard deviation (SD) and coefficient of variation (CV). The 1 μg l-1 group had either complete exposure or exposure up to 30dph (A) or 63dph (B).

Nominal concentration of MDHT (μg l-1)	TWA in μg l-1	% of nominal	SDTWA	CVTWA
0.10	0.107	107	0.019	18
0.32	0.239	75	0.061	25
1.0	0.809	81	0.101	12
1.0 A	1.086	109	0.067	6
1.0 B	0.697	70	0.067	10
EE2 0.01	0.012	120	0.001	7

Hatchability and survival

There was a higher hatching rate (90 to 99 %) in the EE2 and MDHT treatment groups compared to the controls (77 ± 13 %), but this was not statistically significant (Steel's Many-One Rank Test). Survival rates in the control group were between 78 and 100 % at hatching, between 86 and 95 % at 30 dph, between 93 and 100% at 63 dph, and between 92 and 100 % at 114 dph.

Egg survival during the first 24 hours was generally >60%. There were no treatment related differences for survival during the embryonic, larval or juvenile stages up to 114 dph between the various groups including the treatments exposed during a part of the test period (MDHT 1.0 μg l^{-1} for 30 or 63 dph).

Somatic growth (wet weight, standard length and condition factor)

Growth occurred over the course of the experiment in the control as determined by the increase in wet weight (from 34.6 to 917 mg) and standard length (from 13.4 to 36.7 mm) (Table 3.2). Exposure to EE2 had a significant negative effect on body weight after both 63 and 114 dph but none on condition factor (Figure 3.2).

Table 3.2 Means for wet weight and standard length ± standard deviation (SD) of the various groups exposed to methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) at 30 dph (n = 12), 63 dph (n = 20) and 114 dph (n = 20). The 1 μg MDHT l^{-1} group had either complete exposure or exposure up to 30dph (A) or 63dph (B).

Nominal concentration of MDHT (μg l^{-1})	30 dph		63 dph		114 dph	
	Wet weight (mg)	Length (mm)	Wet weight (mg)	Length (mm)	Wet weight (mg)	Length (mm)
Solvent-Control	34.6 ± 12.5	13.4 ± 1.5	276 ± 98	24.5±2.6	917 ± 424	36.7±5.7
0.10	34.9 ± 12.0	13.5 ± 1.4	259 ± 63	23.0±1.8	1086 ± 315	36.8±4.9
0.32	31.4 ± 13.0	13.3 ± 1.6	326 ± 111	23.9±2.7	1247 $^{+}$* ± 366	36.7±4.8
1.0	40.2 ± 11.1	14.0 ± 1.0	308 ± 77	23.8±1.9	977 ± 421	37.3±4.8
1.0 A			208 $^{-}$* ± 68	22.5±2.7	1002 ± 381	36.8±5.3
1.0 B					853 ± 162	33.7±3.1
EE2 0.01	29.1 ± 7.6	13.1 ± 0.8	178 $^{-}$* ± 62	21.2±2.3	565 $^{-}$* ± 202	32.3±2.7

$^{-}$* Significantly lower than the control (α=0.05, two sided)
$^{+}$* Significantly higher than the control (α=0.05, two sided)

Continuous exposure to MDHT resulted in a significantly higher mean body weight at 0.32 μg l^{-1} (114 dph). Calculation of condition factors showed significantly higher factors for fish sampled from continuously exposed MDHT-groups after 63 and 114 dph (Figure 3.2). Condition factors of fish exposed to 1.0 μg MDHT l^{-1} and transferred to dilution water after 30 or 63 dph were comparable to those of the control group fish.

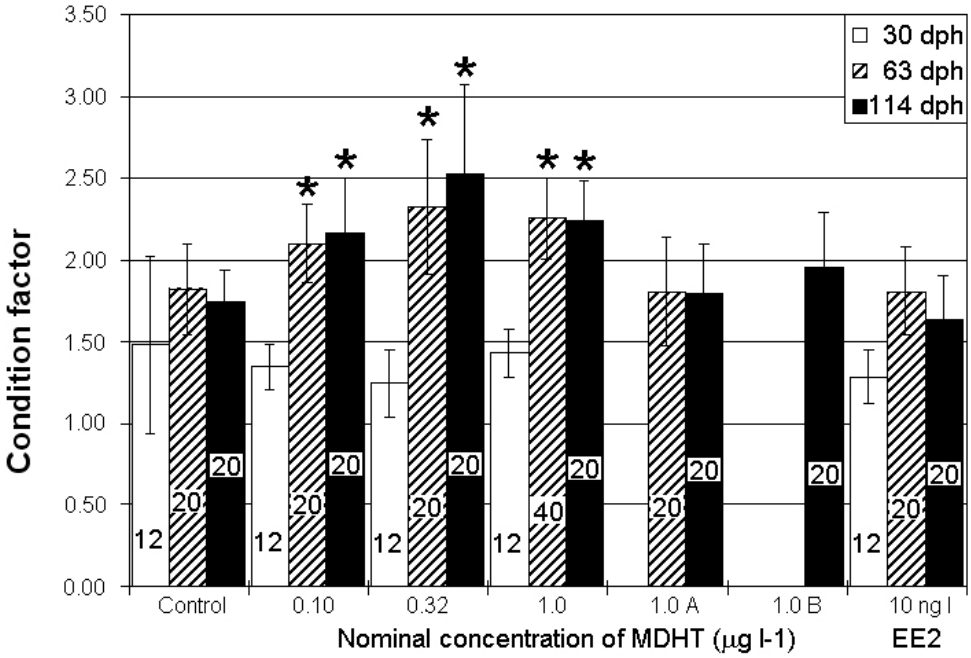

Figure 3.2 Condition factors in fathead minnows (y-axis) determined on 30, 63 or 114 dph at various concentrations of methyldihydrotestosterone (x-axis). Values are means ± standard deviation. The asterisks denote significant difference from the control (SC) at $p<0.05$ (Dunnett's Test, two tailed $\alpha=0.05$). EE2 = ethinylestradiol. The 1 µg l-1 group had either complete exposure or exposure up to 30dph (A) or 63dph (B).

Behavior and appearance

Fish from the solvent-control and from the EE2 treated fish behaved similarly, although, none of the fish exposed to EE2 developed any male appearance. On the other hand, all fish exposed to MDHT developed a male appearance (dark colored head and bands on the body) and showed a noticeable aggressive behavior regardless of the level of MDHT. In almost all vessels at least one male appeared as extremely dominant attacking or pursuing other fish. This behavior weakened when the fish were set in clean water.

At 0.32 and 1.0 MDHT µg l^{-1} in total up to 30 % of the fish developed deformation of the spine (bended tail, data not shown). Comparing the percentages of deformed fish this feature could be related to MDHT exposure. Gross examination of the fish sampled on 63 dph showed that the percentage of fish with a deformed spine was 15 % at 0.1 µg l^{-1}, 45 % at 0.32 µg l^{-1} and almost 40 % at 1.0 µg l^{-1}, while none of the 20 control fish was deformed. At the highest MDHT concentration of 1.0 µg l^{-1} the deformed fish remained visibly smaller compared to other fish in the same vessel and incidentally developed other deformities such as a flattened snout and deformation of the head.

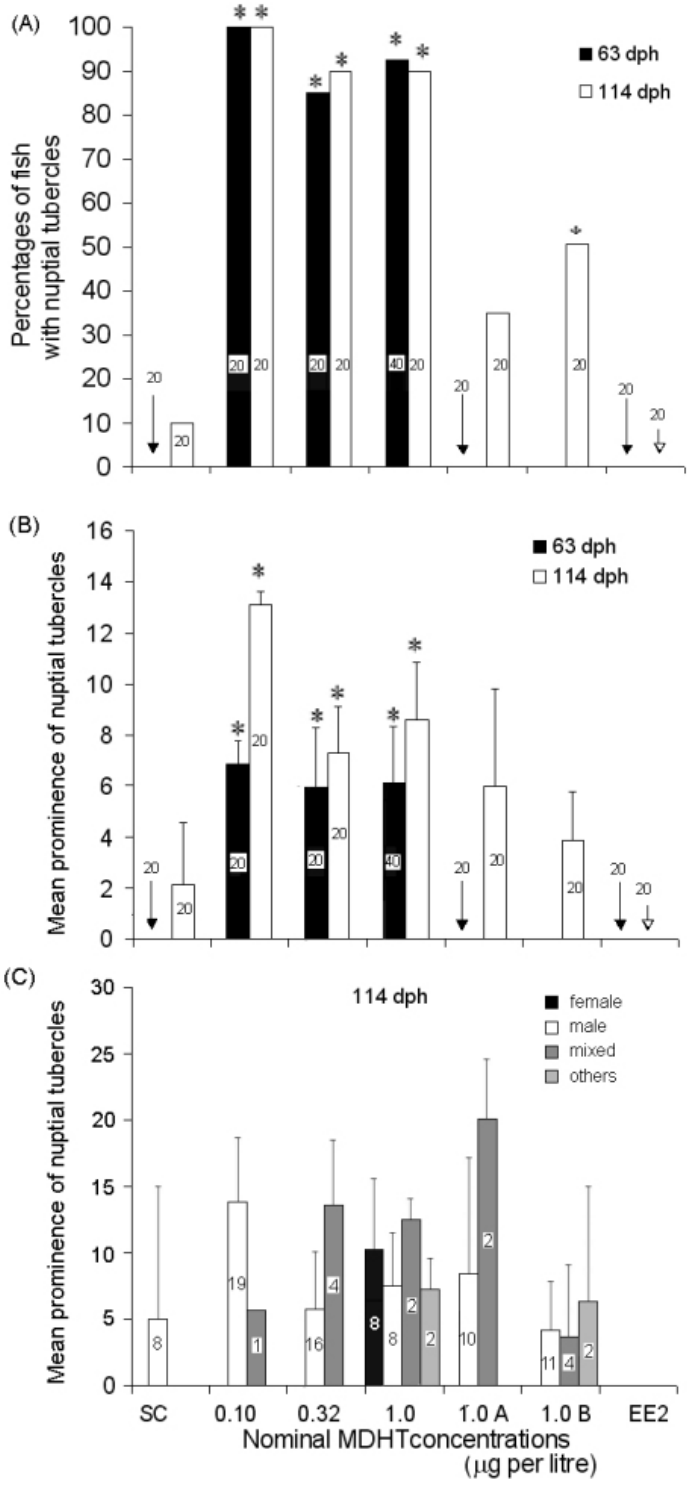
(A)
Percentages of fish with nuptial tubercles
63 dph
114 dph
(B)
Mean prominence of nuptial tubercles
63 dph
114 dph
(C)
Mean prominence of nuptial tubercles
114 dph
female
male
mixed
others
SC
0.10
0.32
1.0
1.0 A
1.0 B
EE2
Nominal MDHTconcentrations
(µg per litre)

Figure 3.3 (previous page):

A: Percentages of fathead minnow with nuptial tubercles (y-axis) versus concentrations of methyldihydrotestosterone (MDHT, x-axis) after exposure up to 63 dph (black bars) or 114 dph (white bars). The 1 μg l^{-1} group had either complete exposure or exposure up to 30dph (A) or 63dph (B). The asterisks denote significant difference from the solvent control (SC) at $p<0.05$ (Wil-coxon's Rank Sum Test, one tailed $\alpha=0.05$). EE2=ethinylestradiol (0.01μg l^{-1}).

B: Grading of nuptial tubercles in fathead minnows (y-axis) versus concentrations of MDHT (x-axis) after exposure up to 63 dph (black bars) or 114 dph (white bars). Values are means ± standard deviation. The asterisks denote significant difference from the control (SC) at $p<0.05$ (Steel's Many-One Rank Test, two tailed $\alpha=0.05$).

C: Grading of nuptial tubercles in fathead minnows (y-axis) versus concentrations of MDHT (x-axis) after exposure up to 114 dph specified for the different sexes. Values are means ± standard deviation.

Secondary sexual characteristics (grading of nuptial tubercles)

Male fish developed nuptial tubercles in the solvent control after 114 dph (Figure 3.3), while they did not in EE2 exposed fish. Exposure to all MDHT concentrations induced the precocious development of nuptial tubercles as of 63 dph (Figure 3.3). Between 80 and 100 % of the fish exposed to MDHT for the whole test period developed nuptial tubercles (Figure 3.3A). This corresponded with the finding that most of these fish had developed testes (Figure 3.5). At 63 and 114 dph, the mean grading of nuptial tubercles in all the MDHT exposed fish was significantly higher than in the controls (Figure 3.3B). Transferring fish to dilution water after 30 dph (1.0 A) or 63 dph (1.0B) resulted in a decrease of the expected proportion (ca 50%). These fish were found to be male based on the histological examination of the gonads (Figure 3.5). The fish that had developed female gonads after continuous exposure to 1.0 μg MDHT l^{-1} up to 114 dph (Figure 3.5) also developed nuptial tubercles (Figure 3.3C).

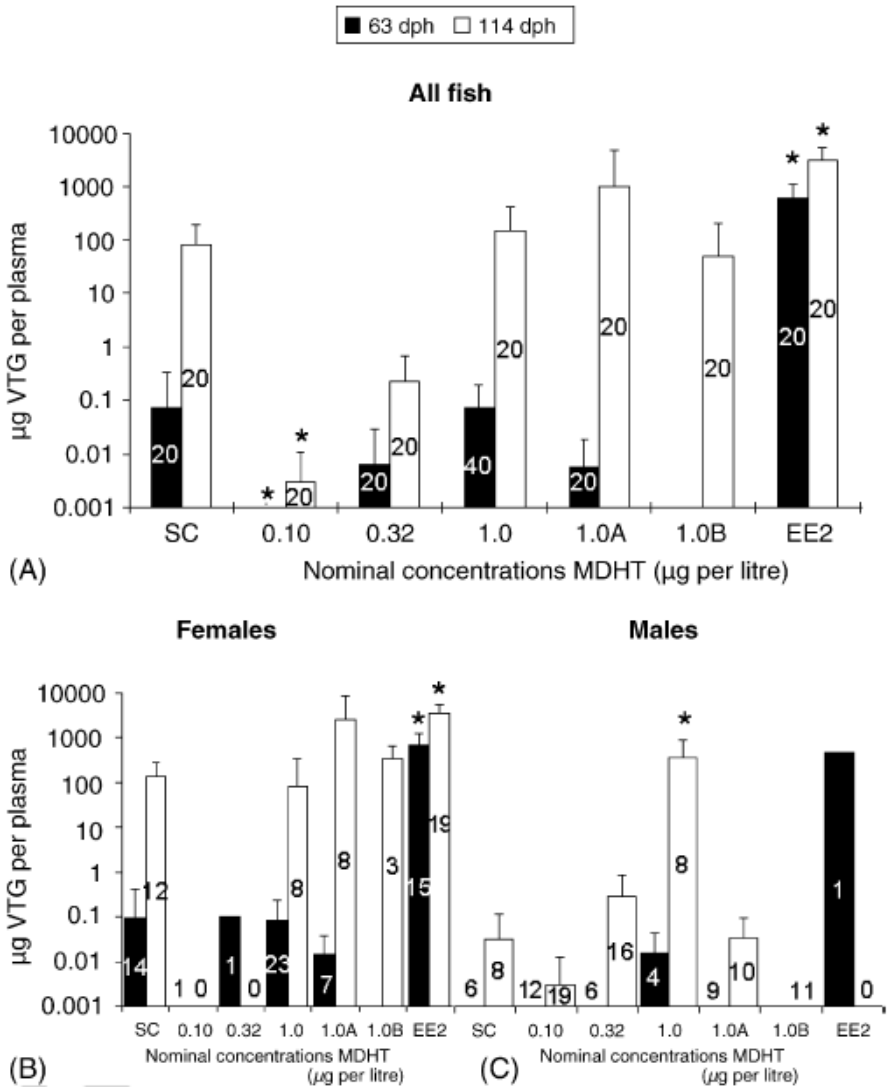

Figure 3.4 Results of measurements on plasma VTG levels in 63 and 114-dph fathead minnow exposed to various concentrations of methyl-dihydrotestosterone (MDHT), A: all fish, B: only females, C: only males. Columns represent mean values with the error bars corresponding with the standard deviations. SC= Solvent Control and EE2 (ethinylestradiol, 0.01µg l^{-1}). Values in the bars represent the number of fish. The 1 µg l-1 group had either complete exposure or exposure up to 30dph (A) or 63dph (B). Asterix means significantly different from the control group (Steel's Many-One Rank Test, α=0.05 two sided). The response in the EE2 group was significantly higher than all other groups for both time points (Kruskal-Wallis ANOVA with Dunn's Multiple comparison at α=0.05).

Plasma vitellogenin concentrations

In control fish plasma VTG increased from 63 to 114 dph (Figure 3.4A) mainly due to maturation of females (Figure 3.4B). VTG levels were significantly higher in fish exposed to 0.01 μg EE2 l^{-1} at 63 and 114 dph as compared to the controls and could be related to 75 or 95 % females, respectively. Lower or no detectable VTG levels were recorded in fish from the MDHT treatments of 0.10 and 0.32 μg l^{-1}. After 114 dph a significantly higher mean VTG level was recorded in male fish exposed to 1.0 μg MDHT l^{-1}.

Histological analysis

After 30 dph, 58 % of the solvent-control fish (Figure 3.5) had developed female gonads (early ovaria stage 1, Figure 3.7, Plate 1B, page 47), while the others still had undifferentiated gonads (Figure 3.7, Plate 1A). Almost all gonads of 30-dph fish exposed to MDHT concentrations of 0.1 and 0.32 μg l^{-1} were sexually undifferentiated; incidences of females were significantly lower compared to the solvent-control (Figure 3.5). At 1.0 μg l^{-1}, ovaria (early ovaria stage 1) were identified in ca. 30% of the fish exposed. In addition, fish exposed to MDHT presented a high incidence of 'cavitation of the gonad' (Figure 3.7, Plate 1C, page 47), especially at the mid concentration of 0.32 μg l^{-1} (75%).

Double attachments to the coelomic wall were present in the majority of the 30-dph fish and differences between treatments and the solvent-control were not statistically significant (Figure 3.6). After 63 dph, a female to male gonadal sex ratio of 2:1 was present in the solvent control group (Figure 3.5), where female gonads (ovaria at Stage 2 - cortical alveolus) had double attachments to the coelomic wall (Figure 3.7, Plate 2B, page 48) and male gonads (mainly at stage 2) had a single attachment (Figure 3.7, Plate 2A, page 48). Of the fish exposed to EE2, 75% had female gonads, while 15 % were still sexually undifferentiated. At the lowest and mid concentration of MDHT, only 5 % of the fish had a female gonad (significant compared with the control), up to 40% at 0.32 μg l^{-1} were still sexually undifferentiated, and up to 25% (at 0.32 μg l^{-1}) had mixed sex gonads (Figure 3.7, Plate 2C, page 48). The remaining fish were male with testes in stage 4 (spermatids/sperm/small lumen) or 5 (abundant sperm/expanded lumen). At the highest MDHT concentration 60 % had a female gonad predominantly in early stages of oogenesis (stages 1 and 2, primary growth and cortical-alveolus, respectively).

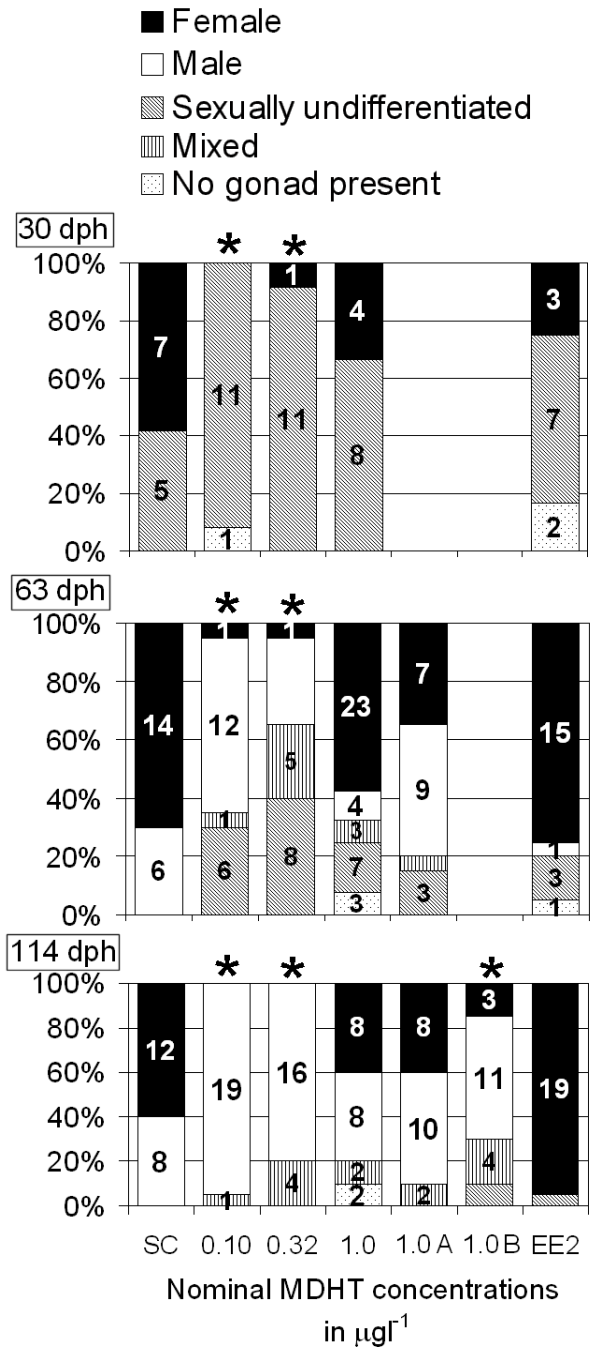

Figure 3.5 Sex ratios of fathead minnow exposed to various concentrations of methyldihydrotestosterone (MDHT, x-axis) at (A) 30, (B) 63 and (C) 114 dph, based on histological analysis of the gonadal tissue in situ. Numbers of fish are given in the bars. See tables. Asterices indicate significant lower female percentages than the control applying the ANOVA-Dunnett test on arcsine-square-root transformations for the various groups exposed to MDHT or ethinylestradiol (EE2). The 1 µg l^{-1} group had either complete exposure or exposure up to 30dph (1.0 A) or 63dph (1.0 B).

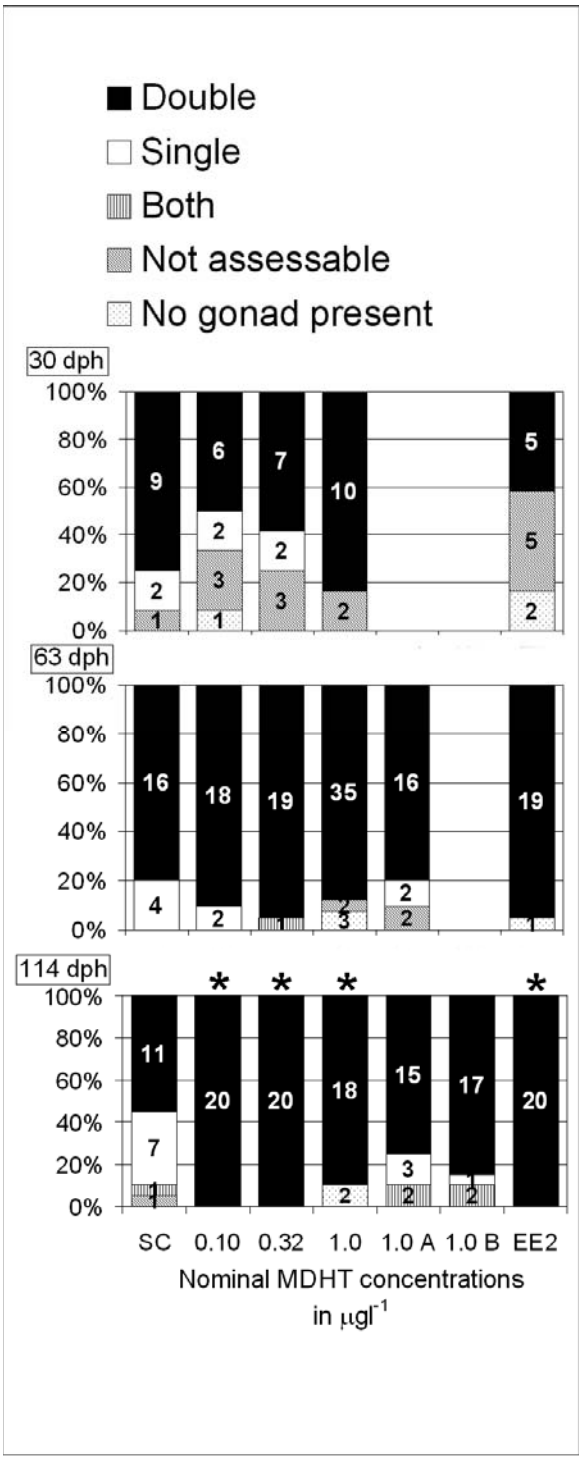

Figure 3.6 Gonadal attachments in fathead minnow exposed to various concentrations of methyldihydrotestosterone (MDHT, x-axis) after 30, 63 and 114 dph. Numbers of fish are given in the bars. Asterices indicate significant higher percentages of fish with double bounds than the control applying the ANOVA-Dunnett test on arcsine-square-root transformations for the various groups exposed to MDHT or ethinylestradiol (EE2). The 1 µg l-1 group had either complete exposure or exposure up to 30dph (1.0 A) or 63dph (1.0 B).

Additional effects of MDHT exposure were defined as 'cavitation' of the gonad (Table 3.3) or as severely impaired growth or dysplasia of gonadal tissue still recognizable as testicular tissue (Figure 3.7, Plates 2D, page 48).

At the end of the exposure period (114 dph) fish of the control-group showed a female-male gonadal sex ratio of 3 : 2 (Figure 3.5), which corresponded well with the ratio of 11 :7 for double or single peritoneal attachment (Figure 3.6). In the control group, not all testes in the males were completely developed, while a majority (83 %) of the female fish had fully developed ovaries with primary and secondary oocytes. The majority of the fish exposed to EE2 developed ovaries, of which 20 % had only small or severely dysplastic ovaries, 60 % had mainly primary pre-vitellogenic oocytes and about 75 % lacked mature secondary oocytes or eggs. In addition, interstitial proteinaceous fluid was observed in the tissues of all these fish.

Table 3.3 Percentages of incidence of the histological findings in tissues of fathead minnow exposed to methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) up to 63 - 114 dph: dysplasia of gonadal tissue and the appearance of 'cavitation'. The 1 μg MDHT l-1 group had either complete exposure or exposure up to 30dph (A) or 63dph (B).

Effect	Sampling dph	Solvent control	EE2 10 ng l^{-1}	Nominal concentration of MDHT in μg l^{-1}				
				0.1	0.32	1	1A	1B
Cavitation (%)	63	0	15	35	90	65	10	30
	114	0	0	30	5	20	0	5
Dysplasia (%)	63	0	10	50	45	65	0	50
	114	0	35	40	35	80	0	10

Fish exposed to 0.1 μg MDHT l^{-1} all had developed testes with one fish showing mixed sex (testis-ova). Exposure to 0.32 μg MDHT l^{-1} resulted in 80 % males and 20% mixed gonads and 45% of the males had markedly smaller testes with enlarged seminiferous tubuli completely filled with sperm. Fish continuously exposed to 1.0 μg MDHT l^{-1} up to 63 dph or 114 dph showed higher incidences of mixed sex, smaller testes or dysplastic gonadal tissue. At 1.0 μg MDHT l^{-1} up to 114 dph extreme dysplasia was observed in 80% of the gonadal tissues examined of which about the half could be identified as testes (Table 3.3).

Changes at the level of the reproductive tracts were significant at 114 dph but for all three concentrations of MDHT (Figure 3.6). Measure of agreement between gonad observation on respectively single, double attachments or non assessable attachments and classifying the fish as male, female or indeterminate (either mixed gonads or undifferentiated) showed an almost complete lack of agreement for all MDHT concentrations and exposure regimes and also for the EE2 treated group (Table 3.4).

Table 3.4 Measure of agreement between gonadal sex assessment and type of reproductive tract in the experiment with methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) after 63 days or 114 days (weighed Cohen's Kappa). The 1 μg MDHTl-1 group had either complete exposure or exposure up to 30dph (A) or 63dph (B). The exposure groups with exposure until the moment of analysis were taken together. This measure is theoretically between -1 (negative agreement) and 1 (perfect agreement). An agreement approximating 0 indicate the absence of any correlation.

Nominal concentration of MDHT (μg l^{-1})	after 63 days		after 114 days	
	estimate	s.e.	estimate	s.e.
Solvent-Control	0.74	0.17	0.97	0.03
0.10	-0.01	0.05	-0.03	0.03
0.32	-0.01	0.01	0.00	0.00
1.0	0.00	0.00	0.00	0.00
1.0 A	0.22	0.14	0.38	0.15
1.0 B			0.10	0.07
EE2 0.01	0.00	0.00	0.00	0.00

s.e. = standard error

Family-wise analyses showed significantly affected ovary stages at 63 dph in relationship to both MDHT treatment and exposure period, whereas testis stages were only significantly affected in relationship to the treatment. The frequency of dysplastic cells was significantly affected by both MDHT treatment and exposure period at 63 dpd and 114 dph. In addition to the gonads, liver and kidney were examined (results not shown). Basophilia of the liver was exclusively apparent in the fish exposed to EE2. Pathology of the kidney included minimal to slight hyaline droplet deposition in the renal tubular epithelium of fish exposed to EE2. Renal tubular dilation was found to be present in 100% of kidneys examined in mid and top-dose MDHT-treated groups (minimal to slight in severity) and was treatment related.

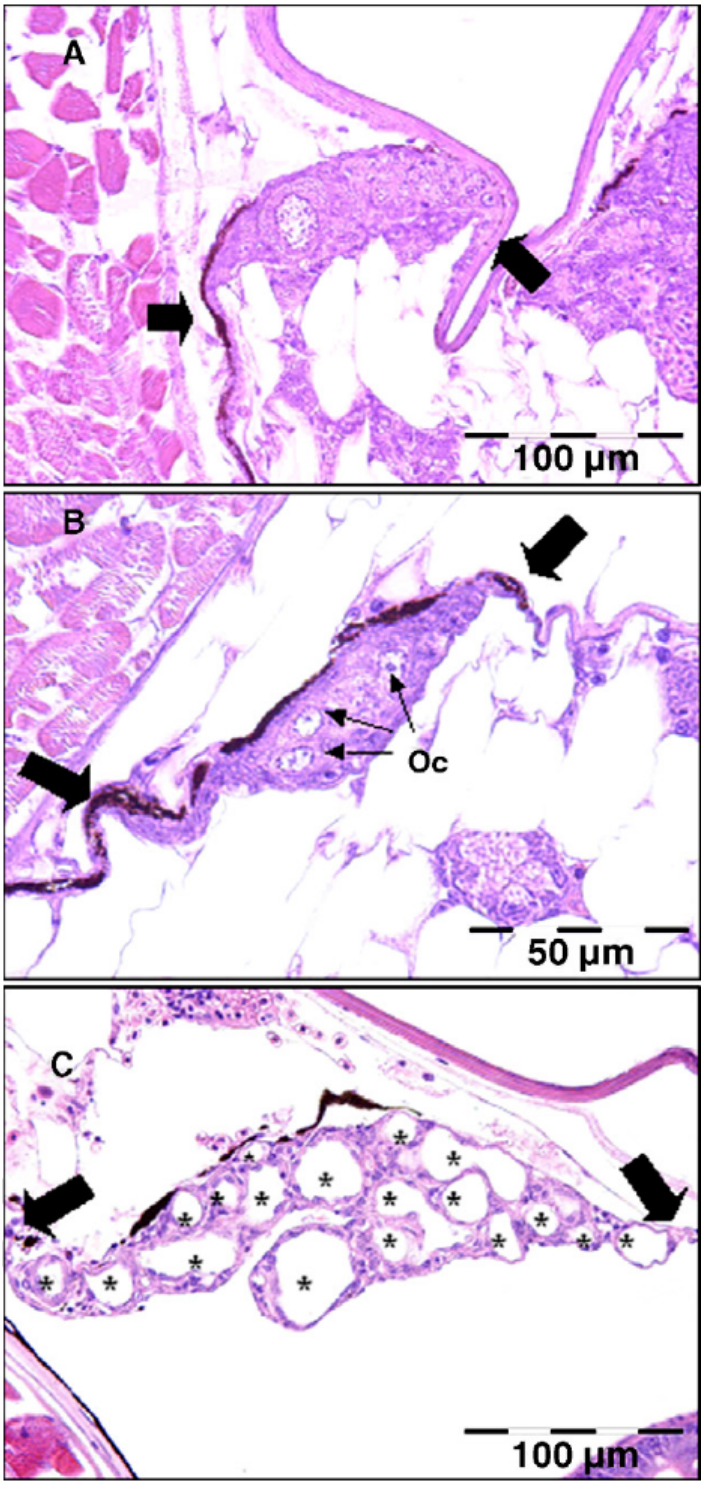

Figure 3.7 Plate 1: Histological cross sections of whole body fathead minnow at 30 dph representative of (A) a sexually undifferentiated gonad from the solvent control group with a double attachment (thick arrows), (B) a female gonad from the solvent control group with a double attachment (thick arrows, note the primary oocyte - Oc) and (C) a sexually undifferentiated gonad from the 0.32µg l^{-1} MDHT-treated group with a double attachment (thick arrows) and cavitation of the seminiferous tubules (asterisks).

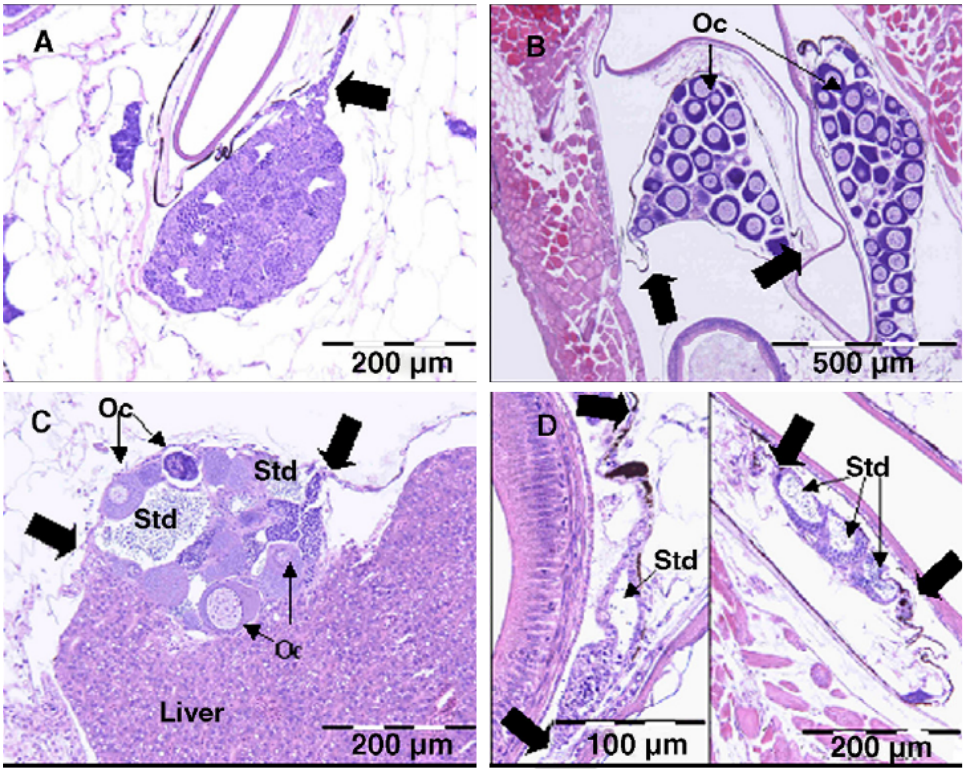

Figure 3.7 Plate 2: Histological cross sections of whole body fathead minnow at 63 dph representative of (A) a male gonad from the solvent control group with a single attachment (thick arrow), (B) a female gonad from the solvent control group with a double attachment (thick arrows, note the primary oocytes, Oc), (C) a mixed gonad from the 0.32µg l^{-1} MDHT-treated group with a double attachment (note the presence of both primary oocytes (Oc) and spermatids (Std) within the lumina). Note also the vacuolation of the surrounding liver, (D) dysplastic male gonads from the 0.1µg l^{-1} MDHT-treated group (left) and from the 0.32µg l^{-1} MDHT-treated group (right), with a double attachment (thick arrows, note the presence of spermatids within the lumina - Std).

Discussion

This paper presents the first sub-chronic study to examine the effects of a non-aromatizable androgen during the early life stages and sexual development of fish. The study further demonstrates that the fathead minnow is an amenable species for use in studies investigating androgenic properties of EACs in addition to estrogenic active compounds. It detected changes at the level of SSCs and gonadal development. The results presented confirm the high androgenic potency of MDHT in fish. Prolonged exposure at 0.1μg l^{-1} resulted in an all male phenotypic population at the end of the test period. Since most endpoints examined were affected rather severely at 0.1 μg MDHT per litre, the lowest effect level for MDHT in fathead minnow will probably be at least 10 times lower.

The observed EE2 related reduction in body weight and length and VTG induction are in agreement with findings in other studies [54, 88]. The fact that a majority of the fish exposed to 0.01 μg EE2 l^{-1} developed ovaries, confirmed the observations in a full-life cycle test [54] showing a female:male sex ratio of 84:5 after exposure to EE2 at 4.0 ng l^{-1} for 56 dph. Complete or almost complete feminization was also recorded for fathead minnow [59] and zebrafish [81] exposed to 10 ng EE2 l^{-1} up to 60 dph. The interstitial eosinophilic proteinaceous fluid recorded in tissue of fish exposed to EE2 in the current study, relates to accumulation of VTG [81, 103, 108].

The significantly increased condition factor and aggressive behavior of fish exposed to MDHT can be related to the stimulating effect of this anabolic steroid on muscle growth and masculine behavior. Although there are no reports of spinal deformations in other studies exposing early life stages of fish to MDHT [102], this feature is probably also related to the effect of MDHT on body development and growth.

Expression of SSCs in fathead minnow has proven to be an easy and promising diagnostic endpoint for both estrogenic [109, 110] and androgenic [87] chemicals and is one of the core endpoints in the fish screening assay for endocrine active chemicals (OECD, 2004). However, earlier studies have shown that SSCs in fathead minnow can be less sensitive than gonadal histology and VTG measurement [109]. In the present study the presence of male characteristics coincided with the gonadal sex, except at the highest MDHT level where fish with ovaries had tubercles on the snout.

The histopathology of the gonadal tissues showed that the sex ratio in the solvent-control samples was biased towards females (6:4 at 30 and 114 dph and 7:3 in 63 dph). Genotypic identification of the sexes could not be performed, as a method for genetic sex determination in fathead minnow is not yet available [111]. This makes it difficult to determine the actual influence of MDHT exposure on sex-differentiation. Normally, in fathead minnow, ovaries differentiate at between 10 and 25 dph and contain early primary oocytes from about 25 dph [106]. Spermatogenesis starts in male gonads not earlier than after about 60 dph [106]. In the present study, MDHT exposure during at least 30 dph induced enhanced testes development in fish that probably would have become normal males without further exposure and induced development of testes in animals that probably would have developed into normal females.

Especially exposure to 1 μg MDHT l^{-1} resulted in severe effects on gonadal tissue including dysplasia and 'cavitation', i.e. 'holes' with only the connective tissue present. These 'holes' appear rather like empty tubuli than being related to the ovarian cavity. In their study, Weber et al. [81] report "testicular acellularities" as having occurred through elimination of the contents of an entire spermatocyst or 'lobule' without the loss of the surrounding structure. In the case of MDHT exposure this would mean an almost complete loss of spermatocysts. In a study with adult zebrafish, van der Ven et al. [103] found changes only at the level of Sertoli cells expressed as hypertrophy and hyperplasia at MDHT concentrations as high as 86.5 μg l^{-1}. The present study shows that early life exposure induces more severe effects on gonadal development at lower concentrations than adult exposure. However, these effects appear to be reversible and it remains important to test whether these histological changes indeed reduce fertility or reproductive capacity of the respective fish including those exposed to the lowest MDHT concentration.

Van Aerle et al. [86] discussed the peritoneal attachment as an endpoint for endocrine disruption at the gonadal level. During early larval development, male gonads develop as two-lobed organs each with a single *dorsal* attachment and protruding into the cavity at the ventral side. Instead, ovaries retain the two-sided attachment and develop the ovarian cavity. Although MDHT induced development of testicular tissue in almost all fish at 0.1 and 0.32 μg l^{-1}, it inhibited the development of the single dorsal attachment.

A powerful end point was found in the measure of agreement between gonad observation on single, double attachments or non-assessable attachments and classifying the fish as male, female or indeterminate (either mixed gonads or undifferentiated). This approach showed that this agreement deviated significantly from the control group even for the MDHT group that was allowed to recover after exposure to 1.0 μg l^{-1} up to 30 dph (Table 3.4).

The effects on liver and kidney induced by MDHT (hepatocyte vacuolation and renal tubular dilatation) were treatment related but reversible.

The observations that relatively high numbers of fish showing mixed sex gonads when exposed to MDHT, a part of the fish exposed to 1.0 μg MDHT l^{-1} still developed ovaries and showed VTG induction, contradict the assumption that MDHT is non-aromatizable. In a study with MT, Seki et al. [112] correlated the appearance of testis-ova with the estrogen activity of MT after aromatization. When MDHT is really not aromatizable, it should have potential to bind directly to the estrogen receptor or it affects the delicate hormonal balance in the neuro-endocrine system, generally referred to as the hypothalamus-pituitary-gonadal axis [51, 87]. Wilson et al. (2004) reported that dihydrotestosterone (DHT) was a partial ER agonist at high concentrations (> 50 nM) in their estrogen-responsive gene expression assay (T47D-Kbluc assay). They found that although the degree of luciferase induction by DHT in this assay was significantly lower than that attained by 0.1 nM estradiol (E2), it appeared to be ER mediated. DHT showed no luciferase induction at a concentrations of ≤ 50 nM.

In addition to the aromatizable androgens such as T and MT [51, 112], other androgens appear to show contrasting effects on both male and female gonadal development of fish dependent on the levels of concentration tested [87]. On our request Xenobiotic Detection Systems (Durham, USA) tested MDHT in an in-vitro test with a genetically engineered cell line

(BG1LucE2) containing a stably transfected estrogen inducible reporter responding to chemicals that are estrogen agonists with the induction of luciferase. MDHT induced luciferase production up to similar levels as the reference E2 but at MDHT concentrations of a factor of 10^4 higher. Induction started at approximately 0.1 μM MDHT. The highest induction was recorded at approximately 1 μM MDHT and decreased up to 50 % at approximately 10 μM MDHT. In the current study, the effects observed at 1.0 μg l^{-1} indicate that at this concentration MDHT reached levels in the fish high enough to stimulate the estrogen receptor significantly without being aromatized. It is possible that such levels were reached by bioconcentration of MDHT in the fish exposed, as it is not easily metabolised. Hence, further testing at lower concentrations will be relevant to obtain the full picture of gonadal effects of MDHT in fish.

Conclusions

This study confirms that the extended early life stage test is suitable to assess androgenic effects in fathead minnow exposed during sexual differentiation. Exposure during the first 30 days was sufficient to induce a significant delay in the female gonadal development. The most sensitive endpoints are related to histological examination of the gonads (gonadal sex and type of reproductive tract).

Table 3.5 Determination of the No Observed Effect Concentration (NOEC) in the experiment with methyldihydrotestosterone (MDHT) after 30, 63 days or 114 days of either complete exposure or exposure up to 30dph (A) or 63dph (B).

Endpoint	NOEC in μg l^{-1} for MDHT at the various exposure scenarios in dph					
	30	63	63 A	114	114A	114B
SSC: % with tubercles		<0.1	≥1	<0.1	≥1	<1
SSC: grading		<0.1	≥1	≥1 ##	≥1	≥1
VTG all fish		≥1	≥1	<0.1	≥1	≥1
VTG males		≥1	≥1	0.32 ###	≥1	≥1
VTG females		?	≥1	?	≥1	≥1
Sex ratio	<0.1	<0.1	≥1 #	<0.1	≥1	≤1##
Gonadal attachments	≥1	≥1	≥1	<0.1	≥1	≥1
Agreement between gonadal sex assessment and type of reproductive tract		<0.1	<0.1	<0.1	<0.1	<0.1

? No or only one female present in MDHT concentrations < 1.0 μg l^{-1}.
Reversible compared to 30 dph.
Reversible compared to 63 dph.
Induction of VTG at 1.0 μg l^{-1}.

MDHT induces the suppression of early gonadal development and gonad maturation, the formation of mixed gonads, gonadal dysplasia, cavitation of the seminiferous tubules/ducts, inhibition of male-type single attachment/efferent duct formation, while it accelerated the development of male gonads and SCCs, and stimulated spermatogenesis. Most of these histopathological effects (including dysplasia and caviation) are reversible, irrespective of the duration of the exposure during sexual differentiation. Other endocrine related endpoints (e.g. VTG induction) were less sensitive and required longer exposures before observing a statistical change.

The no observed effect concentration (NOEC) of MDHT on sexual development of fathead minnow is below 0.10 μg l^{-1} (Table 3.5).

Acknowledgements

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Chapter 4

A pair-breeding study with fathead minnow (*Pimephales promelas*) exposed to an androgenic chemical

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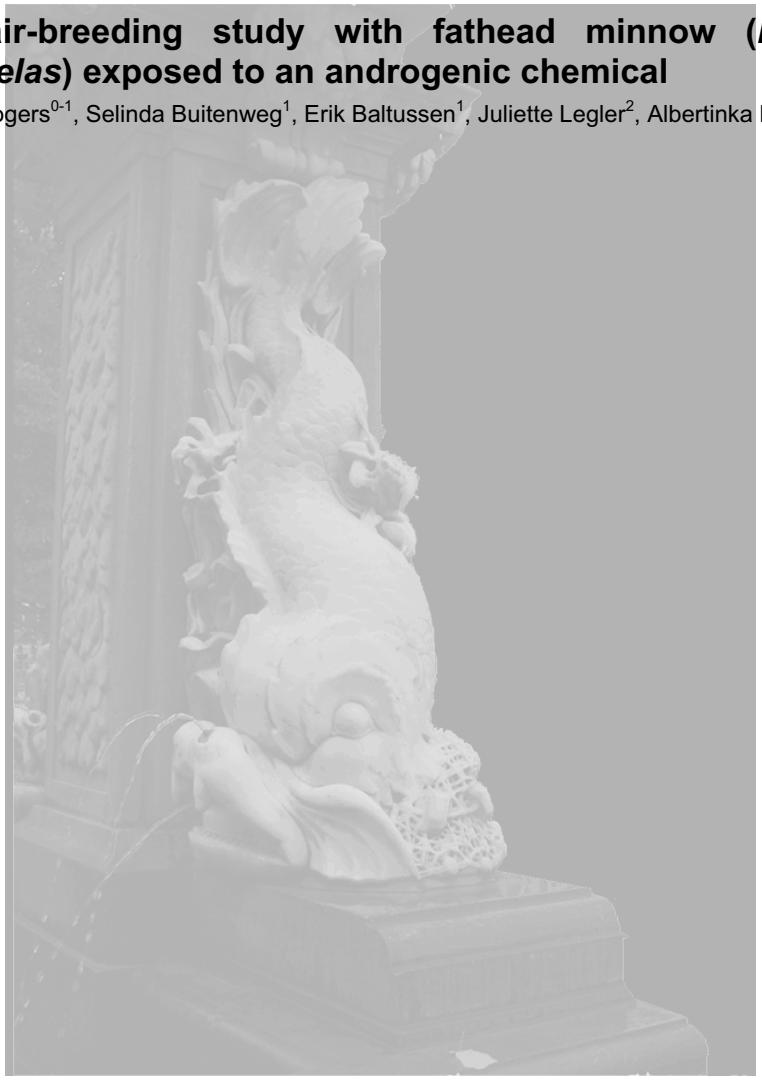

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Abstract

The Endocrine Modulators Steering Group (EMSG) of the European Chemical Industry supported the development of shortened tests as alternatives to fish full life cycle tests. They include a modified fish early life stage test and a fish pair breeding reproduction study. In this paper, the results of two fish pair breeding reproduction studies with methyldihydrotestosterone (MDHT) are presented. The reproductive performance of breeding pairs of fathead minnows was examined during exposure to nominal MDHT concentrations 0.1, 0.32, 1 and 10 µg l^{-1} for 21 or 28 days. Other endpoints examined were somatic growth, the prominence of nuptial tubercles (male related) and vitellogenin (VTG) induction (female related). Ethinylestradiol (EE2) was included as an estrogenic reference substance at 0.01µg l^{-1}. The prominence of the nuptial tubercles was significantly higher in males exposed to 0.1 and 1 µg l^{-1} compared to the solvent-control (SC). MDHT exposure did not significantly affect VTG induction in either males or females, whereas EE2 significantly induced VTG in males and females. Gonadal histology showed testis with enlarged seminiferous tubuli completely filled with sperm combined with a thin germinal epithelium in males exposed to 0.1 and 0.32 µg MDHT l^{-1} and significant oocyte atresia in females exposed to ≥ 0.1 µg MDHT l^{-1}. Relatively high variations of up to 90 % in egg production hampered statistical analysis of differences between experimental groups. During the experimental periods, the pair-bound reproduction in the SC decreased with time. A significant decrease in fecundity was observed in fathead minnow pairs exposed to ≥ 1 µg MDHT l^{-1} when compared with the SC. Comparison of the survival and hatching of offspring (F1) between treatments was hampered by a high variation in egg production in the SC and complete cessation of reproduction in most MDHT treated pairs. Evaluation of trans-generational effects was compromised by the fact that insufficient numbers of F1 batches were available for testing. Before being acceptable as a robust test method the reliability, inter-laboratorial repeatability and consistency of fecundity as endpoint should be improved for fathead minnow as standard organisms for pair wise reproduction tests.

Introduction

As part of its Aquatic Research Programme to assess the effects of endocrine active chemicals (EACs), the Endocrine Modulators Steering Group (EMSG) of the European Chemical Industry has developed an alternative to the fish full-life-cycle (FLC) test by extending the existing fish early life-stage test to assess the effects of EACs on sexual differentiation and early gonadal development [88, 113]. However, this type of study neglects the other sensitive window included in the FLC test, namely the reproductive phase. The pair-wise breeding approach in short-term reproduction tests has been introduced for assessment of endocrine disruption of chemicals on reproduction in fathead minnow (*Pimephales promelas*) [110]. The objective of the present study is to investigate effects of the androgen methyldihydrotestosterone (MDHT) on reproduction and the fertility of the resulting F1 generation of the fathead minnow. and compare the results with an earlier performed extended fish early life-stage test [113]. The synthetic steroid MDHT is a non-aromatizable androgen [97].

The protocol designed for this study aimed at assessing the reproductive performance of breeding pairs of fathead minnows during a pre-exposure phase, followed by exposure to MDHT for 28 days. The pre-exposure period is used to determine the fecundity of each individual pair, for comparison of the fecundity during exposure. As fathead minnows are fractional spawners, the time to egg deposition is an unpractical endpoint to monitor. Therefore, in addition to fecundity, the experimental setup included an assessment of the effects of MDHT exposure of the adults on their secondary sexual characteristics, gonadal histology, and vitellogenin (VTG) production, as well as the hatchability of the F1 generation. Further, the protocol included determination of the sex ratio and gonadal histology of the F1 developed in clean untreated dilution water for 30 or 90 days post hatching (dph). Thus the test was designed to allow both the evaluation of adverse effects on several endpoints related to the reproductive condition of the adults as well as trans-generational development after exposure.

Materials and methods

Test species

Sexually mature fathead minnows (*Pimephales promelas*, Teleostei, Cyprinidae), approximately five months of age, were obtained from the brood stock of the NOTOX Laboratory. Fish were fed daily a mixed diet of pellet food (Bio-Optimal C80, Brande, Denmark) and adult brine shrimp and maintained under a 16:8 h light:dark photoperiod.

Test chemicals

The androgen MDHT (17α-Methylandrostan-17β-ol-3-one, CAS 52-11-19) and the synthetic estrogen EE2 (Ethinylestradiol, CAS 80-46-6) were at least of 98% purity (Sigma Chemical Co. Ltd. ,Dorset, England). Stock solutions of MDHT and EE2 were prepared in Trigol (Triethyleneglycol, CAS 112-27-6).

Pre-exposure periods

The pre-exposure experiments were largely conducted in the same climate room as used later for the exposure phase. Sexually mature male (wet weight between 5 and 9 g) and female (wet weight between 2 and 4 g) fish were selected at random and pairs of fish (1 male and 1 female) were placed in individual replicate test vessels containing a spawning surface (Perspex capture tray with a PVC spawning tile). Fecundicity during the pre-exposure phase was assessed daily based on breeding behaviour, egg number, egg quality (viability and the presence of abnormalities) and spawning ability (production of eggs). Approximately every week, pairs that did not produce any or relatively small numbers of eggs were replaced by new pairs or recombined with a new fish from the stock as long as these were available.

The pre-exposure of the first reproduction test started with 56 pairs. After the first 8 days, the total production of eggs was evaluated. The production of eggs varied largely and 25 pairs had not produced any eggs (Table 4.1). In total 22 pairs were replaced and pre-exposure was continued until the next evaluation 6 days later when 12 pairs had no eggs. Again 17 pairs were replaced and the pre-exposure period was continued for another 7 days reaching a total period of 21 days. After 3 weeks 25 of the initial 56 pairs (45 %) were still present. In total 82 different pairs were pre-exposed for a minimum period of 8 days of which 35 pairs had been monitored for at least 2 weeks (Table 4.1). Finally, 36 pairs were selected for the exposure phase. Hence, not all pairs had the same period of pre-exposure reproduction period but these were divided equally over the six test groups.

Table 4.1 Summary of fathead minnow egg production during the 21-day pre-exposure periods prior to the first (I) and second (II) reproduction test.

Pre-exposure period		Number of eggs produced per pair			Total number of pairs	Pairs with no eggs		Number of pairs lost
		Mean	Minimum	Maximum		Number	%	
I	7 days	177	0	989	82	36	44	0
	14 days	501	0	1510	35	1	3	0
	21 days	696	38	1790	25	0	0	1
II	6 days	63	0	266	18	8	44	0
	21 days	481	0	1677	53	21	40	6

The pre-exposure of the second reproduction test started with 18 pairs. Fish were left for six days to assess fecundity. After six days, 10 pairs had produced one to three spawns, while the other 8 pairs failed to produce any spawns (Table 4.1). Other females from the original stock of fish replaced the females of these failing pairs. An additional 18 pairs were set out for breeding and during the next 14 days 6 pairs were lost due to death of both male and female fish. In total 53 pairs were tested during the whole 20 days of pre-exposure and 21 pairs (40 %) did not produce any eggs (Table 4.1). Finally, 30 pairs were assigned to the exposure study with a mean number of eggs per pair of 481, and a minimum of 0 and a maximum of 1677 eggs per pair (Table 4.1). Because of this high variation, the pairs were not completely randomly assigned to the different

MDHT exposure groups. Instead, they were divided in groups with production of > 50, between 15 and 50 and of < 15 eggs and from each of these groups randomly assigned to the different treatments of the second reproduction study.

Exposure protocol

After selection based on the 21 day pre-exposure phase, breeding pairs were divided over the exposure vessels for a further 21 days (experiment 1) or 28 days (experiment 2) to assess the impact of MDHT on reproductive performance. The fish were exposed in non-chlorinated tap water with a hardness between 180 and 200 mg l^{-1} (measured as $CaCO_3$). Before the dilution water entered the system it passed an ultra-violet light source system to inactivate microbial activity.

Based on a range finding experiment, the MDHT concentrations tested were 0.1, 0.32, 1 and 10 μg l^{-1}. The estrogenic reference group was exposed to 0.01 μg EE2 l^{-1} as in Panter et al. [88] and Bogers et al. [113]. The first study included a dilution water control (DWC) and a solvent control (SC) with Trigol at 0.1 ml l^{-1}, while the second study included only a SC with Trigol at a negligible level of 0.0033 ml l^{-1}. In the second study, no DWC group was included since no endocrine effects of Trigol were observed in the first study with an even 30 times higher Trigol level.

The stock solutions with test substance were pre-mixed with dilution water under continuous stirring via a computer-controlled system consisting of flow meters, dispensers (Gilson, Velliers Le Bel, France), mixing vessels and distribution vessels. Test materials were constructed of glass, stainless steel or Teflon (tubing) with a minimum of other materials, e.g. silicon rubber tubing and adhesive, which did not make contact with the water. The pairs were exposed in tanks of 10 litres (glass: L x W x H: 422 mm x 126 mm x 184 mm). Dosing was performed from the stainless steel mixing vessel into the stainless steel distribution vessel. From the distribution vessel the test solution entered the vessels of the 6 replicates via a overflow tank at an average flow rate of 4.6 l/h. This flow rate results in more than 10 volume exchanges of test solutions per 24 hour. The progeny tanks used for the hatchability trials (four per treatment) had a working volume of 2 litres (stainless steel: L x W x H: 180 mm x 180 mm x 75 mm). After 30 days the young fish were transferred to 22-litre vessels (glass: L x W x H: 490 mm x 214 mm x 210 mm). All systems were checked daily for any technical failure.

The dissolved oxygen, temperature and pH values were measured in all vessels three times a week. The dissolved oxygen concentration remained above 70% of the air saturation value throughout the exposures. The pH was between 7.6 and 8.1 and the temperature 24 - 27 °C. A 16:8 hour light : dark photoperiod (ca. 20 ± 4 $\mu E.m^{-2}.s^{-1}$ or 1200 ± 200 lux) with a staged change of light intensity (20 minutes) for dawn and dusk periods was provided. F0 fish were fed with frozen adult brine shrimp and pellet food (Bio-Optimal C80, Brande, Denmark). Within 1 hour after each feeding, any food residues were removed. Fish were not fed at least 24 h prior to termination of the exposure.

Sampling of fish

At the end of the F0 exposure phase, a maximum of 12 of the remaining adult fish were sampled per treatment, anaesthetized and sacrificed according to the Dutch Act on Animal

Experimentation. Fish were immediately weighed (to the nearest 0.0001 g) and their length measured (to the nearest 1 mm). The condition factor was calculated as the ratio between the weight of the fish and the cube of its length, multiplied by 100. Blood was collected by cardiac puncture in the F0 adult fish from the second reproduction study using a heparinised syringe (1000 units heparin ml-1). The samples were centrifuged for 5 min at 10,000 g (4 °C), and the plasma obtained and stored in a -80°C deep-freezer, until analysis of VTG. After sacrificing, the fish were cut in three parts: head, body and tail. For 24 hours, the head and the body were immersed separately in Bouin's (first study) or Davidson's solution (second study) modified according to Creasy and Jonassen [114] and then stored in 70%-ethanol. The head part was used for determination of nuptial tubercles and the body part for histological processing. The tail part was discarded.

Hatching success and further development

All trials assessing hatching and further development of the F1 generation were conducted in dilution water only. Egg batches produced after seven days of the F0 exposure were tested for hatchability. After 24 hours, all embryos were observed for mortality. Of the live embryos 50 were transferred and kept until complete hatching in sterile petri-dishes (diameter: 15 cm), which were gently shaken on a shaking device with daily renewal of the solutions. The developing larvae were maintained in dilution water until 30 dph in the first and until 90 dph in the second reproduction test. On day 4, all hatched larvae and any remaining embryos were transferred to stainless steel vessels of ca. 1.5 litres in a continuous flow-through system and grown up until 30 dph. In the second reproduction study, the remaining juvenile fish were transferred to 30-litre glass vessels in the flow-through system and grown up to 90 dph. Newly hatched larvae were fed rotifers (*Brachionus plicatilis*) and subsequently *Artemia* nauplii. Juveniles and adults were fed *ad libitum* with adult brine shrimp supplemented with pellet food (Bio-Optimal C80, Brande, Denmark).

Quantification of secondary sexual characteristics (SSCs)

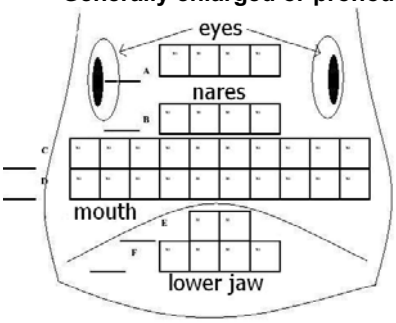
After the 28 days of exposure the SSCs were quantified as a combination of the number and prominence of nuptial tubercles (a male characteristic) which has been shown to be responsive to steroid hormones [107]. Counting and rating of the nuptial tubercles on the fixed heads of the fish sampled was conducted using a binocular microscope partly following Smith [57] using a mapping of the tubercles as shown in Figure 4.1. Each tubercle was rated and the total prominence for each fish equalled the sum of all rates. The rates were defined as follows:

1. Rating 0: no tubercle present,
2. Rating 1: a tubercle has a single point with its height nearly equivalent to its radius,
3. Rating 2: an enlarged tubercle identified by tissue resembling an asterisk in appearance,
4. Rating 3: a pronounced tubercle usually quite large and rounded with less definition in structure.

Using this rating system generally will result in overall tubercle scores of < 50 in a normal control male with a total of 18 to 20 tubercles [58]. Although this kind of rating still is subjective a real bias is prevented as much as possible by performing it double blind.

Figure 4.1 Regions on the snout of fathead minnow for mapping nuptial tubercles:

A - Tubercles located around eye. Mapped dorsal to ventral around anterior rim of eye. Commonly multiple in mature control males, not present in control females, generally paired (one near each eye) or single in females exposed to androgens. B - Tubercles located between nares, (sensory canal pores). Normally in pairs for control males at more elevated levels (2- enlarged or 3- pronounced) of development. Not present in control females with some occurrence and development in females exposed to androgens. C - Tubercles located immediately anterior to nares, parallel to mouth. Generally enlarged or pronounced in mature control males. Present or enlarged in less developed males or androgen-treated females.



D - Tubercles located parallel along mouth line. Generally rated developed in control males. Absent in control females but present in androgen-exposed females.

E - Tubercles located on lower jaw, close to mouth, usually small and commonly in pairs. Varying in control or treated males, and treated females.

F - Tubercles located ventral to E. Commonly small and paired. Present in control males and androgen-exposed females.

Vitellogenin measurement

Blood plasma samples were assayed for VTG using an established heterologous carp (first study) or homologous fathead minnow (second study) enzyme-linked immunosorbent assay (ELISA) both supplied by Biosense Laboratories AS (Bergen, Norway).

Histological processing and analysis

Fixed body parts containing the gonads and intestinal organs of the F0-fish of the second exposure test were embedded longitudinally in paraffin wax and the wax blocks were sectioned with a microtome at 3 μm thickness, mounted on glass slides and stained with haematoxylin and eosin. The resulting whole-body sections were microscopically examined to determine fish sex and condition of testis or ovaries. Fish gonadal sex was histologically defined as 'male' when cells of spermatogenic lineage were present, or 'female' when cells of oogenetic lineage were present. When both male and female germ cells were recognized within a gonad, this was recorded as 'mixed'. Histological examination included gonadal staging, i.e. abnormal spermatogenesis or oocytogenesis visible as deviating distributions of staging or missing stages, and enlarged seminiferous tubuli in testes, oocyte atresia, changes in numbers or appearance of interstitial cells.

Analytical chemistry

Water samples for chemical analysis were taken from the stocks and exposure solutions at the start of the experiment and at regular intervals during the exposure. The aqueous samples were concentrated using solid-phase extraction (SPE). Quantitative analysis was based on the

areas of the MDHT and EE2 peaks in a GC-MS chromatogram divided by the area of an internal standard peak (β-Estradiol). For further details of the analytical method see Bogers et al. [113].

Statistical analysis

Statistical analysis was performed on data for egg production in both reproduction tests and additional data derived from the second reproduction test, i.e. wet weight, standard length, condition factor (body weight/(0.01*length^3)), SSCs and plasma VTG concentrations (log transformed). These data were analysed by analysis of variance (ANOVA). Where assumptions of normality and homogeneity were met, ANOVA was followed by the Dunnett's t test to compare the treatment means with respective controls and/or a multi comparison test (Tukey test) for comparison among groups. Where the assumptions were not met, data were analysed using suitable non-parametric tests (Steel's Many-one rank test and Kruskal-Wallis test). Tests were performed using a two-sided α of 0.05. The decision male or female was based on histological examination. The results are reported as means ±. standard deviations, total numbers or percentages of total.

Results

Analytical chemistry

Concentrations of MDHT and EE2 in the SC were below the limits of detection of <0.0021 MDHT μg l^{-1} and <0.0013 EE2 μg l^{-1}. The time weighed average (TWA) concentrations for MDHT ranged from 40 to 78 % of nominal concentration (Table 4.2). The variability of the analytical measurements expressed as the coefficient of variation (CV) ranged between 13 and 110 % depending on the concentration measured. For EE2 the measurements for the first experiment failed but in the second were successful showing an exposure concentration that was 140% of the nominal concentration with a CV of only 6% (Table 4.2).

F0: Fish survival and somatic growth

For both sexes the percentages of mortality were < 10 % during the pre-exposure phase of the first reproduction test, while they were < 10 % for males and 19 % for females during the pre-exposure phase of the second reproduction test. When fish died, they were often damaged, especially the female fish. This can be explained by the observation that some males were quite aggressive in their effort to stimulate the spawning of their female partners.

During the exposure of the first reproduction test, 25 % of all males and 11 % of all females died resulting in a loss of 31% of the pairs. During the second week of the exposure phase, three males died in both the 10-μg MDHT l^{-1} group and the EE2 group leaving only three pairs for each group. In addition, a relatively high rate of bacterial growth was visible in a some of the vessels especially of the SC resulting in technical failure of the water flow and aeration irrespective of intermittent cleaning of the vessels. This caused almost complete oxygen depletion in some of the test vessels of the SC upon which it was decided to terminate the test and evaluate only the reproduction parameters up to day 21.

Table 4.2 Time weighed average concentrations (TWA) of methyldihydro-testosterone (MDHT) and ethinylestradiol (EE2) measured in the test solutions during the first (lasting 21 days) and second exposure study (lasting 28 days), respectively, with standard deviations (SD) and coefficient of variations (CV).

Nominal (µg l^{-1})	21-day reproduction test			28-day reproduction test		
MDHT exposure	TWA in µg l^{-1}	SD_{TWA} in µg l^{-1}	CV_{TWA} in %	TWA in µg l^{-1}	SD_{TWA} in µg l^{-1}	CV_{TWA} in %
0				<0.0021		
0.10	0.040	0.044	110	0.060	0.012	21
0.32	-	-	-	0.186	0.025	13
1.0	0.608	0.145	24	0.416	0.254	61
10	7.80	2.93	37	-	-	-
EE2 exposure						
0	[a]	[a]	[a]	<0.0013		
0.01	[a]	[a]	[a]	0.014	0.0008	6

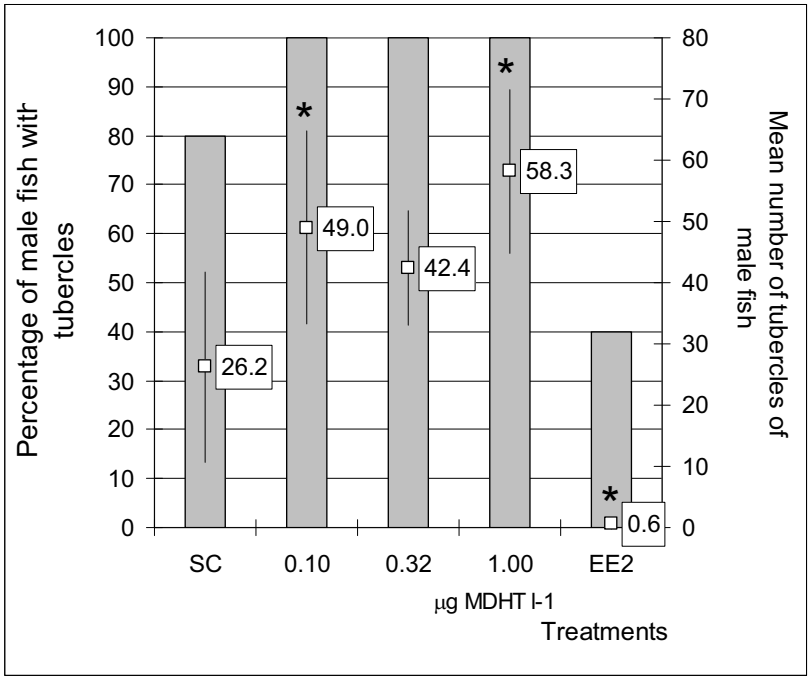
[a] Measured values coud not be accurately quantified due to high variations in the analytical recoveries
- means: not included in the test.

During the exposure of the second reproduction test, 10 % of the males and 7 % of the females died. In total 10% of the pairs used in the test were lost. No significant effects of MDHT on F0 survival were observed. At the end of the exposure phase no exposure-related effects were seen on the somatic endpoints (wet weight, standard length and condition factor) in male or female fish (Table 4.3).

Table 4.3 Means for wet weight, standard length and condition factor with standard deviations (SD) of the adult fathead minnow exposed to methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) during 28 days. SC is the solvent control.

Exposure	Mortality	n	Wet weight (mg)		Standard length (mm)		Condition factor	
(µg l-1)	(%)		Mean	SD	Mean	SD	Mean	SD
Males :								
SC	17	5	7558.0	1481.1	72.2	7.6	2.06	0.58
0.10	0	6	7133.3	2966.3	73.2	7.0	1.74	0.36
0.32	17	5	6433.1	2100.9	75.2	6.0	1.55	0.50
1.0	0	6	8615.6	1182.7	72.8	13.0	2.67	1.83
EE2 (0.01)	17	5	7523.3	1596.8	75.0	8.3	1.80	0.28
Females:								
SC	0	6	2819.6	1064.8	56.0	3.7	1.56	0.35
0.10	0	6	2565.0	642.1	54.0	4.2	1.61	0.15
0.32	17	5	3601.7	767.9	59.0	3.5	1.76	0.34
1.0	0	6	3002.5	588.6	57.6	4.3	1.62	0.47
EE2 (0.01)	17	5	3280.8	295.3	57.6	2.9	1.72	0.18

A. Males



B. Females

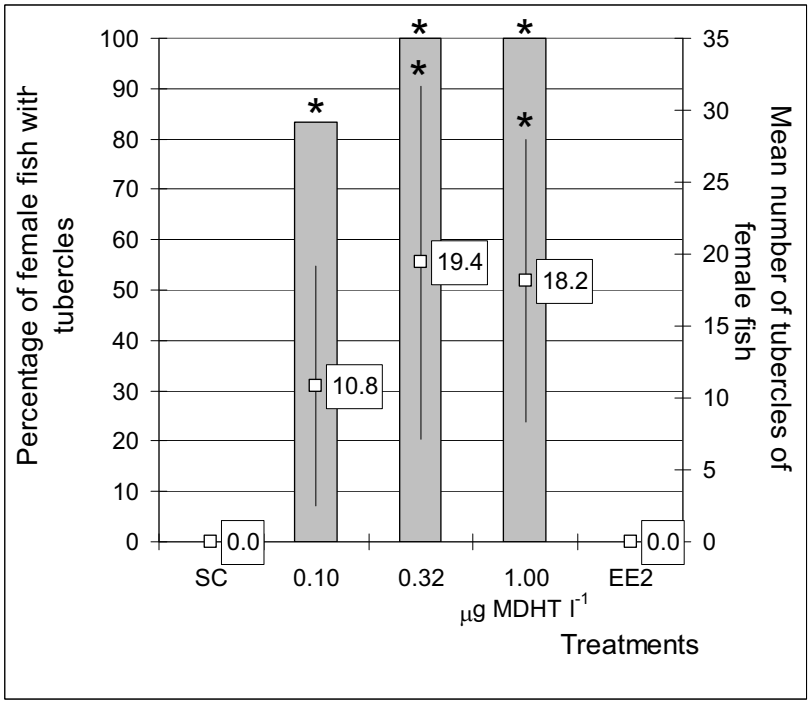


Figure 4.2 (opposite page): Percentage of fathead minnow with prominent nuptial tubercles (columns) in males (Figure 4.2A) and females (Figure 4.2B) exposed to methyl-dihydrotestosterone (MDHT) or ethinylestradiol (EE2) during a 28-day reproduction test. The prominence is indicated as mean number of tubercles per fish (right Y-axis) plus standard deviations (error bars). An asterisk denotes a significant difference compared with the Solvent control (SC) at α=0.05, two sided.

F0: Secondary sexual characteristics and vitellogenin

Nuptial tubercles were prominent in 100% of the males exposed to MDHT during the second study (columns in Figure 4.2A), while 80% of the males in the SC had prominent tubercles. Prominent tubercles were present in 83 to 100 % of the females exposed to the MDHT (columns in Figure 4.2B) compared to none of the females of the SC and the EE2-control.

The prominence of the nuptial tubercles, expressed as number of tubercles per fish, was significantly higher compared to the males in the SC in males exposed to 0.1 and 1 mg MDHT l^{-1} (mean and error bars in Figure 4.2A) and females exposed to 0.32 and 1 mg MDHT l^{-1} (mean and error bars in Figure 4.2B). The prominence of the nuptial tubercles for males exposed to EE2 was significantly lower than for males in the SC and lower than for females exposed to 0.1 ug MDHT l^{-1}.

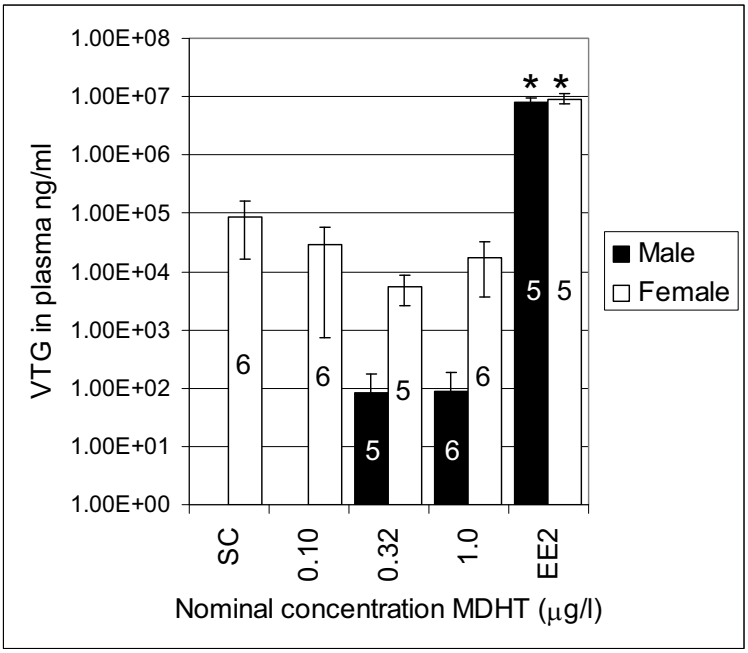


Figure 4.3: Vitellogenine (VTG) concentrations measured in blood of male and female fathead minnow exposed to methyldihydro-testosterone (MDHT) or ethinylestradiol (EE2) during a 28-day reproduction test (error bars correspond with standard deviations). An asterisk denotes a significant difference compared with the Solvent control (SC) at α=0.05, two sided.

Detectable VTG levels were recorded in one male exposed to 0.32 mg l^{-1} and one exposed to 1 μg MDHT l^{-1}, but not in any of the males in the controls (Figure 4.3). High VTG levels were detected in all males exposed to EE2. Although there was a decreasing trend, MDHT exposure did not significantly affect the VTG induction in the females, whereas EE2 increased this significantly.

F0: Histological analysis in fish gonads

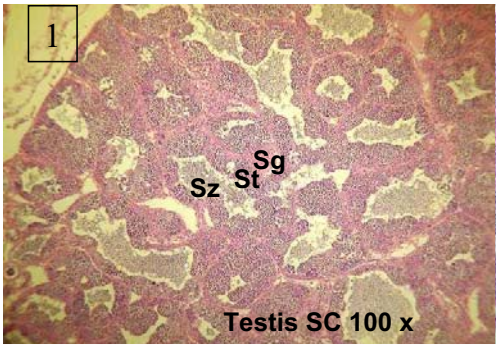
The gonads of most male fish exposed to 0.1 and 0.32 μg MDHT l^{-1} for 28 days had enlarged seminiferous tubuli completely filled with sperm combined with relatively thin germinal epithelium (Figure 4.4 Plate 1, Photo's 3-6). This feature was less clearly observed in some males exposed to 1 μg l^{-1} for 28 days (Figure 4.4 Plate 1, Photo's 7-8). The gonads of male fish exposed to EE2 contained interstitial proteinaceous fluid, which was probably VTG, as well as increased clusters of Leydig cells (Figure 4.4 Plate 1, Photo 9).

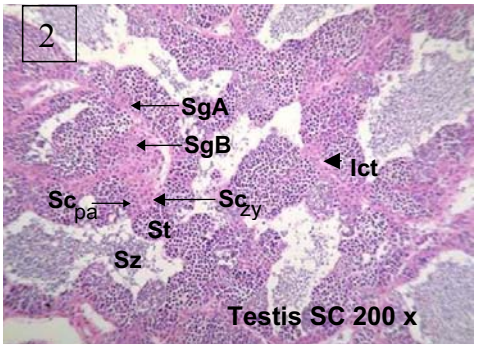
In female fish exposed to 0.1 μg MDHT l^{-1} for 28 days, the highest incidences of oocyte atresia, mature oocyte membrane folding and hypertrophy were observed (Figure 4.4 Plate 2, Photo 11). The gonads of most female fish exposed to 0.32 and 1 MDHT l^{-1} for 28 days completely lacked secondary oocytes and matured oocytes (Figure 4.4 Plate 2, Photo's 12-13). At 0.32 μg MDHT l^{-1}, the highest incidence of immature oocyte atresia was observed (100% grading from moderate to severe). Further, this was the only concentration where females (three out of five) had gonads which contained cavities with hyperthrophic perifollicular theca cells. The gonads of all female fish exposed to EE2 contained interstitial proteinaceous fluid (only incidentally observed in the SC and 0.1 μg MDHT l^{-1}) and moderate to severe immature oocyte atresia (Figure 4.4 Plate 2, Photo 14).

F0: Reproductive parameters

Number of spawns per pair: In the pre-exposure phases, less than 20 % (13 and 17 % for experiment 1 and 2 respectively) of all pairs used produced 2 or more spawns per week. In the first week of the first exposure experiment, 13 % of all pairs produced more than one spawn. Three of the six pairs spawned in the DWC, the EE2 group and the 0.1 and 10 μg MDHT l^{-1} groups (Figure 4.5A). Only 1 pair exposed to 1 μg MDHT l^{-1} spawned (only once), whereas five of the six pairs of the SC did. In the second week, pairs exposed to MDHT concentrations of 1 and 10 μg l^{-1} did not longer spawn, while in the other groups some pairs still did, especially in the SC (5 pairs). In the last week of the first study only pairs of the DWC, SC and 0.1 μg MDHT l^{-1} still spawned. The pairs from these control and low dose groups produced 77 % of all spawns collected during this first exposure study.

The number of spawns in the SC of the second reproduction study was significantly lower than the number of spawns in the 1st study (Figure 4.5B) but the same as in the DWC during the first 2 weeks from the 1st study. The pairs of the second study spawned mainly during the first two weeks, and the pairs exposed to 0.32 and 1 μg l^{-1} only in the first week as was the case for the two highest exposure groups from the 1st study. In week 4 of the second study, only one pair exposed to 0.1 μg MDHT l^{-1} produced a spawn, whereas all other pairs did not produce any spawn, including those of the SC.


1
Sg
Sz
St
Testis SC 100 x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SgA
SgB
Ict
Scpa
Sczy
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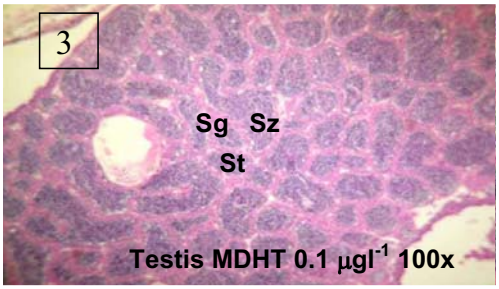
Sz
Testis SC 200 x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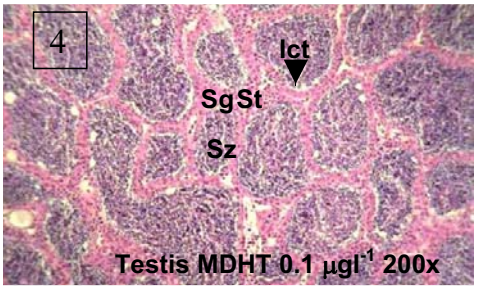
Sg
Sz
St
Testis MDHT 0.1 µgl-1 100x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Ict
Sg St
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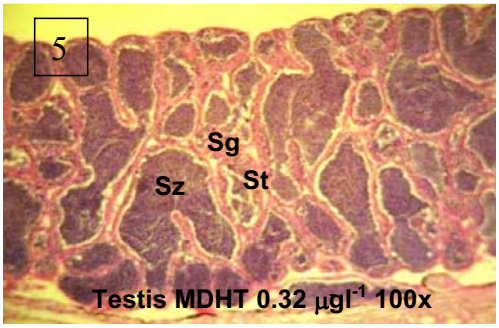
Testis MDHT 0.1 µgl-1 200x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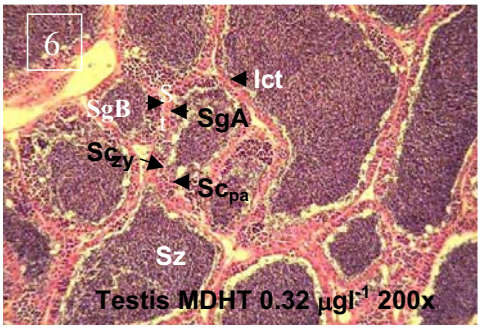
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Ict
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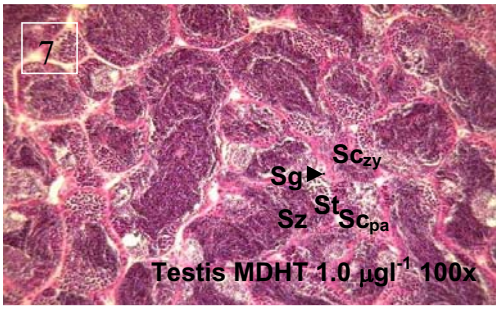
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Sczy
Sg
St
Sz
Scpa
Testis MDHT 1.0 µgl-1 100x

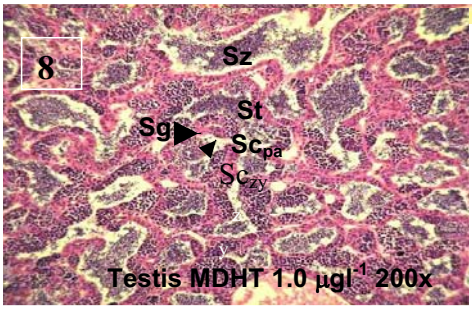

8
Sz
St
Sg
Scpa
Sczy
Testis MDHT 1.0 µgl-1 200x

Figure 4.4 Plate 1 (previous page):

photo 1. Testis of a fathead minnow exposed for 28 days to the solvent-control (SC), enlargement 100x.

photo 2. Testis of a fathead minnow exposed for 28 days to the SC including all the different stages of spermatogenesis normally visible in gonadal tissue of fish, enlargement 200x.

photo 3. Testis of a fathead minnow exposed for 28 days to methyldehydro-testosterone (MDHT) at 0.1 μg l^{-1} (100x), note the seminiferous tubuli completely filled with sperm combined with relatively thin germinal epithelium.

photo 4. Testis of a fathead minnow exposed for 28 days to MDHT at 0.1 μg l^{-1} (200x).

photo 5. Testis of a fathead minnow exposed for 28 days to MDHT at 0.32 μg l^{-1} (100x).

photo 6. Testis of a fathead minnow exposed for 28 days to MDHT at 0.32 μg l^{-1} (200x).

photo 7. Testis of a fathead minnow exposed for 28 days to MDHT at 1 μg l^{-1} (100x).

photo 8. Testis of a fathead minnow exposed for 28 days to MDHT at 1 μg l^{-1} (200x).

photo 9. (next page) Testis of a fathead minnow exposed for 28 days to ethinylestradiol (EE2) at 0.01 μg l^{-1} (100x), note the interstitial proteinaceous fluid, which is in fact eosinophilic vitellogenin, and the increased numbers of Leydig cells (LC).

Figure 4.4 Plate 2 (opposite page):

photo 10. Ovary of a fathead minnow exposed for 28 days to the solvent-control (SC), enlargement 100x.

photo 11. Ovary of a fathead minnow exposed for 28 days to methyldehydro-testosterone (MDHT) at 0.1 μg l^{-1}, enlargement 100x.

photo 12. Ovary of a fathead minnow exposed for 28 days to MDHT at 0.32 μg l^{-1}, enlargement 100x, notice the atresia of the maturing oocytes, no normal mature eggs were observed in slides of this group.

photo 13. Ovary of a fathead minnow exposed for 28 days to MDHT at 1 μg l^{-1}, enlargement 100x, in this group most female fish completely lacked secondary oocytes and matured oocytes.

photo 14. Ovary of a fathead minnow exposed for 28 days to ethinylestradiol (EE2) at 0.01 μg l^{-1} (100x), note the interstitial proteinaceous fluid, which is in fact eosinophilic vitellogenin.

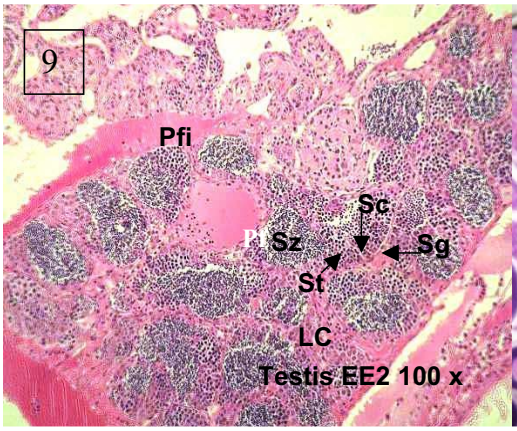
ABBREVIATIONS

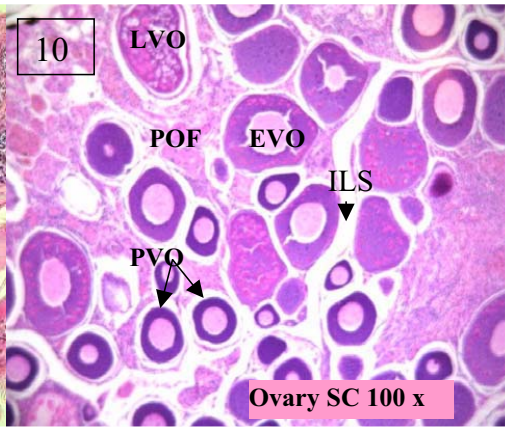
TESTIS :

Sg	= spermatogonium cells
SgA	= Sg A cells (solitary)
SgB	= Sg B cells (small clusters)
Sc	= spermatocysts
Sc_{zy}	= Sc zygotene stage of meiotic prophase
Sc_{pa}	= Sc pachytene primary spermatocytes
St	= spermatids
Sz	= mature spermatozoa
Ict	= interstitial connective tissue
Pf_i	= proteinaceous fluid, interstitial
LC	= Leydig cells

OVARY :

PVO	= previtellogenic oocytes
EVO	= early vitellogenic oocytes
LVO	= late vitellogenic oocytes
POF	= post ovarian follicles
ILS	= interlobular space
Pf_i	= proteinaceous fluid, interstitial
AIO	= atresia of immature oocyte
AMO	= atresia of mature oocyte


9
Pfi
Sc
Sz
Sg
St
LC
Testis EE2 100 x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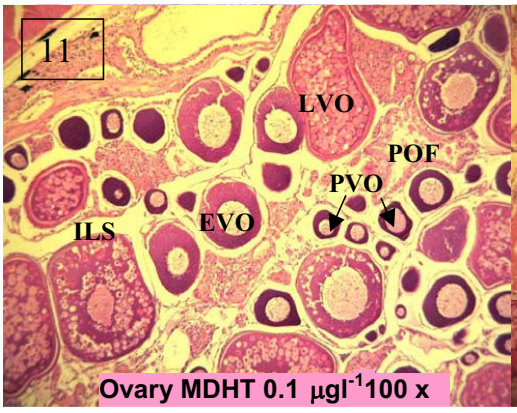
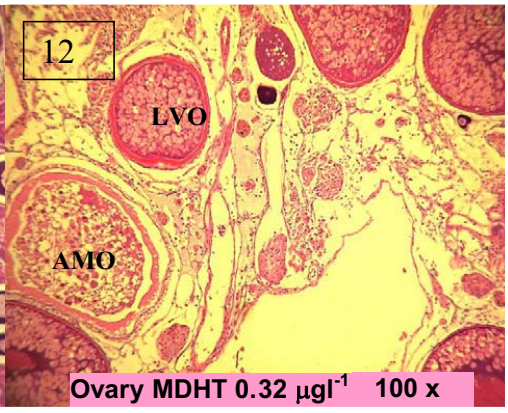
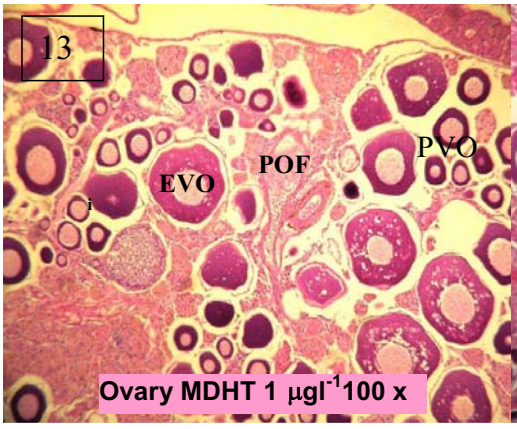
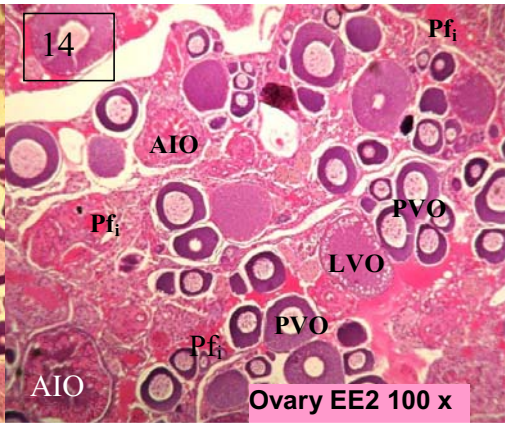
LVO
POF
EVO
ILS
PVO
Ovary SC 100 x


11
LVO
POF
PVO
ILS
EVO
Ovary MDHT 0.1 µgl-1 100 x


12
LVO
AMO
Ovary MDHT 0.32 µgl-1 100 x


13
EVO
POF
PVO
Ovary MDHT 1 µgl-1 100 x


14
Pfi
AIO
Pfi
PVO
LVO
PVO
Pfi
AIO
Ovary EE2 100 x

Number of eggs spawned per pair: During the 21 exposure days of the first reproduction study, egg production was significantly lower in the groups exposed to 1 and 10 μg MDHT l^{-1} compared with the SC (Figure 4.6A). In the second reproduction study, egg production in the SC and EE2 group increased during the first 12 days (Figure 4.6B). However, after this period no additional eggs were produced. The groups exposed to 0.32 and 1 μg MDHT l^{-1} only produced eggs in the first week of the exposure period.

F1: Hatchability and survival

As a consequence of the relatively low numbers of spawns after the first weeks of the reproduction tests, hatchability trials data are rather limited, especially in the second test. No batches of eggs were available for hatching trials from the groups exposed to MDHT concentrations > 0.1 μg l^{-1} after the first weeks of exposure. In the second reproduction test, 2 batches of eggs not originating from the experiment were included as a "zero"-control.

The eggs collected from the DWC and SC animals after week 1 in the first test hatched within 3 to 4 days and survival ranged between 86 and 100 %. The F1-embryos from eggs collected from the pairs exposed to 0.1 μg l^{-1} showed relatively high mortality during the first 1 or 2 days, with percentages of survival between 10 and 77 %. The surviving eggs also hatched after 3 or 4 days. The subsequent 30-day grow up trials showed more than 60 % survival of the hatched larvae, except for one of the two batches originating from the SC, where only 23 % of the hatched larvae survived.

Figure 4.5A

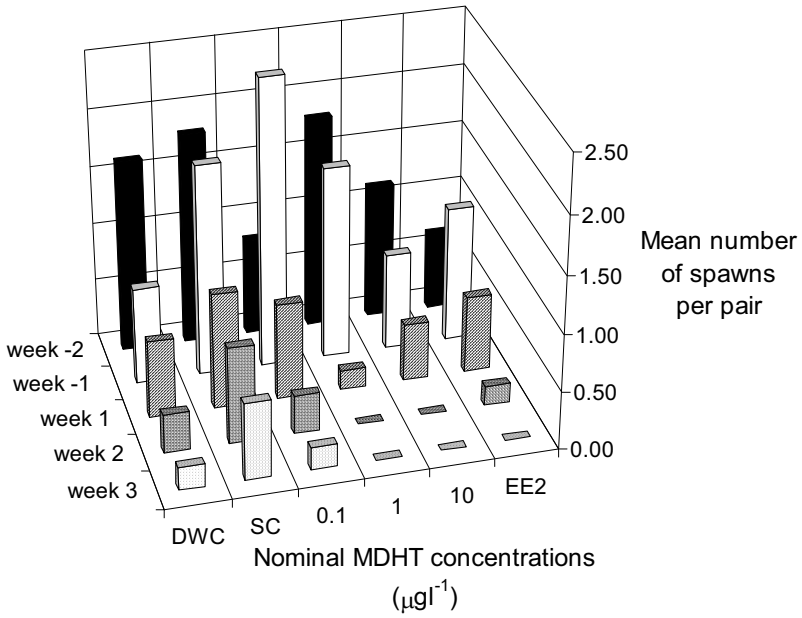


Figure 4.5B

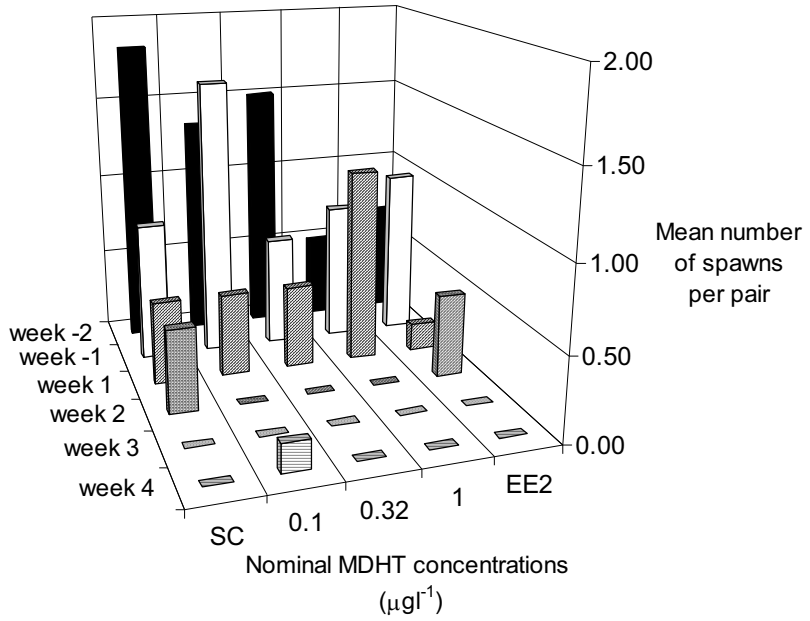


Figure 4.5 Number of spawns produced per week by fathead minnow breeding pairs fish exposed to methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) during the first reproduction test lasting 21 days (4A, opposite page) and the second, 28-day, reproduction test (4B). They also includes the number of spawns of the same pairs during the pre-exposure period (weeks –2 and –1).

Hatching success was 98 and 100 % in the "zero"-control batches added to the second reproduction test. The embryos of one of the two batches of eggs collected from the SC pairs and of the only batch from the EE2-group died within 24 hours, leaving no surviving embryos. The hatching success of the other SC batch was 96 %. The hatching success from the only batch collected from the 0.1 µg MDHT l^{-1} group was 58 %. Because of the very limited number of larvae batches available for grow up trials, it was not considered relevant to perform further analyses of somatic growth, VTG induction or gonadal histology in the F1 generation.

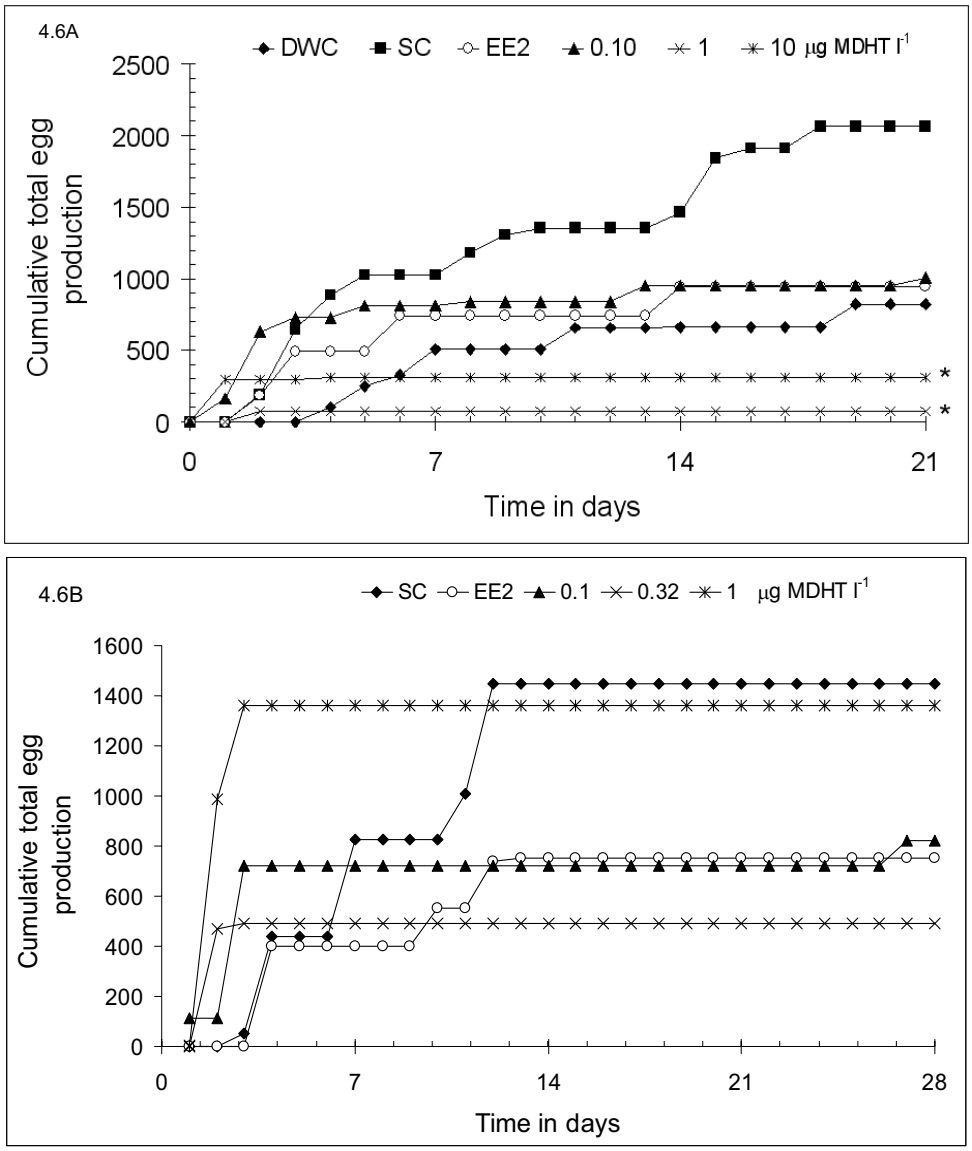


Figure 4.6: Cumulative number of total eggs produced by fathead minnow breeding pairs exposed to methyldihydrotestosterone (MDHT) or ethinylestradiol (EE2) during the first (4.6A) and the second (4.6B) reproduction test. An asterisk denotes a significant difference compared with the Solvent control (SC) at α=0.05, two sided.

Discussion

The fathead minnow is a widely used model to examine the effects of EACs in fish [51,115]. The present study had the objective to assess the effects of exposure to an androgenic compound on fecundity and other endpoints related to the reproductive condition of adult fish, in combination with possible trans-generational effects on (sexual) development of the second generation.

The synthetic and non-aromatizable androgen MDHT (97) was dosed using a flow through system, but final exposure concentrations still deviated up to twofold from the nominal concentration. The variation in exposure concentrations of MDHT is not exceptional, as a similar variation was found for dihydrotestosterone (DHT) in a screening flow through assay with fathead minnow [116] and with MDHT in a 21-day study with zebrafish [103].

As this article also aims at giving more insight in the problems involved with reproduction studies with fathead minnow, we decided to include the data from the first experiment. The relative high mortality of adult fish in various groups due to technical problems, forced the termination of the first reproduction study after 21 days. In the second study no such mortality occurred during the 28-day test period. We replaced several pairs during the pre-exposure period. However, as the replacing pairs had all been maintained under the same conditions for at least two weeks (first experiment) or 20 days (second experiment) this did not compromise statistical comparison with fecundity during the exposure periods The prominent masculinisation of the SSCs of female fathead minnow exposed to the lowest MDHT concentration of 0.1 $\mu g\ l^{-1}$ for 28 days supports the high androgenic potency of MDHT for fish [103]. However, VTG production was much less sensitive to MDHT exposure, as even exposure to 1.0 $\mu g\ l^{-1}$ did not significantly reduce the VTG production in female fish. In contrast, Ankley et al. [87] found significant reduction of VTG concentrations in female fathead minnow exposed to the androgenic growth promoter 17β-trenbolone during 3 weeks. The finding that VTG production in females was not significantly reduced by MDHT exposure was in contradiction to the histological findings in female gonads at 0.32 and 1.0 μg MDHT l^{-1}, in particular the lack of maturation of the oocytes, a process directly related with VTG production. However, van der Ven et al. [103] also recorded a decreased presence of vitellogenic oocytes in zebrafish exposed to MDHT. Harris et al. [117] have previously reported examples of high VTG in the plasma in the absence of oocyte growth and maturation. They hypothesized that this may be due to lack of uptake of VTG into the oocyte, through the VTG receptor. Asanuma et al. [118] found that 11-ketotestosterone (11KT) enhanced E2-induced VTG production in isolated hepatocytes from female Japanese eel, although 11KT alone failed to induce VTG production. Based on this it can be hypothesized that MDHT does not affect hepatic production of VTG but instead may block the receptor-mediated process of uptake of VTG into the oocyte. Hence, this induces accumulation of VTG in liver and blood plasma.

The fact that in most males exposed to MDHT, the VTG concentrations were essentially non-detectable, confirms that MDHT is non-aromatizable into estrogens. For comparison, the aromatizable methyltestosterone caused a significant induction of plasma VTG in both male and female fathead minnow after 12 days of exposure [51] or 21 days of exposure [119]. Panter et al. [116] have shown a distinct stimulation of vitellogenin production in the same fish species treated with non-aromatisable dihydrotestosterone (DHT). MDHT tested in an in-vitro test with a genetically

engineered cell line (BG1LucE2) induced luciferase production up to similar levels as the reference E2 but at MDHT concentrations of a factor of 10^4 higher [113]. This indicates that at certain MDHT levels in the fish exposed may have been high enough to stimulate the estrogen receptor significantly without being aromatized.

MDHT exposure suppressed ovarian maturation and stimulated spermatogenesis. In another study with MDHT, exposure of fathead minnow during early life stages showed that this effect was reversible [113]. Vertebrate Sertoli cells play an important role in spermatogenesis and have receptors for testosterone [120]. Stimulation of spermatogenesis may have been mediated by the increase of the activity of the Sertoli cells in the presence of MDHT [103].

The choice to perform reproduction tests with pair breeding fish was inspired by the fact that such a design can provide measurements of fecundity in individual females as opposed to a "mean" measurement obtained when using groups of more than one female fish exposed simultaneously in the exposure chamber. However, this argument is only relevant when it is possible to compare the fecundity of females with a different experimental history, e.g. to compare exposed to non-exposed females [121]. This type of experimental set-up requires a constant reproduction by the pairs in time, which clearly was not the case in the present studies. The design of a pair-wise reproduction assay with fathead minnow shows several weaknesses, which need careful consideration:

a. the substantial number of pairs that produce little or no eggs and the relatively high risk of low spawning rates in the controls,
b. in a pair-wise breeding set up, aggressive male behaviour can damage or even kill female fish,
c. the large variation in egg production, which hamper statistical analysis at both the pair-wise (vertical) as treatment level (horizontal),
d. a decrease of reproduction with progression of time may not indicate an effect as pair-bound reproduction tends to decrease with time as a rule,
e. an unbalanced comparison of F1 between different treatments due to the high 'background' variations in number of spawns, numbers of eggs per spawn and the timing of the spawns during the exposure period.

All these weaknesses result in relatively large variation in the fecundity endpoint and a low statistical sensitivity of the respective data. Länge et al. [54] confirm this lack of sensitivity of the fecundity endpoint related to egg laying due to a large variation in egg production by individual pairs in the controls and the treatments. As an example they report variations of 0 to 1,977 eggs produced by the individual pairs of fathead minnow in the control group and 0 to 1,763 in a treated group.

According to the international guidelines, fathead minnow can be kept in breeding conditions all year without having to manipulate light/dark regimes or water temperature. However, consultation with different laboratories revealed that in practice there is a relatively high variability in egg production during the adult life stage of fathead minnow. To illustrate this further, the coefficient of variation (CV) of the number of eggs per female in 23 full-life-cycle studies ranged between 1 to 60 % with a median of 15 %, while in 21-reproduction tests performed in three

different laboratories (n=51) the CV ranged from 8 to 137 % [122]. For comparison, the historical data at NOTOX B.V. on the parthenogenetic reproduction of the invertebrate *Daphnia magna* show a CV of 14 % ± 5 %. The results of an OECD ring test [123] with three species of fish, i.e. medaka, zebrafish and fathead minnow, showed that in fathead minnow, comparison of spawning in treated groups was confounded by poor fecundity in control groups. Moreover, comparing the different possible endpoints concerning the reproductive performance or capacity of fish, fecundity is generally considered the less sensitive compared to other endpoints when measured based on the number of eggs deposited over time [124].

In pair-wise testing of reproduction, a CV of 15 % would require at least 8 replicates per treatment to discriminate a ≥ 25 % reduction at a 95 % confidence interval (α=0.05). In the present studies, the CVs were > 25 %, which means that six replicates applied per treatment were insufficient to detect differences < 50 % (α=0.05). We only found differences of >50 % in the first reproduction test at MDHT concentrations of 1 and 10 $\mu g\ l^{-1}$. Ankley et al. [51] recommend the use of groups of spawning minnows to ensure a greater likelihood that eggs will be laid each day in the test chamber during the exposure period, lowering the variability in fecundity estimates. In their evaluation of a short-term (21-day) reproduction test with fathead minnow, they exposed four female and two male fathead minnow in each replicate with four replicates per treatment. The CV of the number of eggs per spawn was 37 % for three replicates in the control. Still they found significant reduction of fecundity by exposure to methoxychlor at 0.5 and 5 $\mu g\ l^{-1}$. At the lowest concentration this was probably due to a lower CV (15 %) in combination with a lower mean value (25 % below the mean of the control). Although group breeding evidently does not increase mean batch size (= number of eggs per spawn) or the mean number of eggs produced per female, it has the potential to decrease the CV in the numbers of eggs produced and thus to increase the statistical power of the test.

In their recent study, Thorpe et al. [115] concluded that high variations in estimates of egg production may relate, at least in part, to the egg collection strategy used. They compared two types of strategies, i.e. the combination of a spawning tile with a capture tray without or with a mesh-screen. The mesh-screen prevented the eggs from being eaten by the parents, and its use decreased the CV for egg production by a factor of two, i.e. from an average CV of 60 % to 30 %. Adopting the mesh-screened tray reduced not only variation in egg number between pairs, but also between experiments. In spite of this improvement, still relatively high numbers of replicates will be necessary to increase the required statistical power of this endpoint in pair-wise reproduction tests. An average CV of 30 % will still require 12 replicates per treatment to discriminate a reduction of ≥ 40 % in egg numbers between pairs before and after treatment. In addition, anticipating an average loss of 25 % of the pairs as described by Thorpe et al. [115], the number of replicates per treatment should be increased to 15.

Another feature observed in our study was a general reduction of spawning in time, suggesting that fish became “spawned out”. Incidentally, this feature occurred already in the pre-exposure phase of the first reproduction test, but in general the pairs that reproduced from the start, continued spawning during the second and third week of both pre-exposure periods. However, in the control groups of both reproduction tests, most pairs stopped spawning after the

additional one or two weeks. A time related decrease in control spawning has also been observed in other studies [54, 121] but not as prominently as presented here. The study reported by Thorpe et al. [115] also showed the exclusion of relatively high numbers of pairs from the experiments (17 to 33 %) due to "egg-bound" females or deviating spawning behavior. "Egg-bound" females are unable to release their eggs, causing their abdomen to become extremely swollen. A possible solution to prevent this effect may be to keep the male and female fish separated by a mesh in the same vessel and allowing them to be together during 1 week in the pre-spawning period and 1 week after e.g. 2 weeks of exposure. This might also facilitate comparison between the egg batches for F1 hatching and the F1-development trials in order to evaluate possible trans-generational effects of MDHT exposure.

Effects on reproduction may not necessarily be a result of an endocrine disrupting mode of action, as systemic toxicity can also play a role. Therefore, a combination of endpoints including fecundity, histology and VTG should be studied to support possible endocrine effects of suspected substances [51, 87, 125]. Based on the results of 21 day tests with fathead minnow, medaka and zebrafish in Phase 1B of the OECD Ring test for endocrine disruptive screening [123], at least one of these three endpoints proved to be the most sensitive depending on the type of substance tested. However, not one of these endpoints could be designated as the most sensitive in all cases.

Despite the relatively high variation in fecundity, the nominal MDHT concentrations of 0.32, 1 and 10 $\mu g l^{-1}$ inhibited reproduction completely after ca. one week of exposure. Ankley et al. [51] reported a complete cessation of spawning when fathead minnow was exposed to methyltestosterone at 200 $\mu g\ l^{-1}$ for 21 days. Based on analytical chemical measurements, the actual 100% MDHT effect concentration for reduction of egg production was ca. 0.19 $\mu g\ l^{-1}$. The results for the F1 trials were insufficient for evaluation of possible trans-generational effects of MDHT exposure.

Exposure to any of the MDHT concentrations did not significantly affect VTG production in female fish, whereas histology of the gonads indicated inhibition of oocyte maturation at the higher concentrations and oocyte atresia at the lowest. In addition, exposure to an actual MDHT concentration of ca. 0.060 ± 0.012 $\mu g\ l^{-1}$ (at nominal 0.1 $\mu g\ l^{-1}$) induced subtle changes in the secondary sexual appearance of the female fish reflected by the emergence of tubercles on their snouts. Although MDHT is not an environmentally relevant chemical, this indicates that SSC's are a sensitive endpoint for identifying significant environmental concentrations of androgens.

Conclusions

Based on gonadal histology and SSC, the lowest observed effect concentration (LOEC) of MDHT in a 28-day reproduction study is 0.1 $\mu g\ l^{-1}$, whereas based on reduction of VTG it is above 1 $\mu g\ l^{-1}$.

Although the results suggest that fecundity is affected at concentrations > 0.1 $\mu g\ l^{-1}$, the overall high variability of egg production hampered a powerful analysis of the data. This high variability of egg production can be reduced by improving the strategy of egg collection, by changing the scheme of allowing the pairs to mate to prevent the naturally reduction of spawning

activity over time, and by increasing the number of replicates. Furthermore, the current design of the pair-wise reproduction tests with fathead minnow requires optimization considering spawning condition of the female fish and sexual behavior of both the male and female fish. Assessment of possible trans-generational effects requires a more complicated experimental design allowing significant amounts of comparable egg batches in the chemical exposed fish. To achieve this, concentrations of the chemical, which should only affect egg production in subtle manner, should be included in the experiment.

Although fathead minnow is a species widely used in FLC or short-term reproduction tests, it is clear that there is still a need for increasing the reliability, inter-laboratorial repeatability and consistency of the endpoint of fecundity when fathead minnow is to be used as a standard species in pair-wise reproduction tests for the assessment of biologically significant and ecologically relevant effects on fish populations.

Acknowledgements

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Chapter 5

An *in vitro* / *in vivo* screening assay as a sensitive tool to assess endocrine disruptive activity in surface water

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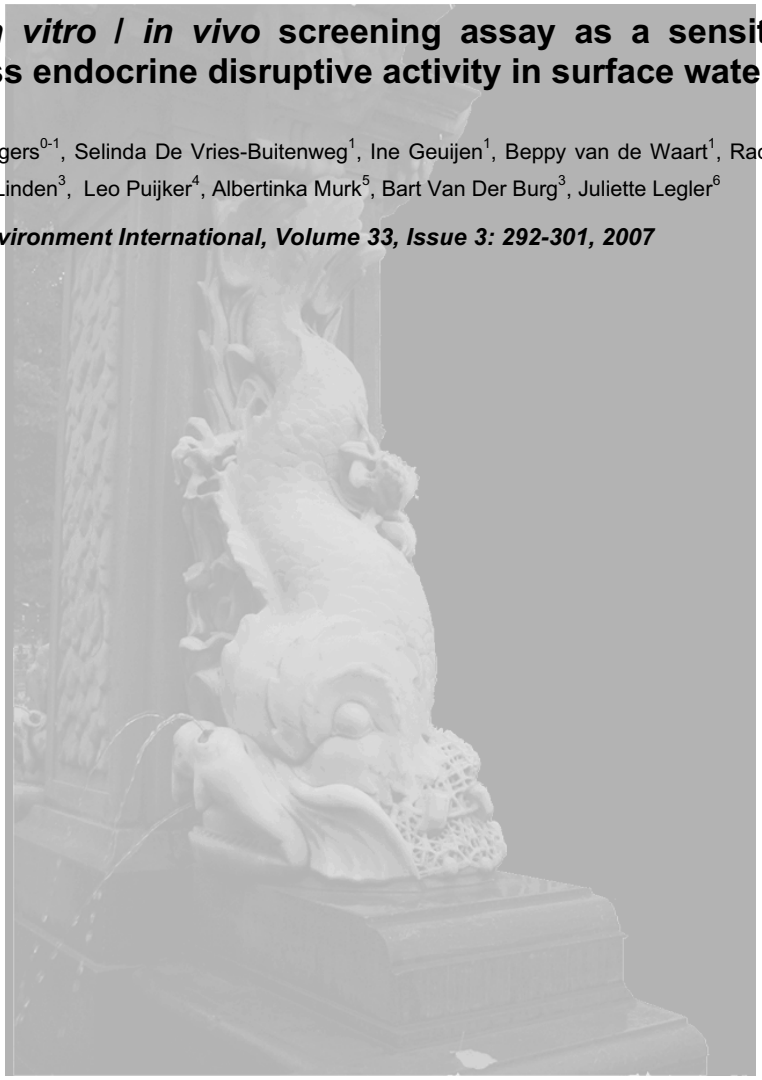

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Abstract

Adult male fathead minnow were exposed for 14 or 28-days under flow-through conditions to undiluted filtered water samples from the rivers Meuse and Rhine in the Netherlands. The experiment included two vessels per treatment each containing 10 fish and samples of five fish were taken after 14 and 28 days. Additional groups were exposed to 17α-ethinylestradiol (EE2) as a reference and untreated drinking water as a negative control. Major endpoints examined included induction of vitellogenin (VTG) synthesis, VTG mRNA activity, hepato- and gonadosomatic indices (HSI and GSI) and gonadal histology. No significant difference was recorded in body weight or mean GSI values between the various treatments. Only exposure to Meuse water resulted in significantly higher HSI means after 14 days. Histological examination showed no apparent effects on gonadal tissue except for eosinophilic blood plasma in fish exposed to Meuse water or EE2. After 14 and 28 days, elevated VTG and VTG mRNA levels were measured in most livers of the fish exposed to Meuse water, but not in the fish exposed to Rhine water. This was confirmed by measuring estrogenic responses in the *in vitro* ER CALUX® assay. Induction of VTG synthesis proved to be the most sensitive endpoint in the Non Spawning Male Fish Assay for *in vivo* detection of bio-available estrogenic activity supplementary to a sensitive *in vitro* assay. The other endpoints examined varied too much and required a higher number of fish or replicates to achieve sufficient power for statistical testing making them less animal friendly.

Introduction

For already almost a decennium significant concern has grown worldwide regarding the risk of synthetic chemicals and environmental contaminants acting as endocrine disrupting chemicals (EDCs). This concern focused especially on the potential of chemicals to adversely affect the reproductive systems of both human and wild life populations around the world. In the Netherlands, a preliminary inventory was started by the Kiwa Water Research, the research institute of the Dutch Waterworks in corporation with the Association of River Waterworks (RIWA) in 1997 [126], which resulted in a project plan to examine the possible presence of estrogenic active chemicals (EACs) in surface waters at locations where water is extracted for drinking water preparation. In 1999, the Dutch National Health Council published a report [127] stating that there were sufficient reasons for concern about the presence of EDCs in especially the aquatic environment in the Netherlands. The Council came up with 34 groups of compounds that were suspected to have the potential to disrupt the endocrine system of the organisms present and thus endangering the reproduction of certain species. In addition to natural and synthetic hormones, these compounds included alkylphenols, bisphenol-A, polybromobiphenyls, polybrominated diphenylethers and to a lesser extent phthalates. In an early study concerning analytical detection of estrogenic hormones in surface and wastewaters, most of the estrogens were below detection limits, however, at some locations levels up to 6 ng l^{-1} were found [127]. As in various other European countries, a nation-wide survey has been performed in the Netherlands (LOES Project) to make an inventory of the presence of estrogenic active substances in Dutch surface waters [25]. This survey showed that at certain locations sewage treatment works (STW) effluents were estrogenic to fish but the Dutch STWs appeared to be very effective in removing natural and synthetic estrogens from the untreated wastewater. A limited sampling performed on wild populations of freshwater fish showed that intersex bream (*Abramis brama*) were found in the only small river included where a municipal STW discharged relatively large volumes of effluent. Follow-up sampling of fish from other small rivers with comparable discharge ratios [128] revealed male bream or roach (*Rutilus rutilus*) with intersex and/or increased levels of the female protein vitellogenin (VTG). However, no intersex fish were found in main rivers like the Rhine and Meuse. The authors of the report recommended that authorities responsible for monitoring the surface water quality should further examine and list locations with a potential for a risk based on endocrine disruption. In the slipstream of the LOES-Project, various research projects started for detection of estrogenic activity in surface water serving as sources for drinking water. The research encompasses projects concerning the various stages of the fresh water cycle, varying from drinking water to waste water treatment and all related environmental aspects (e.g. endocrine disruption in fish). The main target of the present paper was to examine whether the Non Spawning Male Fish Vitellogenin *in vivo* Assay is sufficiently sensitive to confirm *in vitro* determined estrogenic activity by simultaneous *in vitro* and *in vivo* testing of the water phase of surface waters of two major rivers (Rhine and Meuse) sampled at locations where water is extracted for drinking water preparation. The *in vitro* experiments were performed in the ER-mediated chemically activated luciferase gene expression (ER CALUX®) assay [47]. In the ER CALUX® assay, reporter gene expression is a measure of the ER-mediated

cascade of intra-cellular events resulting in activation of genes. The assay is performed with T47D human breast adenocarcinoma cells with endogenous estrogen receptors, which were stably transfected with an estrogen-responsive luciferase reporter gene containing three estrogen-responsive elements. In earlier studies, this assay has proven to be more sensitive than other assays (YES or CARP-HEP) and accurate in detection of estrogen activity in samples of EACs, effluents or sediments [25, 129, 49].

The *in vivo* counterpart asked for a sensitive short-term bioassay in an aquatic organism, preferably fish. Induction of VTG in male fish has proven to be a sensitive endpoint for detection of estrogenic active EDCs as well as of effluents and surface waters [33, 130, 131, 132]. VTG is an oestrogen dependent yolk protein precursor synthesized in the liver and transported by the blood stream to the female gonads where it is incorporated in the developing oocytes. As male fish do not produce VTG except when they are exposed to estrogenic active substances it serves as a biomarker for exposure to estrogens in oviparous vertebrates. VTG levels can be determined in blood or homogenized liver tissue. The *in vivo* non-spawning male fish assay further addressed several other biological endpoints including secondary sexual characteristics, the gonado–somatic index (GSI), the hepato-somatic index (HSI), VTG mRNA levels in liver tissue, and histopathology of excised gonads. Female fish were not included to prevent elevation of the background VTG production in males in response to female excretion of compounds such as 17β-estradiol [133]. The assay was designed to assess estrogen activity in the water samples taken from Rhine and Meuse at extraction sites for the preparation of drinking water, without identifying specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects or disturbance of hormone synthesis or metabolism). An additional objective was to test whether the standard exposure period of 28 days could be shortened without loss of information, which significantly decreases the inconvenience for the test animals.

Materials and methods

Surface water samples

Between October 7th and November 4th 2003, surface water samples were taken from the river Rhine at sampling point Nieuwegein (Lek Channel) and from the river Meuse at sampling point Hedel, both in the Netherlands. Samples were taken with a bucket at 10 to 50 cm under the water surface. The water was transferred to coded 10 or 20 l mess tins and transported to NOTOX three times a week on Monday,Wednesday and Friday in the late afternoon and stored at 7 °C until further use (within 24 h). Incidentally, extra samples were delivered on other or additional days of the week. After emptying the mess tins, they were rinsed with tap water before being re-used for sampling. All mess tins were allocated to a specific location to prevent contamination with sampling water from the other location.

Chemicals and solvents

17α-Ethinylestradiol (EE2) was obtained from Sigma, The Netherlands (Product Number E4876) with a purity N98% (by HPLC). The solvent triethyleneglycol was supplied by Merck, Darmstadt, Germany and of synthesis quality with a purity of ≥99%.

Test organisms

Fathead minnows (*Pimephales promelas*, Teleostei, Cyprinidae) (Linnaeus, 1758) were bred from an in-house culture. Fish were held in tap water (hardness ca. 180 mg $CaCO_3$) at a temperature range of 21–26 °C in a 200-liter tank in a recirculating system with a bio-filter. Physical water parameters, i.e. pH, hardness, nitrate, nitrite and ammonia concentration were measured once a week and temperature every day. The stock was fed with frozen adult brine shrimp and pellet food. The male fish selected for testing were acclimatized to the test conditions from seven days before the start of the test.

Preparation of surface water samples

The river water was used without prior dilution, but filtered under high pressure through a glass filter and a 0.2 µm membrane filter (Schleicher and Schuell) to remove all suspended particles before dosing. Volumes of surface water to be filtered amounted to 50 l per sample per day and an additional 50 l for the weekly renewal of the whole vessels. When the complete samples could not be filtered on the same day of delivery, fractions of the samples were stored in a climate room at maximum 7 °C until they were filtered. Period of storage never exceeded 24 h.

Design of the in vivo assay

The assay was performed with adult male fish from single non-spawning adult male populations. The assay was conducted using undiluted river water from the Rhine and Meuse Rivers, one EE2 exposure concentration (positive control) and untreated tap water (negative control), with two vessels per treatment each containing 10 males. The exposure was conducted for 28-days. Test vessels were 30 l vessels with an actual volume of ca. 25 l. The light period consisted of 16 h photo-period daily, between 10–20 $\mu Em^{-2}s^{-1}$ or 600–1000 lux. As EE2 was not readily soluble in water, stock solutions of 50 µg l^{-1} were prepared in tri-ethyleneglycol (Trigol). The stock solutions with EE2 were dosed, via a computer-controlled system consisting of a dispenser (Gilson 402), into a mixing flask where dilution water (tap water) entered through a flow meter. In the mixing flasks the dosed volumes were mixed with the dilution water under continuous stirring providing a target concentration of 5 ng l^{-1} (in 0.01% Trigol). From the mixing vessels, the test solutions entered a distribution vessel for each replicate separately. From the distribution vessels, the test solution entered the replicates at an average flow rate of 1.1 l/h, i.e. sufficient to reach a one-volume exchange of test solutions per 24 h. The dilution water control vessels were dosed with tap water in a similar set-up as used for the EE2 treated solutions and defined as the blank or drinking water control. The resulting 0.2 µm-filtrates of the river water samples were dosed using tubingpumps at a flow rate up to 1 l/h per replicate. The whole system was checked daily. Weekly samples of ca. 1 l were taken from the replicates of the drinking water control and the EE2 treated group. These samples were shipped to KIWA N.V., Nieuwegein where they were used for preparation and further testing in the ER CALUX® assay at BioDetection Systems, Amsterdam, The Netherlands.

The animals were weighed before they were randomly assigned to the test vessels and allowed to acclimatize for 7 days before the start of the test. During the test period, fish were fed with frozen adult brine shrimp and pellet food. Within 1 h after each feeding, any food residues

were removed by siphoning. Fish were not fed at least 24 h prior to termination of the experiment. Fish were observed daily and dead fish were removed when observed. On day 0, day 14 and day 28, observations of physical appearance were made on among others body colour, coloration patterns, body shape, specialized secondary sex characteristics. After 14 and 28 days of exposure, 10 fish per treatment (five of each replicate) were sacrificed and the complete livers were collected and weighed for determination of the hepatic somatic index (HSI). Thereafter they were treated for determination of VTG levels and VTG mRNA. The gonads were removed and transferred to fixative in pre-weighed vials. The vials with gonads sampled on day 28 were weighed for calculation of the gonadal-somatic index (GSI) and processed for subsequent histological analyses.

Quantification of secondary sexual characteristics

Characteristics of physical appearance in adult fathead minnows potentially important in endocrine disrupter testing include body color, coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor). Nuptial tubercles are located on the head (dorsal pad) of reproductively-active male fathead minnows, and are usually arranged in a bilaterally-symmetric pattern. Mapping, counting and grading of the nuptial tubercles was conducted using a binocular microscope and based on the work of Smith [57] and Jensen et al. [58], where the grading relates to the progressive prominence of the tubercles.

GSI and gonadal histology

Gonadal histology was performed only on gonads collected after 28 days, and gonads were fixed in modified Davidson's solution [114]. After 24 h, the gonads were transferred to 70%-ethanol. Dehydration of the gonad samples occurred in a graded ethanol series, clearing in xylene, paraffin wax infiltration and embedding; sectioning at 4 µm on a rotary microtome and staining with Mayer's Haematoxylin and Eosin. The resulting slides were examined for histopathological alterations in male gonadal tissue applying the following scores:

a) inflammation: 0=absent, 1=present;
b) eosinophilic blood plasma: 1=normal, 2=increased; 3=severe, with apparent resorption vacuoles;
c) necrosis: karyopycnosis in spermatogonia: 0=absent, 1–2=moderateextensive;
d) apoptosis: single cell necrosis in spermatogonia, judging from eosinophilic cytoplasm and pycnotic/rhectic nucleus: 0–1=absent-sporadic;
e) clumped spermatocytes: clusters of spermatocytes in tubular lumen, phagocytes by macrophages/ sloughing of sertoli cells unclear; 0=absent, 3=severe;
f) sertoli cells: 1=normal, 2=nuclear hypertrophy, due to low numbers of sertoli nuclei in sections, these were often based on not more than 2 to 3 nuclei.

Further observations included, if present, Leydig cell hypertrophy, calcified structures in damaged parts of the gonads or local vacuolisation of spermatogonia.

Viltellogenin (VTG) and VTG mRNA analysis

The livers were excised, weighed, and then divided into two subsamples of which one was weighed and stored for VTG analysis, while the other one was stored for VTG mRNA determination. All samples were frozen in liquid nitrogen and stored at −80 °C. VTG concentrations in liver homogenates were measured using a commercially available Enzyme–Linked Immunosorbent Assay (ELISA) supplied by Biosense Laboratories AS, Norway, together with a protocol. The assay is based on a competition for the fathead minnow-Vtg antibody between VTG coated on the wells of the ELISA-plate and free VTG in solution (sample).

The VTG mRNA levels were measured based on binding of isolated-mRNA to a specific probe, resulting in emission of light according to manufacturer's instructions (Molecular Light Technology, Wales, UK). The VTG mRNA levels were normalized relative to β-actin-mRNA contents measured in the same samples. The β-actin mRNA contents are relative constant and not sex related. Further, to a certain extent, it is a measure of the amount of liver tissue analysed.

Water quality parameters

At the start and once per week during the exposure, a water quality check was performed on fresh tap water and river water samples by measuring pH, alkalinity, NO_2^-, NH_4^+, NH_3, chloride, hardness, salinity and conductivity. When values measured deviated from optimal, the content of the whole vessel was renewed and the respective parameter was measured again to confirm it had improved. Dissolved oxygen concentrations were determined (one per treatment) at the start and three times a week during the first week of the test period and daily thereafter. Temperature was measured continuously in one vessel of the negative and one of the positive control, three times a week in both vessels of the river water samples.

Design of the in-vitro assay

Prior to the ER CALUX® *in-vitro* bioassay, organic compounds, possibly including EACs, were isolated from water samples by liquid–liquid extraction with ethylacetate. A water sample of 1 L was filtrated using 0.45 µm cellulose nitrate filters and subsequently extracted three times with successively 200, 50 and 50 ml ethylacetate. The combined extracts were concentrated to nearly dryness, i.e. less than 10 µl in pre-weighed glass point vials. After addition of 50 µl dimethylsulphoxide (DMSO), the extracts were concentrated by a gentle stream of nitrogen to remove the remaining ethylacetate. Finally, DMSO was added up to 50 µl by weight to achieve a final concentration factor of 20,000. The T47D human breast adenocarcinoma cells were cultured as described previously [49] in Nunc 96-well plates (5000 cells/well) in assay medium (1:1 mixture of Dulbeccos's modified Eagle's medium/Ham's F12 medium) supplemented with nonessential amino acids and fetal calf serum (stripped from hormones). After incubation for 24 h, the assay medium was renewed followed by an additional 24 h of incubation. Before exposure, the assay medium was mixed with the extracts of the surface water samples or samples taken from the EE2 and blank solutions used in the *in vivo* test. The cells were exposed to these mixtures in triplicate for another 24 h. In addition, a 17β-estradiol (E2) calibration curve was tested in triplicate exposing cells to 0, 0.3, 0.6, 1, 3, 6, 10 en 30 pM. After exposure, the medium was removed and the cells were lysed in Triton lysis buffer. Luciferin substrate was added to each well and luciferase activity

was measured with a luminometer (LUCYII, Anthos). The estrogenic potency of the samples was expressed as estradiol equivalency (EEQ). The method used was comparable to that earlier described by Murk et al. [49]. The limit of detection (LOD) was 0.007 ng l^{-1} and the limit of quantification was 0.020 ng l^{-1} (lowest point of the calibration curve).

Data handling and statistics

All data on body and tissue weights, HSI and GSI were averaged per replicate and then compared with the combined means of the blank replicates. The values of the two blank replicates were compared using the Student's t-test for two sample analysis. They were only combined when there was no statistical difference. All data were tested for normality (Chi-square test) and homogeneity of variance (Bartlett test). The statistical analyses for pair-wise comparison with the combined control was performed with ANOVA–Dunnet or Bonferroni ttest, α=0.05 two-sided. In addition, a multiple comparison was performed with the Tukey test (α=0.05).

Results

Surface water samples: delivery and quality

The ultra-filtering of the river water samples was challenging due to the relatively large volumes of water that had to be filtered almost daily in order to maintain a constant flow during the experimental period. Although the flow rate of 1 l/h corresponded with one replacement of the actual water volume (ca. 25 l) in each vessel per day, it proved to be insufficient to maintain an optimal quality of the test solutions especially during the first week of exposure. Oxygen concentration generally was above 5 mg O_2 l^{-1} (60% of saturation), except during the first week when incidentally oxygen dropped below this level in one or more vessels (3–4 mg O_2 l^{-1}). The pH values measured generally ranged between 7.6 and 8.4, except on day 28 with three replicates where pH was 8.5 (8.6–8.8). The temperature remained largely between 22 and 24 °C. Difference in temperature between different exposure groups and replicates did not exceed 0.5 °C. Ammonium concentrations reached levels up to 5 mg l^{-1} and were comparable in all test solutions including the untreated controls, although the levels were mostly the highest in the vessels with Meuse or Rhine water. Nitrite concentrations ranged between 0.25 and 1 mg l^{-1} in Meuse water and between 0.1 and 1 mg l^{-1} in Rhine water. Although nitrite levels up to 1 mg l^{-1} were also incidentally detected in the untreated control solutions, the concentrations in the river water were always higher and detectable nitrite levels were already present in fresh samples before introduction into the test vessels. When the quality of the test solutions became below optimal, exposure water was completely renewed in addition to the daily exchange provided by the flow-through system.

Effects of surface water and EE2 exposure in fathead minnows

Clinical effects and survival

Two fish died during the acclimatization period and were replaced by new male fish from the fish stock. One fish was found dead in a vessel of the EE2 treated solution on day 22. Otherwise,

fish did not show any visible effects on behavior or appearance in any of the vessels throughout the test period.

Body and liver weights

At the start of the exposure, mean body weight of the fish was 4.32 ± 1.39 g. After 14 days of exposure the mean body weights of all replicates remained within that range (Table 5.1). Statistical analysis of body weights showed no significant difference between the various replicates (α=0.05, two-sided).

Table 5.1 Mean values for fish body weights on days 14 and 28 in tap water (blank control), surface water of the rivers Rhine and Meuse and the positive control ethynylestradiol (EE2, 5 ng l^{-1}). At the start of the study, mean body weight was 4.32 ± 1.39 g.

Group	Fish body weight in grams			
	Day 14		Day 28	
	Mean	± SD	Mean	± SD
Blank A	4.86	± 2.14	4.12	± 0.82
Blank B	4.37	± 1.01	4.16	± 0.89
Mean Blank	4.62	± 1.67	4.14	± 0.86
Meuse A	4.60	± 1.41	4.84	± 1.83
Meuse B	3.62	± 0.82	4.20	± 0.67
Mean Meuse	4.11	± 1.15	4.52	± 1.38
Rhine A	3.95	± 1.84	3.80	± 1.23
Rhine B	4.49	± 0.60	3.36	± 0.39
Mean Rhine	4.22	± 1.37	3.58	± 0.91
EE2 A	3.78	± 1.29	3.25	± 0.69
EE2 B	3.75	± 1.31	4.40	± 1.70
Mean EE2	3.77	± 1.30	3.83	± 1.30

After 28 days of exposure, the mean body weights of all replicates remained within the initial range of 4.32± 1.39 g, although the means of replicates Rhine-B and EE2-A were near the lower limit of 2.93 g. Statistical analysis showed no significant difference between the various replicates (α=0.05, two-sided). Mean liver weights of fish exposed for 14 days to Meuse, Rhine and EE2 were 45, 35 and 23% higher than that of the control group, respectively (Table 5.2). The mean HSI values of both Meuse replicates were significantly higher than that of the combined blank control replicates, while this was true only for one Rhine and one EE2 replicate (α=0.05).

Table 5.2 Liver and hepatic somatic indices (HSI) measured after 14 days. Replicates contained 5 adult male fathead minnows each, except when otherwise indicated.

	Liver weight (mg)			HSI		
Group Replicates	Mean	±	SD	Mean	± SD	
Blank A	64.0	±	42.1	1.33	± 0.57	
Blank B	69.9	±	6.6	1.63	± 0.32	
Mean Blank	67.0	±	30.2	1.48	± 0.65	
Meuse A	105.6	±	7.9	2.58	± 1.31	*
Meuse B	88.9	±	13.2	2.43	± 0.36	*
Mean Meuse	97.3	±	10.9	2.50	± 0.96	
Rhine A	104.0	±	16.1	2.77	± 0.78	*
Rhine B	76.8	±	6.3	1.71	± 0.24	
Mean Rhine	90.4	±	12.3	2.24	± 0.58	
EE2 A	91.6	±	16.0	2.38	± 0.45	*
EE2 B	73.8	±	13.5	1.94	± 0.22	
Mean EE2	82.7	±	14.8	2.16	± 0.36	

* Value is significantly different from the combined blank control, α=0.05 two sided, data Log base 10 transformed.

After 28 days of exposure, no significant differences were observed both for mean liver weights and HSI values (Table 5.3). One of the fish in replicate EE2-B had a liver with an outlying weight of 149 mg. Fish exposed to Meuse water had the highest HSI values, but this was not statistically significant (α=0.05).

Table 5.3 Liver and hepatic somatic indices (HSI), gonadal weights and somatic indices (GSI) measured after 28 days of exposure. Replicates contained 5 adult male fathead minnows each, except when otherwise indicated.

Liver weight (mg)			HSI			Gonadal weight (mg)			GSI		
Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
53.8	±	26.5	1.29	±	0.47	56.2	±	15.6	1.43	±	0.61
53.4	±	20.5	1.30	±	0.48	75.3	±	23.3	1.90	±	0.78
53.6	±	33.5	1.30	±	0.67	65.8	±	28.0	1.67	±	0.98
90.4	±	29.5	1.94	±	0.39	78.7	±	41.1	1.59	±	0.63
76.3	±	21.1	1.81	±	0.34	49.1	±	19.4	1.16	±	0.36
83.4	±	25.6	1.88	±	0.37	63.9	±	32.1	1.38	±	0.51
59.8	±	23.1	1.56	±	0.25	57.3	±	27.7	1.45	±	0.28
51.6	±	9.9 [#]	1.54	±	0.38[#]	48.3	±	16.1[#]	1.45	±	0.56[#]
55.7	±	17.8	1.55	±	0.32	52.8	±	22.6	1.45	±	0.45
55.5	±	12.7	1.84	±	0.55	36.4	±	12.9	1.14	±	0.45
57.8	±	19.7 [##]	1.61	±	0.46	42.1	±	20.2	0.88	±	0.41
56.7	±	32.6	1.72	±	0.51	39.2	±	16.9	1.01	±	0.43

[#] Data of the one female fish are not included (n=4).
[##] Value excluding an outlying value of 149 mg, with this outlier the mean is 73.5 ± 44.4.

Nuptial tubercles

Exposure to EE2 for 28 days decreased the mean number of nuptial tubercles in both replicates (Figure 5.1), but this was not statistically significant (α=0.05). The mean numbers recorded in the other replicates were all in the same range.

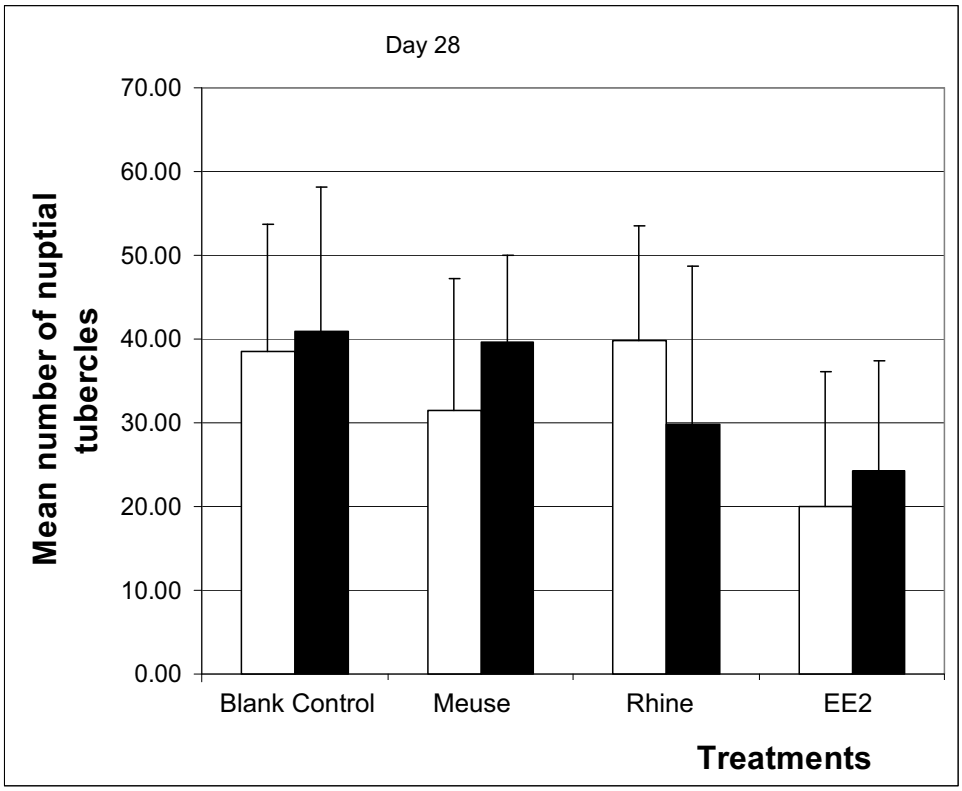

Figure 5.1 Mean number of nuptial tubercles indicative of male sexual characteristic in fathead minnow after 28 days of exposure to surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng l^{-1}) and tap water (blank control). White and black bars represent the two replicates of each treatment.

VTG and VTG mRNA measurements

No detectable VTG induction was recorded in any of the male fish of the blank control or Rhine water replicates after 14 or 28 days of testing (Figure 5.2). Only in one liver sample of a fish from Rhine replicate B after 28 days of exposure VTG induction was measured, and this corresponded with the histological finding that this was a female fish instead of a male. In liver samples, elevated VTG levels were measured in 9 of the 10 fish sampled from the two Meuse

replicates and from the two EE2 replicates after 14 days exposure. This was also observed in 8 samples from each of these groups after 28 days.

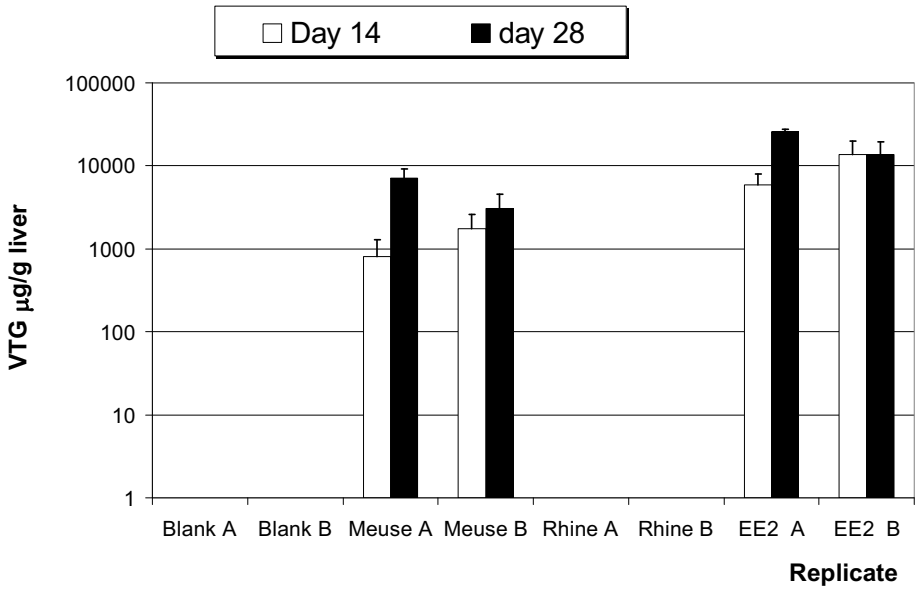

Figure 5.2 Mean hepatic vitellogenin concentrations in liver homogenates of male fathead minnows (n=5) exposed in duplicate to surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng l^{-1}) and tap water (blank control) for 14 and 28 days.

The mean VTG levels induced by EE2 exposure were about a factor of 10 higher than those recorded in the Meuse samples. The mean ratio's between VTG mRNA and β-actin mRNA were generally below 1 in the blank and river Rhine exposure groups indicating absence of VTG mRNA induction (Figure 5.3). The higher responses in the Meuse and EE2 exposure replicates were not statistically significant when sampled after 14 days due to relatively high variations. After 28 days, however, the mean VTG mRNA values normalized for β-actin mRNAwere significantly higher in all replicates of the Meuse and EE2 exposure groups compared to those of the blank control.

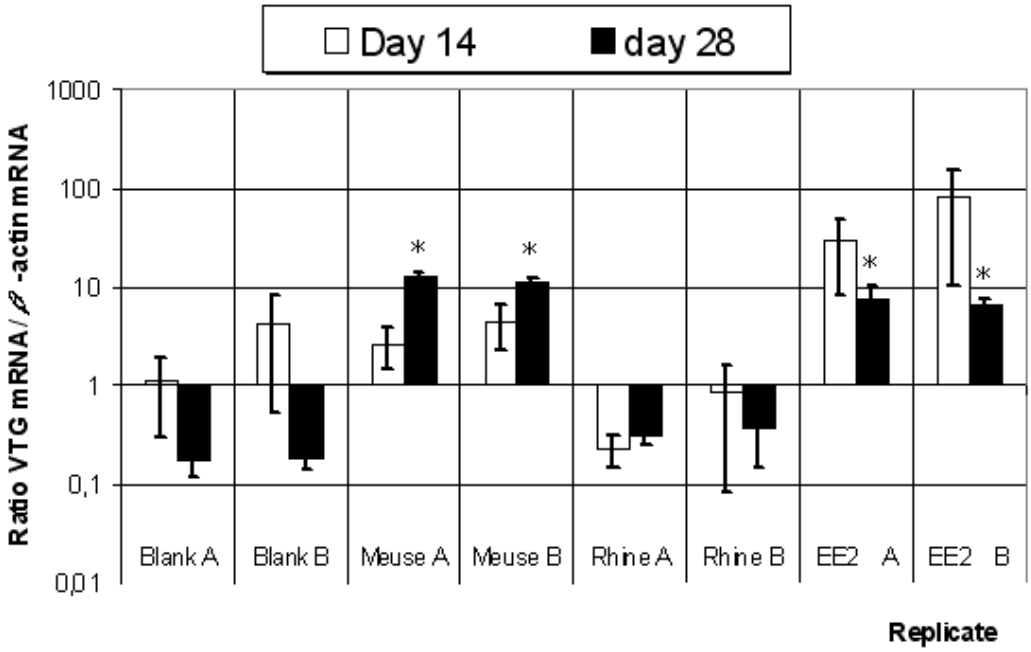

Figure 5.3 Mean hepatic VTG mRNA normalized for β-actin mRNA induction (y-axis) in extractions of liver homogenates of male fathead minnow after 14 or 28 days exposure to surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng l^{-1}) and tap water (blank control). Asterices represent statistically significant differences compared to the combined controls.

Estrogenic activity in surface waters with in vitro ER CALUX® assay

The estrogenicity was above the level of quantification (LOQ) of 0.020 ng l^{-1} in all 39 water extracts (Table 5.4). The mean value for the EEQ of Rhine water samples was in the same range as the mean value of the blank control (tap water). Both mean values are one order of magnitude above the LOQ of 0.020 ngl^{-1}.

In all Meuse samples, the EEQ was relatively high and ranged between 3.6 and 9.4 ng l^{-1}. The mean EEQ for the Meuse was slightly higher than the mean measured in the samples taken from the 5-ng l^{-1} EE2 positive control of the *in vivo* experiment. The EEQ's levels in the river water samples were relatively constant over time with a standard deviation of 18% (Meuse) to 36% (Rhine).

Table 5.4 The individual and mean estrogenic equivalencies (EEQs in ng l^{-1}) measured with the ER CALUX® assay for samples from surface water of the rivers Rhine and Meuse, the positive control ethynylestradiol (EE2, 5 ng l^{-1}) and tap water (blank control) tested in the *in vivo* experiment.

Date	Rhine	Meuse	EE2	Blank
3-Oct	0.08	6.90	nm	nm
6-Oct	0.20	5.00	nm	nm
8-Oct	0.08	3.60	2.70	0.32
10-Oct	0.25	5.30	nm	nm
13-Oct	0.23	4.80	nm	nm
15-Oct	0.24	3.80	6.90	0.47
17-Oct	0.27	4.10	nm	nm
20-Oct	0.30	7.10	nm	nm
21-Oct	-	9.40	nm	nm
22-Oct	0.27	6.80	nm	nm
23-Oct	-	-	4.10	0.10
24-Oct	0.36	3.90	nm	nm
27-Oct	0.19	6.10	nm	nm
29-Oct	0.25	7.70	4.00	0.09
31-Oct	0.12	-	nm	nm
4-Nov	-	-	3.30	0.06
Mean	0.22	5.73	4.20	0.21
SD	0.08	1.77	1.61	0.18

- = below the limit of quantification (0.02 ng l^{-1})
nm = not measured (no sample available)

Discussion

Already within 14 days of exposure the *in vivo* nonspawning male fish assay detected significant estrogenic activity in surface water samples of the Meuse and EE2-spiked water but not in surface water samples of the Rhine. The ER CALUX® assay confirmed these findings. This is one of the first attempts to examine surface water samples simultaneously in *in vitro* and *in vivo* laboratory assays to assess estrogenic activity. Filtration of the surface water samples excluded particulate and suspended matter as these were suspected to possibly contain adsorbed estrogenic compounds (49, 69). The experiment was aimed to assess the possible estrogenic activity exclusively present in the water phase of the river water in the context of its application as source of drinking water.

The present assay included different endpoints of which especially measurement of VTG in liver homogenates, but also, to a lesser extent, VTG mRNA and gonadal histology were sensitive enough to assess significant changes in fish exposed to Meuse water.

Chemical analyses on EE2 concentrations in the positive control were performed but failed to provide reliable values (results not shown), because the target concentration of 5 ng/ l was near the detection level of the method used. Still this concentration was applied as it approximated or was just one magnitude above the range of environmental concentrations detected in surface waters [25, 69].

Water quality was not always optimal, especially during the first week of exposure. However, this had no dramatic effect on any of the fish exposed, supported by the high survival rate (only 1 dead fish), the absence of visible effects on behavior and appearance and the constant body weights during the 28-days of exposure.

Mean body weights in the control vessels A and B were in the same range: 101 and 113% of the initial weight (4.32 g) on day 14 compared with 95 and 96% on day 28. Since fish were adults, they did not grow significantly during the test period. The HSI is frequently included in experiments with fish, as the condition of the liver is thought to be closely related to estrogen exposure, e.g. to substantial VTG synthesis [33, 133, 135]. However, increased HSI can also relate to exposure to other chemicals, e.g. P450 enzyme inducers also increase liver weight [136]. Although the HSI is a relative measure of liver and body weight, the sensitivity of this parameter is rather limited due to the relatively high variation between individual fish as seen in the present study after 14 and 28 days. The significantly higher means of HSI found in fish exposed to Meuse or Rhine water after 14 days of exposure did not correlate with the differences in VTG levels measured in the liver samples. Hence, the cause for higher HSI means in fish exposed to the river waters was probably not limited to estrogenic active compounds. Increased HSI values related to EE2 exposure were also reported in other studies [33, 133].

The GSI values in our study are in the range of the GSI values between 1 and 2% reported to be typical for adult male fathead minnows [58]. As for the HSI, the GSI varied considerably between fish with higher variations within vessels than between vessels. In our study, the average of the relative vessel-SEMs (n = 5) was 17% (9–21%), while in a study of Parrott et al. [33] with fathead minnow, this was 21% (13– 36%) with n varying between 9 to 35. Gimeno et al. [137] reported even higher CVs than observed in our study, i.e. up to 107% for GSI in unexposed male carp (n = 7 or 8). The high CVs for GSI makes this endpoint too insensitive to detect significant differences with sufficient power when not more than five fish are exposed per vessel. Although not statistically significant, exposure to EE2 reduced the average GSI by 31 and 47% in the two replicates, respectively. This is consistent with findings in other studies at higher concentrations of EE2 [138]. However, no apparent exposure related GSI differences were recorded for the river water treated groups, except a rather large difference between the vessel means of the Meuse group.

The major histopathological finding was eosinophilic blood plasma observed in slides originating from the fish exposed to EE2 or Meuse water. This feature has been reported earlier in fathead minnow [139], but also in carp [137], rainbowtrout [140], medaka [109], and zebrafish [81] exposed to estrogens like E2 or EE2. In some cases eosinophilic fluids were reported also in cavities between as well as within organs [108, 137] and this was also observed in fathead minnows exposed in an extended fish earlylife stage test to EE2 at 10 ng l^{-1} in our laboratory [113]. Some researchers attributed this response to estrogen related induction of VTG secretion, which accumulated in the blood and liver due to inadequate deposition in oocytes. Extensive appearance of eosinophilic fluids appears to be related to more chronic exposure of fish to estrogens [113].

Eosinophilic fluid or plasma was not present in fathead minnow after 14 days exposure to E2 [110]. However instead, these authors reported a dose dependent Sertoli cell proliferation and

degeneration. No such finding was recorded in the tissues of the fatheads exposed to 5 ng EE2 l^{-1} during 28 days in our study. The presence of eosinophilic plasma in the histological slides of fish exposed to Meuse or EE2 can be related to the relative high concentrations of VTG detected in their livers. Exposure to Meuse water induced elevated hepatic VTG levels in fish already within 14 days while livers of male fish exposed to Rhine water and the control fish completely lacked VTG. Induction of VTG synthesis in the liver or blood plasma of male fish is generally accepted as a sensitive indicator of estrogenic activity of industrial chemicals or pharmaceuticals [51, 53, 141] or effluents [135, 142, 143, 144].

The reference compound EE2 is a highly potent inducer of VTG synthesis in various fish species [53, 54, 55, 133]. In our study the EE2 concentration of nominal 5 ng l^{-1} (estimated actual value of 3.5±1.3 ng l^{-1}, see below) induced significant induction of VTG in the male fatheads exposed already after 14 days of exposure. Länge et al. [54] found a NOEC of 2.80±1.05 ng l^{-1} (nominal 4 ng l^{-1}) for VTG induction in their full life cycle test with fathead minnow exposed to EE2 and Panter et al. [53] found significant VTG induction in juvenile fathead minnow at levels down to 5±0.5 ng l^{-1}. Since VTG levels in all males of the control group remained below the lowest limit of quantification, this parameter is very sensitive.

The VTG mRNA assay confirmed the VTG induction results, including the high response in the one female fish exposed to Rhine water (excluded from calculations). However, the responses recorded after 14 days were not statistically significant when compared to the negative controls. Interestingly the VTG mRNA levels had increased from day 14 to day 28 in the Meuse water fish indicating an increase of the internal dose of the compounds responsible for the VTG-induction. This higher internal dose may reflect possible bioconcentration of estrogenic compounds or fluctuations in the concentrations of compounds in the river water samples. Detection of hepatic VTG mRNA levels in fish has been used before as a biomarker of estrogenic contamination of effluents [145] or surface waters [146]. Aravindakshan et al. [146] found a relation between hepatic VTG mRNA levels and spermatogenic staging in immature shiners caught in St. Lawrence River near the island of Montreal (Canada). Folmar et al. [147, 148] found clear concentration-response and time-response relationships for VTG levels in plasma of fish exposed to various estrogenic compounds. However, the induction of VTG mRNA was quite variable for the estrogens E2 and diethylstilbestrol (DES) with only EE2 showing a clear concentration-response relationship. In addition, none of the estrogens showed a clear time-response relation for VTG mRNA. Retention of VTG in the plasma is much longer than that of the VTG mRNA in the liver [149].

Hemmer et al. [150] found hepatic VTG mRNA rapidly diminishing after cessation of estrogenic exposure in sheepshead minnow, but plasma VTG clearance was at the level of days and appeared to be dependent on both concentration and exposure time. However, Brock and Shapiro [151] showed that the halflife of vitellogenin-mRNA increased from 16 h to 3 weeks in the presence of estrogen.

The ER CALUX® assay could be applied to quantify the estrogenic potency of the different media the fish were exposed to. Assuming that 1 unit EE2 equals 1.2 estradiol equivalents [69] the average measured EEQ of 4.2±1.6 ng l^{-1} (Table 5.4) corresponded with an average EE2

concentration of 3.5±1.3 ng l^{-1}, i.e. 70% of the target concentration. Recoveries of EE2 below nominal have also been reported by others, i.e. down to 40% of nominal [53, 145].

The ER CALUX® results also showed a stable and substantial estrogen activity in the river Meuse water over the three week period. An attempt was made to identify possible estrogenic compounds in the samples by chemical analyses. Detectable concentrations of EE2 were found in two out of three samples taken from the EE2 treated medium and in one river Meuse sample (results not shown). An estrone (E1) response was found in another sample of the river Meuse. However, the results were only indicative and not considered as the actual concentrations present in the samples. In their extensive project Vethaak et al. [25] found E1 more prominently present in the river Meuse (up to 4 ng l^{-1}) than in the river Rhine. Other steroids like E2 and EE2 were not detected except for E2 in the Meuse at Eijsden where the river enters the Netherlands. Concentrations of bisphenol-A were higher in the Rhine while more dimethyllpropylphtalate (DMPP) was found in the Meuse.

The ER CALUX® assay showed estrogenic activity in the samples from the drinking water control and the river Rhine above the LOQ, whereas no detectable estrogen related effects were recorded in the *in vivo* part of the studies. This response could be an indication of a false positive in the *in vitro* test or, a false negative in the *in vivo* test. Samples of normal drinking water originating from respective surface water sources did not induce significant responses above the detection limit (unpublished data provided by KIWA). The slightly positive ER CALUX® responses in the blank control could be caused by a possible contamination with xeno-estrogens from the experimental set-up including the flow-through system. However, the significant response in the river Rhine samples was in the same range as in the blank control and these samples did not originate from the flow-through system, but from separate samples from the sampling location. The lack of any response in the *in vivo* experiment for the blank control and Rhine samples might relate to the fact that the ER CALUX® extracts were prepared on 0.45 µMfiltered samples, whereas the fish were exposed to river Rhine water filtered through 0.2 µM filters. This difference in type of filters used was discovered only after the *in vitro* and *in vivo* studies had been completed, due to the fact that they were performed at different laboratories. However, this difference was not relevant for the blank control samples (not filtered in the *in vivo* test) and had no effect on the corresponding responses between the *in vitro* and *in vivo* test for the Meuse samples. More likely, the significant responses in the blank control and Rhine samples could be a result of the extraction step applied in the *in vitro* test. The extraction of relatively large water samples in small volumes of organic solvents may concentrate traces of organic compounds present in these samples inducing a response in the *in vitro* test. The fact that the responses of the blank control and Rhine samples were in the same range in the *in vitro* test, indicate that these were not related to estrogenic compounds but were probably false positives.

The average EEQ-levels for the Meuse water and EE2 positive control determined by the ER CALUX® were comparable (Table 5.4) while the hepatic VTG-induction in the EE2-exposed fish was about 10-fold higher (Figure 5.2). This is probably related to the fact that EE2 is several magnitudes more potent in fish compared to *in vitro* assays with mammalian cells and to other estrogens like E2 and E1 [69], which are believed to be the main estrogens present in the Meuse

water [25]. Further Legler et al. [79] reported that relatively stable and accumulating compounds such as EE2 and o,p′-DDT were more estrogenic *in vivo* than *in vitro*, while on the other hand NP and BPA were more estrogenic *in vitro* than *in vivo*.

The positive result for estrogenic activity found in both the *in vitro* and *in vitro* test impelled KIWA to perform further research in the ER CALUX® assay on drinking water samples origination from the Meuse location. These results were not included in this publication as they fell outside the scope of the study, but no response was found in any of the samples tested. Hence, it could be safely excluded that there is any significant leaking of the estrogenic potential ofMeusewater in the resulting drinking water.

The potential of the Meuse water samples to significantly induce VTG production in male fish reached levels comparable to an EE2 level of 5 ng l^{-1} within a relatively short exposure period of 28 days. Although this could indicate a possible risk for fish in the Meuse, the results of the present study did not show adverse effects on gonadal tissue after 28 days other than those related to increased amounts of extra-cellular VTG levels. Induction of elevated VTG levels in male fish, or even the induction of testisova does not automatically relate to a decrease in fertility or fecundity of the adult fish exposed [56].

Conclusion

Among the various endpoints examined, VTG-protein induction proves to be the most sensitive and robust parameter in a Non Spawning Male Fish Assay requiring no more than 14 days of exposure to detect significant estrogenic activity in surface water samples. The other endpoints, including nuptial tubercles, HSI and GSI were less sensitive even after 28 days. Further, a high control variability of HSI and GSI makes that these endpoints require a higher number of fish or replicates to achieve sufficient power for statistical testing. As a result, measurement of VTG-protein induction shortens the exposure period to 14 days without loss of information. This makes the Non Spawning Male Fish Vitellogenin Assay an important candidate as an *in vivo* short term (14 days) screening test for the detection or confirmation of estrogenic active substances or estrogenic potential of effluents and surface waters, by itself or in combination with *in vitro* assays like the ER CALUX® assay. Although the ER CALUX® assay has already proven its high sensitivity and ability to detect estrogen receptor related responses, a sensitive *in vivo* assay should be added in testing programs to avoid false positives or negatives based on the results of the *in vitro* test alone.

The Non Spawning Male Fish Vitellogenin Assay has also potential as a reliable assay to address effluents or surface water sources as low estrogenically active. Finally, the ecological consequences of the current findings need to be studied further in chronic (full life cycle) tests with exposure to EEQ-levels in river water.

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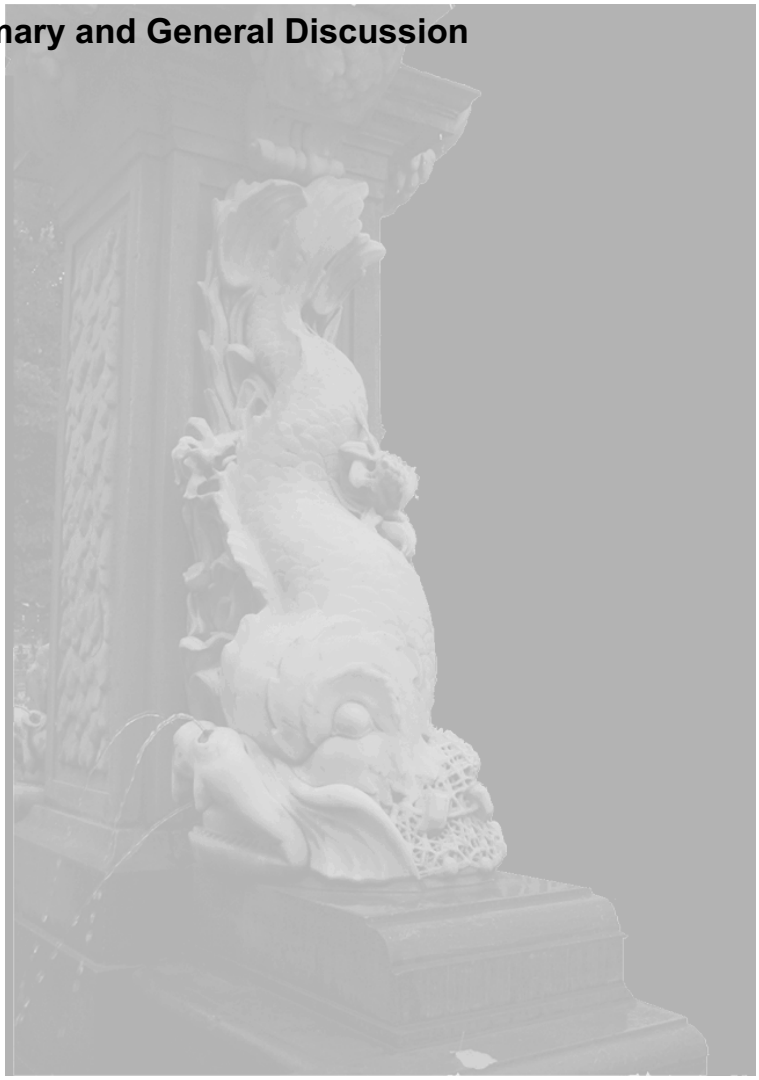
Chapter 6

Summary and General Discussion



As outlined in Chapter 1 of this thesis, endocrine disrupting compounds (EDCs) dispersed in the environment are associated with a global decrease in male or female reproductive capacity in both humans and wildlife. Effects observed in wildlife may provide early warnings of effects produced by EDCs, which may as yet be unobserved in humans. In addition, wildlife should be protected in its own right. In various countries, fish exposed to sewage treatment discharges show sex related anomalies due to the presence of EDCs such as steroidal estrogens and chemical substances in the sewage effluent [22, 26, 27, 28]. As a result, legislation had been developed both nationally and internationally to provide the tools for identification and assessment of endocrine disrupters. In first instance, several *in vitro* bioassays were developed that were useful as tools to prioritize chemicals or environmental matrices for more extensive *in vivo* studies. It was outlined in Chapter 1 that *in vitro* bioassays for detecting EDCs do not account for interactions of the EDCs with the various processes related to the hypothalamus-pituitary-gonadal axis, gonadal maturation, synthesis of sex steroids and *in vivo* processes like bioconcentration, bioactivation and metabolism. Therefore, testing should eventually include *in vivo* verification of potential effects and as such, *in vivo* tests are essential for further screening of potential EDCs. At present, many different *in vivo* assays with different fish species as the test organism and a wide range of possible endpoints are being developed. This raises the need for more insight into the relative sensitivity and applicability of these different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals. Such insight, would add considerably to the definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs. Therefore, the aim of the present thesis was to investigate the relative sensitivity and applicability of different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals, in order to contribute to the definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs. To this end, in the different experimental chapters of this thesis, different endpoints for testing EDCs in *in vivo* experiments with fish were investigated and compared, ultimately providing an overview of their promises and pitfalls.

The study described in Chapter 2 incorporated specific endpoints for estrogenic activity, namely vitellogenin (VTG) levels, as an extra parameter in an early life stage (ELS) test carried out as described in the guideline 210 of the Organisation for Economic Co-operation and Development (OECD) and traditionally used for toxicity screening of chemicals. The sensitivity of this classical endpoint was compared to that of a newly developed endpoint based on the use of a transgenic zebrafish model expressing an estrogen receptor-mediated luciferase reporter gene. The transgenic zebrafish were exposed to ethinylestradiol (EE2), and luciferase activity as well as VTG was measured. Concentrations of EE2 were tested at 1, 3 or 10 ng $l^{-1}$for 30 d from fertilization or during only the last 4 d with dimethylsulphoxide (DMSO) as pre-solvent (0.01 %). Exposure to EE2 induced no toxic effects. Mean body weights were significantly higher in groups exposed for 30 d in the presence of DMSO, but condition factors were not affected. . Significant luciferase and VTG induction occurred at 10 ng EE2 l^{-1} following the 4 and 30-d exposure, while only VTG levels were affected at 3 ng EE2 l^{-1} in the 30-d exposure. This study demonstrated the usefulness of incorporating estrogenic endpoints in the OECD ELS test, fitting the requirements for screening

estrogenic activity of chemicals. Quantitative measurement of both VTG and luciferase activity proved to be rapid and sensitive. Additional value of using transgenic zebra fish lies in combining VTG measurement with the more mechanistic approach of tissue-specific luciferase induction in one experiment, although the VTG endpoint appeared to be more sensitive with longer exposure durations.

In addition to extending the OECD test guideline 210 by adding an extra parameter like VTG levels as done in Chapter 2, the Endocrine Modulators Study Group (EMSG) of the European Chemical Industry has proposed to develop an extended fish ELS test based on OECD test guideline 210 and combining it with a short-term fish pair-breeding reproduction study as a possible alternative for fish full life cycle testing. In this case, parameters quantifying effects on early life stages as well as fish pair breeding success could be used as endpoints for evaluating endocrine disrupting activity. This approach and these possible endpoints were investigated in Chapters 3 and 4 of the thesis, respectively. In Chapter 3, the testing is described of the androgen methyldihydrotestosterone (MDHT) in an extended ELS test with fathead minnow supplemental to the weak estrogen 4-*tert*-pentylphenol (4TPP). Main endpoints affected were secondary sexual characteristics (SSC), plasma VTG induction and gonadal development. Early blastula embryos were exposed to 0, 0.10, 0.32 and 1.0 μg MDHT l^{-1} for up to 114 days post-hatch (dph). A batch of fish exposed to 1.0 μg l^{-1} was transferred to clean water after 30 or 63 dph for the remainder of the study. EE2 was included as estrogenic reference substance at 0.01 μg l^{-1}. Exposure to MDHT had no significant effect on hatching success or survival, but significantly increased the condition factor of fish exposed for 63 and 114 dph (up to 150% of the control). At 63 dph, MDHT exposure induced appearance of tubercles on the snout (a male SSC) of more than 80% of fish. Compared to the controls, plasma VTG was not detectable or significantly lower in fish exposed to MDHT at 0.10 μg l^{-1}, but not significantly affected at higher MDHT concentrations. Both lower levels of MDHT significantly inhibited the development of female gonads as of 30 dph. Fish exposed to MDHT at 0.32 and 1.0 μg l^{-1} showed higher incidences of mixed sex gonads (10–25%) and smaller testes or dysplasia of gonadal tissue. Dysplasia was present in 80% of the fish continuously exposed to 1.0μg l^{-1} up to 114 dph, but reversible when fish were transferred to dilution water after 30 dph and 63 dph, respectively. Results indicate that suppression of ovarian development was the most sensitive endpoint for MDHT exposure after 30 dph. Other endpoints (e.g., growth and SSC) required exposure during at least up to 63 dph to yield a significant effect. Androgenic effects on VTG production required even longer exposure, i.e. until sufficient numbers of females had matured. Thus, while VTG induction appeared to be a very sensitive endpoint in a study with an estrogen as described in Chapter 2, the results presented in Chapter 3 show that the additional gonadal histology was a more sensitive endpoint in the case of androgen exposure of fish.

Given the sensitivity of the endpoints used in the extended ELS test described in Chapter 3, the studies described in Chapter 4 were undertaken to investigate endpoints based on pair-breeding reproduction studies with the same species of fish. The results of two fish pair breeding reproduction studies with MDHT are presented. The reproductive performance of breeding pairs of fathead minnows was examined during exposure to nominal MDHT concentrations 0.1, 0.32, 1 and 10 μg l^{-1} for 21 or 28 days. Other endpoints examined were somatic growth, the prominence of

nuptial tubercles (male related) and VTG induction (female related). EE2 was included as an estrogenic reference substance at 0.01µg l^{-1}. The prominence of the nuptial tubercles was significantly higher in males exposed to 0.1 and 1 µg l^{-1} compared to the solvent-control (SC). MDHT exposure did not significantly affect VTG induction in either males or females, whereas EE2 significantly induced VTG in males and females. Gonadal histology showed testis with enlarged seminiferous tubuli completely filled with sperm combined with a thin germinal epithelium in males exposed to 0.1 and 0.32 µg MDHT l^{-1} and significant oocyte atresia in females exposed to ≥ 0.1 µg MDHT l^{-1}. Relatively high variations of up to 90 % in egg production hampered statistical analysis of differences between experimental groups. During the experimental periods, the pair-bound reproduction in the SC decreased with time. A significant decrease in fecundity was observed in fathead minnow pairs exposed to ≥ 1 µg MDHT l^{-1} when compared with the SC. Comparison of the survival and hatching of offspring (F1) between treatments was hampered by a high variation in egg production in the SC and complete cessation of reproduction in most MDHT treated pairs. Evaluation of trans-generational effects was compromised by the fact that insufficient numbers of F1 batches were available for testing. Altogether, the results presented in Chapter 4 reveal that, before being acceptable as a robust test method, the reliability, inter-laboratorial repeatability and consistency of fecundity in fish pair breeding reproduction studies should be improved. In spite of the advantages of pair-wise reproduction, the results presented here in fact indicate that this method and the related endpoints do not provide the best standard method for testing endocrine disruption in fathead minnow.

In an additional study, presented in Chapter 5, the usefulness of fathead minnow in the Non Spawning Male Fish Assay was investigated with respect to the possible endpoints in this fish model for testing endocrine disruption. Based on the earlier results presented in Chapter 2, VTG was considered to be a sensitive endpoint in a test with exclusively male fish. Furthermore, VTG mRNA levels were examined as an additional endpoint and compared with VTG protein induction. The experimental setup also included the endpoint of nuptial tubercles development tested in Chapter 3 and 4, which appeared to be a sensitive sex-related characteristic of fathead minnow. Adult male fathead minnow were exposed for 14 or 28-days under flow-through conditions to undiluted filtered water samples from the rivers Meuse and Rhine in the Netherlands. The experiment included two vessels per treatment each containing 10 fish and samples of five fish were taken after 14 and 28 days. Additional groups were exposed to EE2 (5 ng l^{-1}) as a reference and untreated drinking water as a negative control. Additional major endpoints examined were hepato- and gonadosomatic indices (HSI and GSI) and gonadal histology. No significant differences were recorded in body weight or mean GSI values between the various treatments. Only exposure to Meuse water resulted in significantly higher HSI means after 14 days.

Histological examination showed no apparent effects on gonadal tissue. Effects were found only on eosinophilic blood plasma in fish exposed to Meuse water or EE2. After 14 and 28 days, elevated VTG and VTG mRNA levels were measured in most livers of the fish exposed to Meuse water, but not in the fish exposed to Rhine water.

VTG protein levels appeared to be a more sensitive endpoint than VTG mRNA activity, as the VTG-mRNA responses recorded after 14 days did not significantly differ from controls. The

estrogenic activity in Meuse water was confirmed by measuring estrogenic responses in extracts of river water in the *in vitro* ER CALUX assay. Induction of VTG synthesis proved to be the most sensitive endpoint in the Non Spawning Male Fish Assay for *in vivo* detection of bio-available estrogenic activity supplementary to a sensitive *in vitro* assay. The other endpoints examined varied too much and required a higher number of fish or replicates to achieve sufficient power for statistical testing making them less animal friendly. Altogether the data presented in Chapter 5 confirm the sensitivity of histopathological markers and VTG as appropriate endpoints in *in vivo* studies for EDC activity.

Table 6.1 Summary of all endpoints (markers) examined in the various chapters of this thesis

	Chapter				Evaluation
Endpoints or markers	2	3	4	5	
Vitellogenin (VTG)	X	X	X	X	Sensitive endpoint in various species for estrogenicity; easy to apply but hardly applicable in studies with exposure to an androgen
Vitellogenin messenger RNA (VTGmRNA)				X	Less sensitive than VTG
Behaviour & appearance		X	X		Responsive endpoints especially upon exposure to androgens
Secundary sexual characteristics (SSC)		X	X	X	Sensitive endpoint for both estrogenic and androgenic exposure but less sensitive than VTG for estrogenic exposure
Body weight-length-condition factor	X	X	X	X	Generally less sensitive than other endpoints studied
Hepatic somatic weight (HSI)				X	Non-sensitive endpoint due to high intra-individual variation and less specific for assessment of endocrine disruption
Gonadal somatic weight (GSI)				X	Non-sensitive endpoint due to high intra-individual variation
Gonadal histology		X	X	X	Highly sensitive endpoint in various species, but requires expert knowledge and effects can be reversible
Peritoneal attachment of gonads		X			Highly sensitive irreversible endpoint, and requires no real expert knowledge
Luciferase induction in transgenic zebrafish	X				Moderately sensitive endpoint, only in selective transgenic species. Allows for evaluation of estrogenic activity in specific tissues. Suitable for mechanistic studies and screening with short-term exposure periods.
Fecundity (egg & spawn production)			X		Non reproducible endpoint in fathead minnow; remains to be investigated in further detail, also in other species
Egg-survival	X	X	X		Non-sensitive endpoint in comparison to the others.
Hatching	X	X	X		Non-sensitive endpoint in comparison to the others.
Overall survival	X	X	X		Non-sensitive endpoint in comparison to the others.
Sex-ratio		X			Moderately sensitive endpoint but can be affected by an uneven sex-ratio in the control group

Table 6.1 presents an overview of the different endpoints thus investigated in the various chapters also providing an evaluation of their relative sensitivity. VTG production was examined in transgenic and wild-type zebrafish (Chapter 2), during the early-life stages until adulthood in fathead minnow (Chapter 3) and in adult fathead minnow (Chapter 4 and 5). In every case VTG induction proved to be a sensitive marker for estrogenic exposure (Chapters 2 and 5), but

appeared to be hardly indicative in studies with exposure to an androgen (Chapters 3 and 4). It should be mentioned that the androgen tested in Chapters 3 and 4 was non-aromatizable, so it is not surprising that VTG was not highly induced. The VTG mRNA assay was less sensitive compared to VTG induction as it needed a longer exposure period before it was affected significantly.

Behaviour and appearance were responsive markers during exposure to the androgen MDHT. In the extended early life stage test (Chapter 3), both features were dramatically affected, especially at the higher concentrations.

The SSCs proved to be a sensitive marker for both estrogenic and androgenic exposure in fathead minnow (Chapters 3 and 4). The development of nuptial tubercles in female fathead minnow or the decline of nuptial tubercles in male fish is a species-specific marker, which responds rather sensitively to either estrogenic or androgenic exposure. However, it appeared to be less sensitive compared to VTG induction in the Non Spawning Male Fish Assay (Chapter 5).

The somatic endpoints of body weight, -length and the condition factor were also evaluated in all studies. Body weight appeared to be a responsive marker especially in the extended early life stage study (Chapter 3), which could be related to the masculinizing effects of prolonged exposure of the young developing fish to MDHT. However, in general these markers proved to be much less sensitive compared to the others.

The relative weights of liver and gonads (HSI and GSI) showed high variation between individual fish. This hampered statistical analysis of these data. Significant differences in HSI were found only in incidental cases (Chapter 5). However, due to the less specific character of this marker, it was not possible to relate this feature to possible endocrine effects.

Gonadal histology proved to be an important endpoint in combination with the other sensitive endpoints. The anomalies observed in the fish gonads reached relatively high incidences at the higher MDHT concentrations, but they proved to be reversible except for peritoneal attachment of gonads. The latter appeared to be a powerful marker in the measure of agreement between gonad observation on single, double attachments or non-assessable attachments and classifying the fish as male (testis), female (ovaria) or indeterminate (either mixed gonads or undifferentiated). Hence, the effect on this endpoint appeared to be irreversible, while the effects on most other endpoints were reversible.

In Chapter 2, the more specific mechanism of luciferase induction triggered by activation by the respective chemical to the estrogen receptor was examined in transgenic zebra fish in comparison to VTG production . Although VTG production was a more sensitive marker during prolonged 30-d exposure, a 4-day exposure during the last larval stages of the zebra fish was sufficient for causing significant effects on both endpoints in one of the dosages applied. Hence, the transgenic zebra fish model can be used to examine whether VTG induction is related with binding of the respective chemical to the estrogen receptor. This model appears to be particularly useful for screening of chemicals with short-term exposure periods.

The endpoints related to fecundity in pair-wise reproduction studies (Chapter 4), e.g. egg production and the number of spawns, showed high variation up to 90 %, thus hampering powerful statistical analysis. Further, significant numbers of pairs failed to produce any broods already

during pre-exposure in clean water. It was therefore concluded that there is still a need for increasing the reliability, inter-laboratorial repeatability and consistency of the endpoint of fecundity when fathead minnow is to be used as a standard species in pair-wise reproduction tests.

Egg-survival and hatching success were less sensitive markers, as they were not significantly affected in both the extended early life stage test and the reproduction tests. Sex-ratio could only be evaluated successfully in the extended early life stage test, although incidentally, the ratio was biased to females in the control group. Sex-ratio was based on gonadal histology since genotypic identification of the sexes could not be performed. MDHT exposure during at least 30 dph induced enhanced testes development in fish that probably would have become normal males without further exposure and induced development of testes in fish that probably would have developed into normal females. Sex-ratio proved to be a sensitive marker showing statistically significant differences at the lowest concentration of 0.1 $\mu g\ l^{-1}$. However, it was not significantly affected at the highest concentration of 1 $\mu g\ l^{-1}$. This marker should therefore be interpreted with caution and should be always evaluated at different test concentrations and in combination with other markers.

Altogether, the overview presented in Table 6.1 provides insight in the relative sensitivity and applicability of different endpoints in fish for *in vivo* screening of endocrine disrupting activity of chemicals, thereby contributing to the definition of a better methodology for using fish as a test organism to assess the potential endocrine disruptive activity of environmental EDCs.

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Samenvatting voor niet-vakgenoten

Iedereen heeft wel eens een artikel in de krant of een nieuwsbericht op tv gezien waarin gewag gemaakt werd van stoffen in het milieu die de vruchtbaarheid van mens en dier zouden bedreigen. Epidemiologische onderzoeken naar de vruchtbaarheid van mannen in de Westerse samenleving gaven in een aantal gevallen een in de tijd verminderde vruchtbaarheid te zien. Onderzoeken in het milieu om ons heen toonden aan dat bijvoorbeeld vissen erg gevoelig zijn voor effecten van stoffen op de geslachtsontwikkeling. Er werden mannelijke vissen gevangen met vrouwelijke eicellen in hun geslachtsorganen en vrouwelijke vissen die de uiterlijke kenmerken van hun mannelijke soortgenoten vertoonden. Dergelijke ontdekkingen werden meestal gedaan in de buurt van bepaalde industrieën, zoals de papierindustrie, en in kleinere rivieren waarin water van stedelijke waterzuiveringsinstallaties terecht kwam. Landelijke onderzoeken toonden onder meer aan dat vooral natuurlijke oestrogenen, zoals die uit de mest van koeien, en kunstmatige oestrogenen, met name die afkomstig uit de pil, in het milieu aanwezig zijn. Verder onderzoek toonde aan dat vissen heel gevoelig zijn voor ethinylestradiol (EE2), een synthetisch oestrogen uit de pil dat via de urine en ontlasting van pilgebruikende vrouwen uiteindelijk in het milieu terechtkomt als het bij de waterzuivering onvoldoende wordt verwijderd. EE2 blijkt ongeveer 100 keer effectiever in vissen dan het natuurlijke estradiol (E2) omdat het in de vis minder snel wordt afgebroken. Zeer lage concentraties tot 10 nano (10^{-9} !) gram per liter bleken al effect te hebben op mannelijke vissen.

Andere door de mens gemaakte chemische stoffen die uiteindelijk in het milieu terechtkomen, blijken soms ook hormoonverstorende effecten op vissen en andere dieren te geven. Zo is er de groep van alkylfenolen (AP's) die in de industrie worden gemaakt om alkylfenolethoxylaten (APE's) te produceren. Nonylfenolethoxylaten bijvoorbeeld, werden op grote schaal toegepast als toevoeging in kunststoffen en als oppervlakte-actieve stoffen in industriële reinigingsmiddelen voor textiel- en tapijtreiniging en als emulgeermiddelen in oplosmiddelen. Diverse onderzoeken hebben aangetoond dat AP's de werking van natuurlijke oestrogene hormonen kunnen nabootsen. De gevoeligheid van vissen voor deze stoffen is duidelijk minder dan die voor het natuurlijke estradiol, maar in het milieu zijn concentraties van AP's gevonden die in het laboratorium significante effecten op de geslachtsontwikkeling in vissen gaven. In Europa zijn intussen de meeste toepassingen van APE's vervangen door alcoholethoxylaten, die minder milieuvervuilend lijken te zijn.

Al deze bevindingen hebben de autoriteiten en overheden in diverse landen ertoe aangezet de regelgeving voor hormoonactieve stoffen aan te passen en nieuwe richtlijnen hiervoor te ontwikkelen. Om in de toekomst chemische stoffen op hun eventueel hormoonactieve potentie te onderzoeken, zijn aangepaste testsystemen en –protocollen nodig. De belangrijkste aanpassingen in bestaande protocollen betreffen vooral de zogenaamde eindpunten of 'markers' die in de testen moeten worden bestudeerd. Eindpunten zijn die onderdelen van het onderzoek waarvan de resultaten bepalend zijn voor de uiteindelijke conclusies van dat onderzoek, omdat ze een bepaald effect of mechanisme voorspellen, b.v. hormonale verstoring. Daarbij wordt meestal een onbehandelde controlegroep meegenomen in het onderzoek, waarmee de resultaten van de

behandelde groepen kunnen worden vergeleken. Als voorbeeld: een belangrijk eindpunt bij kankeronderzoek in ratten is de hoeveelheid tumoren die ratten ontwikkelen gedurende een bepaalde periode van hun leven. De aantallen tumoren geteld in groepen die aan een bepaalde stof zijn blootgesteld, worden statistisch vergeleken met de aantallen geteld in de controlegroep. Statistisch significante verschillen in aantallen tumoren worden dan toegeschreven aan de blootstelling aan de stof omdat dit het enige verschil is tussen de blootgestelde groepen en de controlegroep.

Het doel van het onderzoek in dit proefschrift was het bepalen van de gevoeligheid en bruikbaarheid van verschillende 'markers' voor onderzoek naar de hormoonverstorende potentie van chemische stoffen in toxiciteitstesten met de vis als testorganisme. De testen die kandidaat zijn voor onderzoek naar hormoonverstorende werking van stoffen zijn gebaseerd op celkweken (*in vitro*) of worden uitgevoerd in levende organismen (*in vivo*). Hoewel er intussen bruikbare *in vitro* testsystemen zijn ontwikkeld, is de relevantie hiervan beperkt omdat de regulering van de geslachtsontwikkeling in het levende organisme complex is en uiteenlopende organen hierbij betrokken zijn, zoals de hypothalamus (onderin de hersenen), de daarmee verbonden hypofyse en de lever (zie hoofdstuk 1). Daarom zijn *in vivo*-testen onvermijdelijk. De vis is een voor onderzoek van milieueffecten een veel gebruikt testorganisme.

Voor het testen van de hormoonontregelende eigenschappen van chemische stoffen in vissen zijn diverse eindpunten mogelijk. In tabel 8.1 zijn deze eindpunten gegeven met verwijzing naar de hoofdstukken van het proefschrift waarin ze zijn onderzocht.

Tabel 8.1 De eindpunten onderzocht in de diverse hoofdstukken van dit proefschrift.

Eindpunten of markers	Hoofdstuk			
	2	3	4	5
Vitellogenine (VTG)	X	X	X	X
VTGmRNA				X
Gedrag & uiterlijk		X	X	
Uiterlijke sexuele kenmerken		X	X	X
Lichaamsgewicht, -lengte en conditiefactor	X	X	X	X
Relatief levergewicht (HSI)				X
Relatief gonadengewicht (GSI)				X
Histologie van de gonaden		X	X	X
Aanhechting van de gonaden aan het buikvlies		X		
Luciferase inductie in de transgene zebravis	X			
Vruchtbaarheid (productie van ei en broedsel)			X	
Overleving van eieren	X	X	X	
Uitkomen van eieren	X	X	X	
Algehele overleving	X	X	X	
Sex-ratio		X		

Vitellogenine (VTG) is een door vrouwelijke vissen geproduceerd eiwit dat wordt aangemaakt in de lever en dat via het bloed wordt getransporteerd naar de ovaria. Daar wordt het opgenomen in de groeiende eicellen als voedsel voor de zich in het eitje ontwikkelende larve. Onder invloed

van oestrogeen werkende stoffen wordt de lever aangezet tot het aanmaken van VTG. Indien mannelijke vissen aan oestrogene stoffen worden blootgesteld gaat hun lever ook VTG aanmaken. Daarom is dit een bruikbaar eindpunt voor onderzoek naar de mogelijke oestrogene activiteit van chemische stoffen. VTG-productie wordt echter tevens beïnvloed door de complexe processen via de hypothalamus, de daarmee verbonden hypofyse, de lever en de geslachtsorganen. Het is daardoor minder specifiek voor de identificatie van daadwerkelijke binding van stoffen aan de oestrogen receptor in levercellen. In hoofdstuk 2 is daarom de gevoeligheid van VTG als 'marker'vergeleken met die van een specifiek eindpunt in transgene zebravissen. Dit specifieke eindpunt is de meting van luciferase[3] dat wordt geproduceerd in vissencellen waar een stukje extra DNA kunstmatig is ingebracht. Dit stukje DNA wordt geactiveerd na binding van een stof aan de oestrogen receptor, die zich in de cel bevindt. Dus alleen stoffen die ook daadwerkelijk aan die receptoren binden en deze activeren, zetten de cellen aan tot productie van luciferase. In hoofdstuk 2 werden normale en transgene vissen blootgesteld aan EE2 met de bedoeling een duidelijke reactie te induceren van de productie van zowel het VTG in beide type vissen als de luciferase in transgene vissen. Daar de vissen in de eerste fasen van hun leven extra gevoelig zijn voor hormoonverstorende stoffen, werden ze blootgesteld vanaf het eistadium tot 30 dagen na het uitkomen van de eitjes. Daarnaast werden andere visjes pas blootgesteld aan EE2 vanaf dag 26 tot dag 30 na het uitkomen van de eitjes. Het eindpunt VTG bleek gevoeliger dan luciferasemeting met name in de visjes die tot 30 dagen na het uitkomen van de eitjes waren blootgesteld. Dit kwam waarschijnlijk doordat luciferase sneller wordt afgebroken in het lichaam van de vis dan vitellogenine. Dit blijkt ook uit het resultaat dat er geen verschil in gevoeligheid tussen deze twee markers werd gevonden bij kortstondige blootstelling (4 dagen).

In hoofdstuk 3 werden de eindpunten voor het testen van een hormoonontregelende stof uitgebreid met o.a. de histologie van de geslachtsorganen (gonaden) en werden uiterlijke kenmerken gekoppeld aan het geslacht van de vis. De vissoort die hiervoor gebruikt werd is de Amerikaanse dikkop-elrits die ook in België voorkomt en daar ook wel "mona-lisa" wordt genoemd. De mannetjes van deze vissoort ontwikkelen een karakteristieke donker gekleurde bult boven op de kop en knobbels op de snuit. Als teststof werd methyldihydrotestosteron (MDHT) gebruikt, een kunstmatig hormoon dat onder meer gebruikt wordt door bodybuilders als spierontwikkelend middel. Het voordeel van MDHT is dat het niet gemakkelijk kan worden omgezet of afgebroken in een levend organisme. Natuurlijk testosteron kan in het organisme omgezet worden in het vrouwelijke estradiol waardoor waarneming van effecten van specifiek testosteron bemoeilijkt wordt. Ook hier werden vissen blootgesteld vanaf de eifase tot het jong volwassen stadium. Enkele groepen werden na een bepaalde periode van blootstelling naar onbehandeld water overgebracht teneinde te onderzoeken of mogelijk waar te nemen effecten al dan niet omkeerbaar zouden kunnen zijn. Bij alle geteste concentraties MDHT (0.10, 0.32 and 1.0 micro (10^{-3}) gram per liter) werden effecten waargenomen. Vrouwelijke vissen ontwikkelden de typisch mannelijke knobbels op de snuit en microscopisch onderzoek van de mannelijke en vrouwelijke

[3] Luciferase is een algemene naam voor een klasse enzymen die in de natuur voor een lichtreactie zorgen door het versnellen van de oxydatie van luciferinen waardoor de zogenaamde bioluminescentie ontstaat die voorkomt in bijvoorbeeld vuurvliegjes, glimwormen en de lantaarnzwam.

geslachtsorganen gaf duidelijke afwijkingen in uiterlijk en ontwikkeling te zien. Een belangrijke verandering betrof de aanhechting van de gonaden aan het buikvlies. Bij mannelijke vissen ontwikkelt deze van een dubbele naar een enkelvoudige aanhechting. Echter in mannelijke vissen, blootgesteld aan MDHT tijdens de vroege ontwikkelingsfasen,vond die verandering niet plaats en dit effect bleek onomkeerbaar. De effecten op de meeste andere eindpunten bleken wel weer te verdwijnen na overplaatsing in onbehandeld water . VTG productie bleek als eindpunt pas zinvol tegen de tijd dat vissen volwassen waren, in deze opzet het eind van het experiment.

Hoewel de effecten op VTG productie, uiterlijke geslachtskenmerken en geslachtsorganen duiden op een mogelijk hormoonderegulerende werking van een stof is daarmee nog niet aangetoond dat dit ook uiteindelijk effecten heeft op de voortplanting. Daarom zijn in hoofdstuk 4 studies gedaan waarbij de effecten van stoffen op de daadwerkelijke productie van eieren zijn onderzocht. De dikkop-elrits-vissen die gebruikt werden bleken echter minder geschikt voor dergelijk onderzoek. De vissen werden paarsgewijs getest, dus één mannetje met één vrouwtje in hetzelfde aquarium. Hierdoor werd het mogelijk per paar de eiproductie tijdens de behandeling te vergelijken met die voor de behandeling.

Om een beeld te krijgen van de methode van testen volgt hier een korte beschrijving ervan. In het aquarium wordt een kunstmatig substraat in de vorm van een in de lengte doorgezaagde stuk pvc-buis gebracht. Het mannetje probeert vervolgens het vrouwtje binnen die buis te lokken en door heftig tegen haar lijf te drukken probeert hij de eitjes uit haar lichaam te “kloppen”. Doordat die eitjes erg plakkerig zijn blijven die aan de pvc-buis vastkleven. Het mannetje jaagt daarna het vrouwtje de buis uit en bevrucht de eitjes met zijn sperma. De buis kan vervolgens uit het aquarium gehaald worden en de eitjes geteld. Verder kunnen die eitjes ook weer uitgezet worden om te zien of blootstelling van de ouderdieren een effect heeft op de jonge visjes. In een aantal gevallen bleek echter dat de vrouwtjes niet in staat waren regelmatig voldoende eitjes te produceren. Soms werd, zelfs na een week of langer, geen enkel eitje gevonden. Hierdoor moesten aan het begin van de proef beduidend meer paren worden uitgezet dan uiteindelijk in de daadwerkelijke test nodig waren.

In de test bleek dat MDHT een versterkend effect had op de agressiviteit van de mannelijke vissen waardoor de vrouwtjes soms beschadigd werden of zelfs dood gingen. Tevens bleek dat de eiproductie ook bij onbehandelde dieren afnam in de tijd en de variatie in de aantallen geproduceerde eitjes zo groot was, dat het niet mogelijk was om goede conclusies te trekken over het effect van MDHT. Wel bleken de hogere concentraties MDHT de productie van eitjes al na de eerste week sterk te verminderen, eerder dus dan bij de controle dieren. Het was oorspronkelijk de bedoeling om de eitjes van alle dieren te laten opgroeien in een nieuw aquarium om te zien of blootstelling van de ouderdieren een effect had op de jonge visjes. Dit bleek echter niet mogelijk vanwege de grote onderlinge verschillen in ei-productie en het uitblijven van eitjes in de hoge dosis groepen. Geconcludeerd werd dat het protocol voor paarsgewijs testen van de voortplanting van de dikkop-elrits herziening behoeft en dat het mogelijk beter is over te stappen naar een opzet waarin één mannetje met meerdere vrouwtjes wordt uitgezet.

Zoals eerder aangegeven blijkt VTG productie in met name mannelijke vissen een gevoelige ‘marker’ voor blootstelling aan oestrogeen-actieve stoffen. In hoofdstuk 5 wordt onderzocht in

hoeverre deze 'marker' geschikt is om eventuele oestrogene activiteit in rivierwater te kunnen detecteren, en de mate van gevoeligheid in vergelijking met een specifiek daarvoor ontwikkelde *in vitro* test. De 'marker' in deze *in vitro* test is luciferaseproductie zoals hierboven beschreven voor transgene vissen, maar dan in gekweekte humane cellen (ER-CALUX assay). Voor het *in vivo* onderzoek werden mannelijke dikkop-elritsen gehouden in een doorstroomsysteem met Maas- of Rijnwater dat vooraf was gefiltreerd om de fijne zwevende deeltjes te verwijderen. De controlegroep werd gehouden in drinkwater en een positieve controlegroep werd blootgesteld aan 5 nanogram EE2 per liter water. Een dergelijke concentratie benadert die welke in rivierwater is aangetoond. Zoals beschreven in hoofdstuk 5 bleken de mannelijke vissen die waren blootgesteld aan Maaswater of aan EE2 sterk verhoogde VTG gehalten te bevatten, terwijl de VTG gehalten in de Rijnwater vissen en de controlegroep onder de detektiegrens bleven. De juistheid van deze resultaten werd bevestigd door de *in vitro* test. In de vissen werd tevens de VTG-mRNA-productie gemeten, maar deze bleek als eindpunt minder gevoelig dan het VTG gehalte, waarschijnlijk door de kortere verblijftijd van VTG-mRNA in de cel[4].

In tabel 8.2 worden de diverse eindpunten die zijn onderzocht in dit proefschrift geëvalueerd voor hun gevoeligheid en bruikbaarheid voor onderzoek naar de hormoonontregelende potentie van chemische stoffen. Dit overzicht geeft inzicht in de relatieve gevoeligheid en toepasbaarheid van de verschillende eindpunten in de vis als proefdier en de bijdrage hiervan aan het verder verbeteren van testmethodes voor *in vivo*-onderzoek naar de hormoonontregelende potentie van chemische stoffen in het milieu.

[4] Het VTG-mRNA brengt de genetische codering voor de productie van VTG van het DNA in de kern over op de ribosomen buiten de celkern. In de ribosomen vindt de eiwit-productie plaats door koppeling van aminozuren (= bouwstenen van de eiwitten).

Tabel 8.2 Samenvatting en evaluatie van alle eindpunten (markers) onderzocht in the verschillende hoofdstukken van dit proefschrift

	Hoofdstuk				Evaluatie
Eindpunten of markers	2	3	4	5	
Vitellogenine (VTG)	X	X	X	X	Gevoelig eindpunt in vis voor estrogeniteit; gemakkelijk toe te passen echter minder geschikt voor gebruik in testen met androgeen werkende stoffen.
VTGmRNA				X	Minder gevoelig dan VTG.
Gedrag & uiterlijk		X	X		Responsieve eindpunten vooral in het geval van androgenen.
Uiterlijke sexuele kenmerken		X	X	X	Gevoelig eindpunt voor zowel estrogene als androgene blootstelling maar minder gevoelig dan VTG voor estrogene blootstelling.
Lichaamsgewicht, -lengte en conditiefactor	X	X	X	X	In het algemeen minder gevoelige eindpunten.
Relatief levergewicht (HSI)				X	Minder gevoelig eindpunt door de hoge individuele variatie en minder specifiek voor onderzoek naar hormoonontregelende stoffen.
Relatief gonadengewicht (GSI)				X	Minder gevoelig eindpunt door de hoge individuele variatie.
Histologie van de gonaden		X	X	X	Zeer gevoelig eindpunt, maar vraagt meer deskundigheid in de beoordeling er van en kan omkeerbaar blijken te zijn.
Aanhechting van de gonaden aan het buikvlies		X			Zeer gevoelig onomkeerbaar eindpunt dat redelijk gemakkelijk microscopisch is waar te nemen.
Luciferase inductie in de transgene zebravis	X				Matig gevoelig eindpunt, echter alleen in selectieve transgenetische organismen. Maakt evaluatie van estrogene activiteit in specifieke weefsels mogelijk. Bruikbaar voor mechanistische studies en screening met kortdurende blootstellingperioden.
Vruchtbaarheid (productie van ei en broedsel)			X		Niet echt reproduceerbaar eindpunt in paarsgewijs testen met de dikkop-elrits minnow; vraagt om verder onderzoek.
Overleving van eieren	X	X	X		Minder gevoelig eindpunt dan andere.
Uitkomen van eieren	X	X	X		Minder gevoelig eindpunt dan andere.
Algehele overleving	X	X	X		Minder gevoelig eindpunt dan andere.
Sex-ratio		X			Matig gevoelig eindpunt maar kan worden vertroebeld door ongelijke sex-ratio's in de controle groep.

Profiel

Rinus Bogers werd geboren in Breda op 14 mei 1953. Na zijn lagere school ging hij naar de MULO alwaar hij in 1969 zijn diploma haalde. Hij besloot vervolgens in Weert een opleiding tot sergeant te volgen. Echter na 6 maanden was hij overtuigd dat dit voor hem niet de juiste keuze was. Na verschillende baantjes startte hij een avondopleiding VWO en haalde het diploma hiervoor in 1979. Hij besloot, in overleg met zijn echtgenote Lia, de opleiding Biologie aan de Universiteit in Utrecht te volgen. Daar maakte hij kennis met de wereld van de toxicologie. Hij werd gegrepen door enerzijds het gevarieerde en multidisciplinaire karakter van dit vakgebied en anderzijds de maatschappelijke relevantie ervan. Na zijn studie begon hij in 1987 bij het toen nog jonge NOTOX in Den Bosch als studieleider inhalatie- en ecotoxicologie. Later richtte hij zich op de ecotoxicologie en introduceerde nieuwe testen en testsystemen, leidde analisten en studieleiders op en nam deel aan diverse nationale en internationale werkgroepen. In 1998 werd hij officieel geregistreerd als toxicoloog door de Nederlandse Vereniging van Toxicologen en EUROTOX. In 2001 werd gestart met de voorbereidingen voor een promotietraject. Het onderzoek op het gebied van hormoonverstorende stoffen was toen al zeer actueel en vroeg om nieuwe inzichten, technieken en testmethodes. Zowel binnen als buiten NOTOX werkte Rinus samen met diverse teams van medewerkers, zonder wiens ondersteuning dit proefschrift nooit was voltooid. In 2006 werden de eerste artikelen gepubliceerd en nu heeft u het eindresultaat in handen. Het onderzoek evalueert en valideert de diverse eindpunten voor gebruik in de bepaling van de mogelijke hormoonverstorende eigenschappen van stoffen die we, door ons zelf gemaakt, weer tegenkomen in het milieu, ons milieu.

In juli 2008 startte Rinus met een eigen bedrijf BOGERSCO. Zijn meer dan 20 jaar ervaring in de toxicologie wil hij ten dienst stellen ten behoeve van de registratie van chemische stoffen en industriële producten binnen het bereik van de EU Richtlijn 91/414, REACH of EMEA. Naar gelang de wens van de klant omvat dit werk ondermeer de controle van de volledigheid van dossiers en hiaatanalyse van de gegevens van toxicologische en milieu-secties van dossiers voor de registratie. Daarbij adviseert hij in de keuze van de type studies om eventuele hiaten in gegevens in te vullen.

Cover:

Photo front page: detail of the Fish Fountain at the Fish market in Leiden, The Netherlands (Rinus Bogers)

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