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Identification of Endocrine Disruptive Effects
in the Aquatic Environment
a Partial Life Cycle Assay in Zebrafish

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Samenvatting

In dit rapport wordt de ontwikkeling en toepassing beschreven van een methode om effecten van hormoonverstorende stoffen in het aquatisch milieu te onderzoeken. Het project is uitgevoerd in opdracht van het Ministerie VROM (DGM / SAS) en is gesponsord door de Europese Commissie (DG SANCO).

De primaire doelstelling betrof de ontwikkeling en verdere uitwerking van een onderzoeksprotocol om in kleine laboratoriumvissen effecten te detecteren van hormoonverstorende stoffen. In dit protocol werden zebravissen gedurende een korte maar essentiële periode van de voortplanting en ontwikkeling blootgesteld, te weten 21 dagen voor volwassen dieren en 42 dagen voor nakomelingen (Partial Life Cycle Study, PLC). Blootstellingsconcentraties werden gekozen op basis van een voorafgaande range-finding test van 4-10 dagen. De parameters waren voortplanting (eiproductie, bevruchting, uitkomen van de eieren, ontwikkeling van juvenielen, waaronder geslachtsdifferentiatie), vitellogeninegehaltes (VTG), en histopathologische afwijkingen van relevante doelwitorganen. Voor de bepaling van VTG zijn histologische methoden ontwikkeld als alternatief voor de gebruikelijke ELISA. Deze methoden hebben een vergelijkbare gevoeligheid, er kunnen semi-kwantitatieve bepalingen mee worden uitgevoerd op grote aantallen monsters, en bovendien wordt door toepassing efficiënt gebruik gemaakt van de geteste dieren.

Een tweede belangrijke doelstelling betrof de ontwikkeling van een digitale atlas van histopathologische veranderingen die werden waargenomen als gevolg van blootstelling aan hormoonactieve stoffen in gevoelige organen, in het bijzonder de geslachtsorganen, van zebra- en andere kleine laboratoriumvissen. De gegevens voor de atlas zijn verkregen uit de experimenten die in dit project zijn uitgevoerd. Deze atlas is vrij beschikbaar op Internet (http://www.rivm.nl/fishtoxpat/) ten behoeve van research, testen en training.
Het onderzoeksprotocol werd toegepast met een reeks bekende hormoonactieve stoffen, voornamelijk zoals voorgesteld door de Validation and Management Group eco (VMG eco), onderdeel van de Organisatie voor Economische Samenwerking en Ontwikkeling (OESO, OECD). Deze waren het oestrogeen 17β-oestradiol (E2), het anti-oestrogeen tamoxifen, het androgeen methyldihydrotestosteron (MDHT), het anti-androgeen flutamide en de

schildklierremmer propylthiouracil (PTU). Ook is een veldmonster getest, te weten een effluent van een rioolwaterzuiveringsinstallatie en een synthetisch analoog hiervan, in het kader van LOES, het Landelijk Onderzoek naar oEstrogene Stoffen in het aquatisch milieu. De bevindingen bij de individuele dieren werden voorts getoetst in een populatiemodel om een schatting te maken van de effecten op populatieniveau.

Het *oestrogeen* werd getest tot een concentratie van 1 nM omdat 10 nM E2 in een voorstudie volledige blokkade van ovarium activiteit te zien gaf. Deze concentratie van 1 nM gaf afname in het aantal legsels (maar wel gecompenseerd door een toename van de legselgrootte) en bij nakomelingen een groeibevordering en feminisatie (verschuiving in de geslachtsverhouding), waarbij soms zelfs mannelijke dieren ontbraken. Bij ouderdieren werd verhoging van VTG-gehaltes gezien en de testis vertoonde remming van de spermatogenese. Deze effecten werden gezien vanaf 0,32 nM, niettemin bleek de voortplanting niet beïnvloed.

Het *anti-oestrogeen* tamoxifen gaf een afname in het aantal eilegsels, bevruchting, uitkomen van eieren, overleving en groei van juvenielen te zien. De histologische bevindingen waren karakteristiek, namelijk plooivorming in de eicelmembraan, eidegeneratie, en VTG-verlaging bij vrouwtjes. Bij mannetjes werd verstoring in de synchronisatie van de spermatogenese gezien en stimulatie van de Leydigcellen. Daarnaast werd met verhoging van de dosis een toename in ontstekingsprocessen gezien in buikorganen wat zou kunnen wijzen op verstoring van immunologische afweer. De nakomelingen ontwikkelden zich vrijwel allemaal tot mannetjes. Effecten werden gezien vanaf 10 µg/L.

Als *androgeen* is gekozen voor de niet-aromatiseerbare vorm MDHT omdat methyltestosteron in een voorstudie overwegend oestrogene effecten te zien gaf. Bij 10 µg/L werd al spoedig geen eileg meer gevonden en histologisch bleek ovulatie geremd te zijn, wat aangeeft dat bij deze concentratie geen voortplanting mogelijk is. Bij ouderdieren en nakomelingen (afkomstig van niet behandelde ouders) werd bij hoge concentraties VTG-inductie gezien. Deze oestrogene effecten zijn mogelijk het gevolg van directe activatie van de oestrogeenreceptor bij deze hoge concentraties. De testis vertoonde verstoring van spermatogenese en afwijkingen van de Sertoli- en Leydigcellen. Nakomelingen bleven achter in de groei. Bij lagere concentraties werden lagere eiproductie en achterblijvende groei van nakomelingen gezien. Vanaf de laagste concentratie (0.1 µg/L) trad volledige masculinisatie op bij het nageslacht, hetgeen een kritisch effect is voor het voortbestaan van de populatie.,

Bevruchting, uitkomen van eieren en overleving en conditie van nakomelingen waren niet beïnvloed.

Het *anti-androgeen* flutamide veroorzaakte een afname in het aantal eilegsels en in de overleving van nakomelingen. Histologisch werden in de testis veranderingen gezien als toename van Leydigcellen, stimulering van spermatogoniën en Sertolicellen en remming van de vroege spermatogenese. Er waren geen aanwijzingen voor effecten op VTG of op de vrouwelijke dieren. Na blootstelling van alleen de ouderdieren werd masculinisatie gevonden.

Met de *schildklierremmer* PTU werden tot 100 mg/L geen effecten op reproductie gezien. Vanaf 1 mg/L werd bij ouderdieren en juvenielen struma waargenomen, afname van schildklierhormoon (adulten) en glycogeengehalte in de lever. In het nageslacht werd remming van de groei en ontwikkeling (metamorfose) gezien.

Uit een veldstudie is een *veldmonster* van een verdachte locatie getest, samen met een analoog synthetisch mengsel en E2 als controles. Het monster en het synthetisch mengsel gaven alleen bij vrouwelijke adulten verhoging van VTG te zien en een verschuiving naar vrouwelijke ontwikkeling bij het nageslacht. De effecten waren minder dan bij E2, waar ook VTG-inductie bij mannelijke dieren werd gevonden. Blootstelling aan het synthetisch mengsel veroorzaakte veranderingen in geslachtsorganen van adulten die overeenkwamen met die bij anti-oestrogeen tamoxifen. Geconcludeerd werd dat het effluent enige oestrogene activiteit vertoont, en dat het nettoresultaat van een mengsel kan verschillen van wat men op grond van de individuele oestrogene componenten zou verwachten.

In het PLC- protocol werd een semi-statische blootstelling aan een referentiestof toegepast met tweemaal per week een verversing. Behalve bij PTU werd bij chemische analyse een – soms snelle – afname in de testverbinding gezien; hierdoor kunnen de uitkomsten een onderschatting zijn in relatie tot nominale concentraties.

Met het populatiemodel werd vastgesteld dat, met inachtneming van de testopzet (keuze van de concentratiereeks, spreiding in de uitkomsten, beperkte testduur, soortspecifieke voortplantingsstrategie, etcetera), overlevingskansen van de populatie van zebravissen verminderen bij blootstelling aan MDHT en tamoxifen, ten gevolge van veranderde

geslachtsverhoudingen. Geslachtsdifferentiatie bleek bepalend voor overlevingskansen van de populatie.

De conclusie luidt dat het zebravis-PLC-protocol een bruikbare methode is om de verschillende effecten van hormoonactieve stoffen te identificeren. Histopathologische evaluatie is hierbij cruciaal omddat het een hoge specificiteit en gevoeligheid heeft, en tevens aanwijzingen kan geven voor een werkingsmechanisme. Bovendien zijn voor de histopathologie minder dieren nodig dan voor evaluatie van reproductieparameters. De inductie van VTG is met name een bruikbare methode voor het detecteren van risico's van verbindingen met een hoge oestrogene activiteit, omdat duidelijk waarneembare VTG-veranderingen daar gepaard gaan met schadelijke andere hormonale effecten.

Summary

Here is described the development and application of a detection method for pollutants with endocrine activity in the aquatic environment. This project was sponsored by the European Commission (DG SANCO) and the Dutch Ministry of the Environment (VROM).

The principal objective was the development and further validation of a detection method of endocrine effects by means of a test protocol in small laboratory fish. This protocol was designed to expose zebrafish during a limited but critical window in the reproductive and developmental stages; for the selected species, this was 21 days for reproductive adults, followed by 42 days for progeny (Partial Life Cycle Study, PLC). Exposure concentrations were determined on the basis of a pilot range-finding test of 4-10 days. Parameters were reproductive endpoints (egg production, fertilisation, hatching, juvenile development and sexual differentiation), vitellogenin (VTG) levels, and histopathology of (endocrine) target organs. For VTG analysis, histological methods were developed and tested, as an alternative for ELISA. These methods allow identification and semiquantitative determination of VTG almost equally sensitive as ELISA, with a high throughput, and maximising the informative output of a minimised number of animals.

Another major objective was the development of a digital atlas of histopathological changes in small laboratory fish, zebrafish in particular, induced by endocrine active substances, notably changes in endocrine target organs / tissues. Data were obtained from the experiments conducted in this project. The atlas, publicly available on the Internet (http://www.rivm.nl/fishtoxpat/), .is intended for use as a reference in research and testing and for educational purposes.
The designed test protocol was applied to a spectrum of reference endocrine active compounds, in line with the proposal by the Validation and Management Group eco (VMG eco) functioning under the Organisation for Economic Co-operation and Development (OECD). These were 17β-estradiol (E2) as estrogen, tamoxifen as anti-estrogen, methyldihydrotestosterone (MDHT) as androgen, flutamide as anti-androgen and propylthiouracil (PTU) as anti-thyroid agent. Also a field sample (sewage treatment works effluent and its synthetic analogue) was tested as part of a national field trial (LOES). The

data from the experiments were used in a mathematical fish population model, to estimate the effect of the detected endocrine disruption in individuals at the population level.

The results for *estrogen,* where exposure levels were tested up to 1 nM E2 (10 nM causing histologically complete ovarian inactivation in a pilot test), showed a reduction in number of clutches, which was however compensated by increased clutch size, and enhancement of juvenile growth in exposed groups. There was also feminisation in the offspring, sometimes leading to complete absence of males. In adult males and females, a clear induction of VTG was seen, and testis morphology indicated inhibition of spermatogenesis. Effects were noted from 0.32 nM onwards; nevertheless, reproduction was largely uneventful up to 1 nM

Results from the *anti-estrogen* (tamoxifen) study showed the number of egg clutches, fertilisation, hatching, survival and length / weight to decrease. Histologically, typical effects on the gonads were seen, such as wrinkling of the oocyte membrane; other degenerative changes took place, and VTG was decreased in females. In the testis, asynchrony of spermatogenesis was seen, together with activation / proliferation of Leydig cells. Remarkably, enhancement of abdominal inflammatory processes was observed with increasing dose, which may point towards an (in)direct effect on the animals' immune competence. In juveniles, tamoxifen induced sex reversal, indicated by a nearly 100% male population. The overall lowest effect concentration was 10 μg tamoxifen /L.

In the *androgen* study, methyltestosterone was initially tested, but the clear, induced, estrogenic effects, attributed to aromatisation, of the preliminary study led to investigation of the non-aromatisable MDHT. In the 10 μg/L group, spawning was inhibited within a few days, associated with histologically observed inhibited ovulation; for this reason, it is anticipated that this concentration is incompatible with reproduction. VTG was induced in adults and juveniles. These estrogenic effects were possibly due to direct interaction with the estrogen receptor at high concentrations. Testis morphology indicated disturbance of spermatogenesis and effects on Sertoli / Leydig cells. In juveniles, growth (body weight and length) was reduced. At 1 μg/L, egg production was reduced (concentration related); there was also a gain in body weight in juveniles. At low concentrations (0.1 μg/L and higher) complete masculinisation was induced in developing juveniles. This is considered as the critical effect with respect to extrapolated survival chances of the population. At higher

concentrations gonad development was inhibited. No effects were seen on survival, fertilisation rate, hatching and the condition factor (juveniles) in any of the groups.

Concerning *anti-androgen*, flutamide at 1 mg/L caused a reduction in egg clutches and in the condition of juveniles, and caused a concentration-dependent reduced juvenile survival. Histological changes in the testis included an increase in interstitial cells, hypertrophy of spermatogonia and Sertoli cells, and the inhibition of early spermatogenesis. No effects were seen in VTG, and there were no histological changes in females. In juveniles, paradoxally enough, masculinisation (partial) was seen after parental exposure.

In the study with the *anti-thyroid* drug PTU, it was shown that even at 100 mg/L no adverse effects on reproductive parameters were evident. In both adult and juvenile zebra fish, struma was observed for 1 mg/L and above. In plasma of adults, a dose-dependent decrease in thyroid hormones was indeed measured. Liver glycogen was reduced as well, this being attributed to the known glyconeogenetic activity of thyroid hormones. Developmental effects were limited to reduced growth and metamorphosis.

A *field sample* (a sewage treatment plant effluent) was tested from a so-called hot spot and a synthetic analogue was examined, E2 being used a positive control. The field sample and the synthetic analogue induced VTG in females but not in males; in juveniles there was a shift towards the female phenotype. The effects were less prominent than in the E2-exposed fish, where males too exhibited VTG induction. By contrast, histological effects of the synthetic analogue in adult gonads of both sexes were identical to those from the tamoxifen study (anti-estrogen). From the study it can be concluded that the effluent had an endocrine disruptive potency (shift in sex differentiation). Results also showed that the net effect of a mixture could differ from expected effects of the individual estrogenic constituents.

The PLC-protocol included a semi-static exposure regime for the reference compound, with biweekly renewal of the exposure medium. With the exception of PTU, chemical analysis showed a decline in test compound concentrations, which, in some cases, was fairly rapid. The detected effects may therefore be an underestimation of the actual hazard of the compounds at the given nominal concentrations.

Population modelling showed that under the conditions of the test method (selection of concentrations, variation in results, limited duration, reproduction strategy of zebra fish, etc.) an increase in the risk of extinction of the zebrafish population took place after exposure to MDHT and tamoxifen due to skewed sex ratios. Sex differentiation was critical for chances of survival of the population .

The zebra fish PLC protocol is concluded to be a useful method for identifying various effects of endocrine disrupting chemicals. Histopathological evaluation, with its high specificity and sensitivity, is essential; it can also contribute in identifying the mode of action. Fewer animals are needed for histopathological evaluation than for evaluation of reproduction parameters. VTG induction appears to be useful in specific identification of risk from compounds with a high estrogenic potential, since appreciable VTG increase is associated with adverse, other, endocrine effects.

1. Introduction

Environmental effects of endocrine active substances have raised many concerns world-wide since the early nineties (Colborn et al., 1993; Vos et al., 2000). This concern was raised after abnormalities in sexual differentiation in certain wildlife populations, and it has triggered research, hypotheses and speculation towards insidious effects in wildlife and man, such as population decline in wildlife, reduction in sperm counts, gonad abnormalities and endocrine related tumours in humans.

Most of the concerns and research was focussed towards the aquatic environment, and the need was felt for more specific or dedicated toxicity test protocols. Indeed, current ecotoxicity testing guidelines (OECD TG 201 Fish early life stage and OECD draft TG 212 Egg and sac fry) are not able to identify mechanistic pathways, including the endocrine system, necessary for the characterisation of EDC effects. The emphasis in development of predictive tests for endocrine disruption currently was predominantly on *in vitro* bioassays, while the *in vivo* effects on the (mainly reproductive) functioning of organisms and populations remained relatively unattended. Therefore, the qualitative and quantitative significance of the data from such *in vitro* bioassays is not well known and, thus, for proper risk assessment (and consequently risk management) *in vivo* studies are indispensable. Various international scientific, regulatory and industrial groups have identified the lack of adequate *in vivo* models and testing protocols for endocrine disrupting chemicals (EDCs) in the aquatic environment (EU, OECD, EMWAT, EDSTAC, CEFIC). Since in human risk assessment histopathological screening of various organs and tissues of laboratory rodents is the cornerstone in hazard identification, it is proposed to extend the current test protocols using fish with histopathology; this will not only cover the detection of EDCs but also other categories of toxic compounds.

Thus the aim of the present project is twofold:

1. development and testing of a reproduction study in zebrafish, with the principle aim to identify effects indicating endocrine disruption (in case of an estrogenic action, increased levels of circulating vitellogenin), and relate these to reproductive performance. Both parent and offspring animals are monitored for relevant parameters such as reproduction indices, sex distribution, development and histopathology. This will enable the interpretation of laboratory and field data (e.g. increased vitellogenin levels, testicular and

thyroid abnormalities), in regard to reproductive hazard and will help to estimate consequences for population dynamics and ecology.

2. development and validation of an *in vivo* total body histopathological screening of small fish by exposure to a variety of known EDC's. Such a screening has been developed in the past by our group, using guppy (*Poecilia reticulata*) and medaka (*Oryzias latipes*) as test species; the small size of the test fish allows group-wise whole body histopathological examination with the organs still *in situ*. This cost-effective methodology enabled screening of structural effects, induced by various environmental contaminants in a variety of relevant organs including endocrine organs and endocrine responsive tissues. Not only a range of target organs can be identified by this protocol, adequate knowledge of pathophysiology and toxicological pathology may also give indications for organ interactions, mechanism of action and consequently functional impact on the organism (Wester and Vos, 1994; Wester et al., 2002; Van der Ven et al., 2003b). Histopathology has been mentioned by several bodies (OECD, CEFIC) as an important need in further development and validation of test methods to detect EDCs. In the present project the aim is to introduce this histopathological screening protocol for the oviparous zebrafish *Danio rerio*, a widely used laboratory species for which more information on reproductive physiology is available and which is more suitable for reproduction studies than e.g. the live bearing guppy. From a variety of established EDCs a number is selected to be used as reference compounds, and the attention is focused on (but not limited to), effects on endocrine responsive tissues. Such a protocol with fish is intended to be incorporated in future ecotoxicity testing guidelines. The results of this investigative work on histopathology will be issued as a digital histopathology atlas available through internet to aid researchers and students in training and harmonisation of terminology and interpretation.

2. Method development

2.1.   Vitellogenin analysis

This chapter is excerpted from a paper which will be published in Aquatic Toxicology:
Vitellogenin expression in zebrafish Danio rerio: evaluation by histochemistry, immunohistochemistry, and in situ mRNA hybridisation (LTM Van der Ven, H Holbech, M Fenske, EJ Van den Brandhof, FK Gielis-Proper, PW Wester).

Introduction

Vitellogenin (VTG) is an important biomarker for assessing endocrine disruption, in particular estrogenic stimulation in aquatic vertebrates. This yolk precursor protein is produced in the liver after stimulation of hepatic estrogen receptors, secreted to the blood, and incorporated in the developing oocytes. Hence, under physiological conditions, VTG is mainly present in sexually active females, since males do not produce appreciable levels of estrogen (Kime, 1998). The presence of pollutants with estrogenic activity in the field may cause elevated VTG levels in aquatic vertebrates, and similarly, field samples can be tested for estrogenic activity by laboratory models employing VTG expression as an endpoint (Sumpter and Jobling, 1995; Kime, 1998).

The most widely used detection method is the VTG ELISA with antisera specific to or cross-reactive with the species used in the model (Kime et al., 1999). This method enables quantitative analysis of VTG contents in blood plasma or whole body or organ homogenates. Alternatively, changes in expression and levels of VTG can be detected with sophisticated histological techniques, which may offer substantial advantages over ELISA and other extraction methods:

- more information may be retrieved from the same animal, thereby reducing the number of animals needed for analysis,
- increased quality of information, since it integrates VTG expression with other histological endpoints,
- cost-effectiveness since most of these techniques can be completed on routine sections within a short time,
- it can be applied on very small samples and routine and archive material.

We have explored the employability of immunohistochemistry, histochemical stainings, and *in situ* mRNA hybridisation for analysis of VTG expression as an indicator of estrogenic activity in our zebrafish by comparing them with traditional ELISA.

Materials and methods

Animals, exposures, and histological pre-processing

The zebrafish were exposed to 17β-estradiol (E2; Fluka, Buchs, Switzerland), for 10-21 consecutive days in a semi-static system (see Chapter 2.2). Experiments were performed with serial dilutions in concentration ranges of 0 - 10 nM and 0-1 nM. Standard chemical analysis showed actual exposure levels of 40-80% of nominal values. Male fish were exposed to all concentrations, females only to the control and the highest concentration.

After the exposure period, four fish of each group were bled from the tail vein as described in Chapter 2.2: Partial Life Cycle Study, protocol design. In the narrow range experiments, blood of several fish was pooled, in the wide range experiments blood samples were stored individually. A typical blood yield of normal sized adult fish was 5 μL in males, 7 μL in females. An equal volume of a 6 μg/mL aprotinin (protease inhibitor, Sigma) in phosphate buffered solution was added to the blood. All fish were submitted to routine histological processing, including fixation in Bouin's fixative, embedding in paraffin, and preparation of horizontal sections (5 μm), the latter on amino-acyl silane (AAS) coated glass slides for special histological techniques (see below).

Histology, histochemistry, immunohistochemistry, and in situ hybridisation

Tissue sections were submitted to the following techniques:

- standard H&E staining,
- histochemical staining of VTG, making use of the typical high concentration of phosphate groups in VTG (see Annex 2, histology procedures),
- immunohistochemistry with a zebrafish-specific rabbit anti-lipovitellin polyclonal antiserum (generous gift of Dr. Holbech, see also the ELISA section),
- *in situ* mRNA hybridisation using a 275-bp probe, which is a digoxin labelled PCR transcript of a vector construct containing a VTG PCR product from female zebrafish liver (Dr. Juliette Legler and Dr. Bart van der Burg, the Netherlands Institute for Developmental Biology (NIOB), Utrecht, the Netherlands (Legler et al., 2002).

For objective measurement, staining intensities of representative areas of the tissue structure of interest were quantified on digital images (20x magnification) with standard image analysis software.

ELISA

The blood/aprotinin-diluted samples were centrifuged in a micro-hematocrit centrifuge (600 rpm, 5 min), and stored at -20°C until analysis in an ELISA using a polyclonal rabbit IgG raised against lipovitellin (yolk protein) purified from zebrafish ovaries (Holbech et al., 2001), or with a polyclonal rabbit antiserum raised against plasma VTG purified from female zebrafish stimulated with ethynylestradiol (Fenske et al., 2001).

Statistics

Differences between control and exposed animals were tested for statistical significance with an ANOVA or T-test. Linear relationships between data sets (mean exposure group values) were calculated using the Pearson product moment correlation coefficient (r).

Results

Histological techniques

With routine H&E histology, the cytoplasm of hepatocytes in control males was pale eosinophilic; after stimulation with E2, liver cell cytoplasm became clearly basophilic, as a result of increased mRNA levels (Fig. 2.1.1a). The increased staining intensity was reproducibly measurable at a level of exposure of 1 nM E2/L, and overall basophilic staining intensity increased significantly with concentration of exposure (Fig. 2.1.2). Phosphoprotein staining, analysed in plasma compartments on the sections, showed a low intensity in non-exposed males, which progressively and statistically significantly increased with increasing E2 exposure levels (Fig. 2.1.1b, Fig. 2.1.2). Again, the lowest level of detection was at exposure to 1 nM E2.

VTG mRNA was detected in males only exposed to E2 and in all females. The signal of the hybridisation was limited to hepatocytes, visible as a diffuse or punctuated pattern throughout the cytoplasm. Notwithstanding morphological drawbacks, the measured signal intensity increased significantly with the level of exposure of E2, with a lower signal detection limit at the exposure level of 1 nM/L (Fig. 2.1.2). Furthermore, there was a linear correlation between VTG mRNA *in situ* signal and the other method indicative of mRNA (liver basophilia;
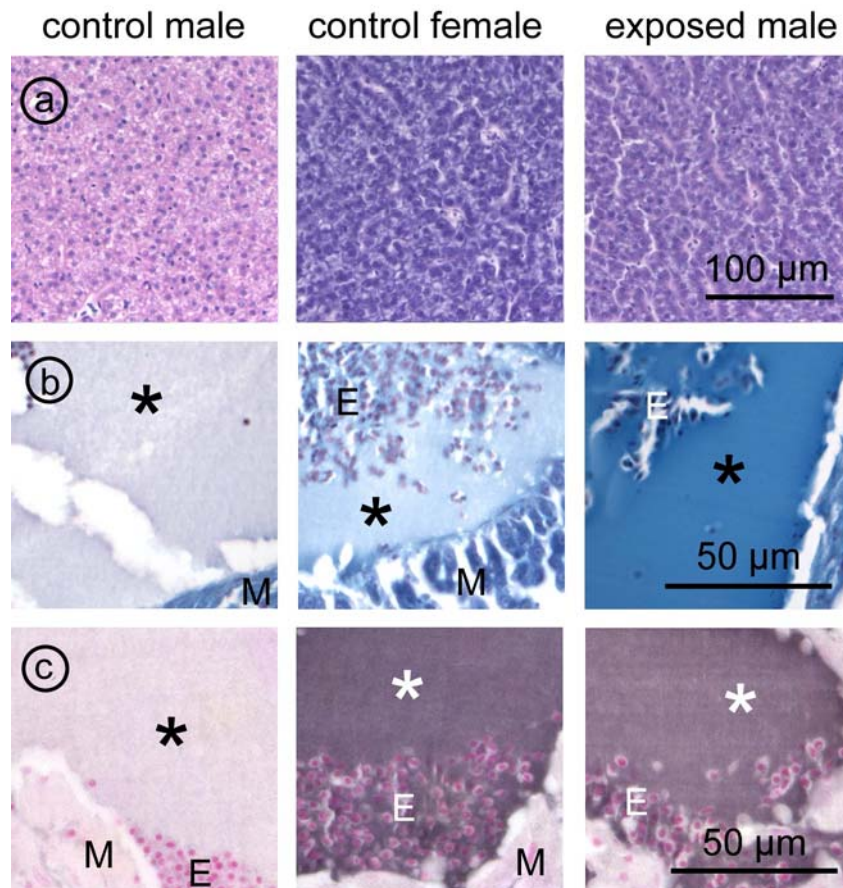
Fig. 2.1.1 Microphotographs of (immuno)histochemical detection of VTG
a Liver sections, representing pale acidophilia in control male zebrafish (left), and dark basophilia in control female (middle) and male exposed to 1 nM E2 (right).
b,c Blood plasma in the heart,
b histochemical staining for phosphoproteins. High intensity staining is found in the male exposed to E2 (1 nM), faint staining in the control female, and virtual absent staining in the control male. Measurements of colour or staining intensity of these three parameters are given in Fig. 2.1.2.
c strong immunostaining intensity for vitellogenin in control female (middle) and male exposed to 1 nM E2 (right); the control male represents the intensity of background staining.

r=0.94, Table 2.1.1), and also between the mRNA *in situ* staining and the methods detecting VTG protein in the plasma (ELISA, immunohistochemistry, and phosphoprotein staining, r=0.79, r=0.80, and r=0.86, respectively; Table 2.1.1).

Immunohistochemistry for VTG showed well localised intracellular compartments in the hepatocytes, but only in females and estrogen exposed males (1 nM and higher), not in control males (Fig. 2.1.3). These localised areas, suggestive of RER/Golgi regions, were too small to yield a significant overall increase in measurable staining intensity at any level of exposure to E2 (no dose response, Fig. 2.1.2), suggesting that the protein does not accumulate in the cells. This is further supported by additional immunopositivity in extracellular, perivascular spaces in the liver, which most likely are spaces of Disse (Fig. 2.1.3).
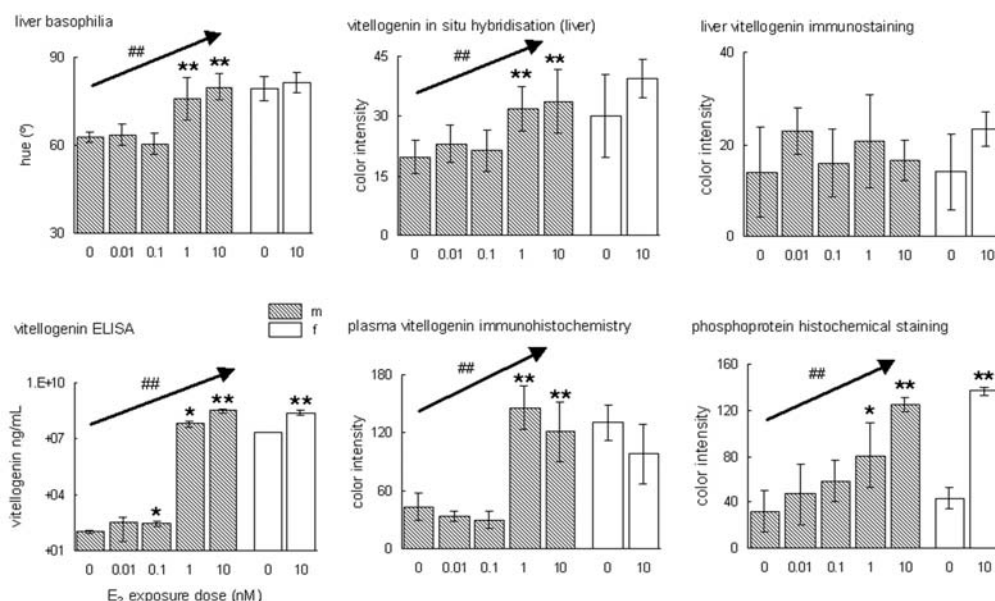
Fig. 2.1.2 - Comparison of semiquantitative representations of various histological detection methods of vitellogenin mRNA (liver basophilia, in situ mRNA hybridisation) or peptide (immunohistochemistry, phosphoprotein staining) with the quantitative vitellogenin ELISA on plasma. Each parameter was measured in males (m), exposed to a logarithmic dilution range of E2 (0-10 nM), and in females (f), control as well as exposed to a high concentration of E2 (10 nM). Each concentration group contained six animals. Statistical significance of differences between exposure and control groups was calculated in a Student's T-test (* $p<0.05$; ** $p<0.001$); statistical significance dose-dependent effects was calculated in a single factor ANOVA (## $p<0.001$).

In the histological sections, the circulatory system showed areas of cell free plasma, particularly in large vessels and in the heart. In these areas, there was also immunoreactivity with the anti-VTG antiserum in females and estrogen exposed males, from a concentration of 1 nM E2 (Fig. 2.1.1c). The intensity of immunostaining was significantly related to the dose of exposure to E2 (Fig. 2.1.2), confirming accumulation of VTG in the circulation. Measurement in an exposure range between 0.1 and 1.0 nM confirmed 1 nM as the lower limit of detection (not shown).

ELISA

The ELISA for VTG used in a wide exposure range detected an increase of VTG in male plasma at an exposure level of 0.1 nM E2; further increase was dose dependent (Fig. 2.1.2). The lower detection limit in the ELISA used in the narrow exposure range was at an exposure level of 0.32 nM E2 (not shown). An ANOVA could not be performed on this latter series, since the plasma samples were pooled in this experiment. Both ELISAs detected VTG in female plasma at high levels.

Table 2.1.1 - Correlation coefficients (R)

	in situ hybridization	H&E	ELISA	plasma immunohistochemistry
H&E	0.94			
ELISA	0.79	0.72 (0.86)		
plasma immunohistochemistry	0.76	0.89 (0.88)	0.48 (0.81)	
Phosphoprotein	0.86	0.70	0.94	0.46

Results of application of the various detection techniques for vitellogenin mRNA or protein on male zebrafish exposed to a wide range of concentrations of E2 were compared. The values in brackets represent the data from the narrow range series.

Correlations for methods to detect VTG protein

The correlations between the tested VTG parameters, liver H&E, plasma ELISA, plasma immunohistochemistry, and plasma phosphoprotein staining, were calculated on pooled average group data of both sexes. Table 2.1.1 shows that there were consistent high correlation coefficients between all these parameters, in both test ranges of exposure to E2.

Discussion

Several histological tools are available to identify and quantify VTG, which, to date mainly has been detected by immunochemical methods in plasma samples. Advantages of these histological methods were mentioned in the introduction of this chapter, and they have satisfactory specificity, sensitivity and validity.

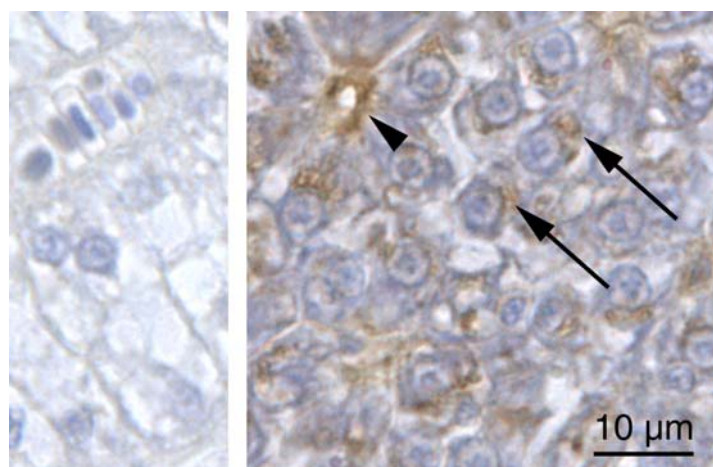*Fig. 2.1.3 - Immunohistochemical staining for vitellogenin in the liver of a male zebrafish exposed to 1 nM E2 (right), as well as a control male (not exposed, left).*
Vitellogenin is present in the cytoplasm of hepatocytes in a perinuclear area reminiscent of RER/Golgi (arrows). Apparently excreted vitellogenin is also present in the space of Disse (arrowhead), between hepatocytes and the sinusoid lining.

The specificity of the employed methods is indicated by staining patterns and locations, as well as correlations of staining intensities with exposure to (endogenous) E2 and correlations between methods. With respect to specificity, the histochemical staining of phosphoproteins (phosvitin, which is part of the VTG complex), is independent of the fish species, since this method will stain all phosvitins, and was equally successfully applied on histological sections of guppy (Wester et al., 1985).

The sensitivity of the histological methods was comparable. VTG mRNA and peptide were both reproducibly detected in male zebrafish after exposure to 1 nM E2. Digitised measurements excluded subjective bias which may play a role in visual inspections.

The sensitivity of the ELISA was higher than of the histological methods, although not more than one dilution factor of of E2, compared to the histological detection of VTG. For quantitative analysis of VTG expression, all methods have their specific (dis)advantages.

applications

These experiments were conducted to analyse whether histological evaluation of VTG expression, either at the level of mRNA or of the peptide, can be useful in the histopathogical analysis of biological effects of exposure of (xeno-)hormones in the aquatic environment. From these results, routine H&E, focusing on liver basophilia, appears to be an acceptable method to screen for estrogen-like stimulation, at least in males. For purpose of validation, H&E could be supplemented with the relatively simple immunohistochemical detection of VTG in an area in a large vessel or the heart, devoid of erythrocytes. An alternative validation method, when no VTG antiserum for the species under study is available, is provided by the phosphoprotein staining method. The *in situ* mRNA hybridisation is a more laborious method, yielding inferior results from the morphological viewpoint, without additional value compared to the other histological methods. Immunohistochemical detection of VTG in the liver could be useful for mechanistic studies, i.e. it indicates qualitative responses after estrogen agonist or antagonist stimulation.

ELISA and immunohistochemistry both have a high throughput, i.e. many samples can be processed within a relatively short time. An important advantage of immunohistochemistry is that the slides remain available for review, and embedded fish for other histological determinations. The limit of exposure to E2 yielding detectable VTG expression by histological methods is below or at the level at which histopathological effects are found (VTG accumulations, alterations in the gonads). These histological methods for the detection of VTG can therefore be considered as valid markers for the induction of clinical

(histopathological) effects, and thus as valuable for hazard identification. In perspective of employing correlates for clinical relevance, there is only limited value in higher sensitivity of the biochemical analysis of plasma by ELISA compared to the other described methods, since ELISA detects VTG induction below an E2 exposure level that yields clinically relevant effects.

2.2.   Partial Life Cycle Study, protocol design

The *partial life cycle (PLC)* protocol as designed was aimed to detect endocrine effects with emphasis on the reproductive system, in both parents and offspring of laboratory fish. Such a protocol is more time and cost effective compared to a multi-generation or full life cycle assay, although the latter has the potential of generating more information, particularly on reproductive parameters in next generations. The central parameters measured in the PLC were reproductive success, juvenile development, histopathology of target organs and vitellogenin (VTG) levels. The test was further developed based on experience gathered during the various tests carried out in the course of the project. To validate the protocol, various reference EDCs were tested.

Selection of the species

The species used is *Danio rerio* (zebrafish). This species was selected as an easy-to-breed laboratory fish for which extensive knowledge from toxicology (Meinelt and Staaks, 1994; Kime, 1995; OECD, 1993) and developmental biology is available (Laale, 1977). These small fishes (approximately 3-4 cm, 0.55-1.0 g for males and females, respectively) are particularly suitable for whole body histology, while a sufficient volume of blood can be sampled for biochemical analysis (4-10 μL). The fish has a short life cycle, is sexually mature after approximately 3-4 months, and there is some sexual dimorphism. They are continuous (non-seasonal) breeders, and eggs are normally produced every 3-5 days under laboratory conditions (Niimi and LaHam, 1974; Laale, 1977; Westerfield, 2000).
Our stock was initially (1998) obtained from a commercial supplier, and after an initial antibiotic treatment subsequently bred in our facility and kept successfully under apparently disease-free conditions. Details on husbandry are described in the zebrafish atlas (http://www.rivm.nl/fishtoxpat).

Test conditions

During the tests, adult fish were kept at a density of 2 L medium per fish in full glass containers covered with glass plates. Juveniles were kept at 150 mL per 5 fish for 21 days, thereafter at 300 mL per 5 fish. Animals were fed *ad libitum* for 5 minutes twice a day with defrosted artemias (commercially obtained).
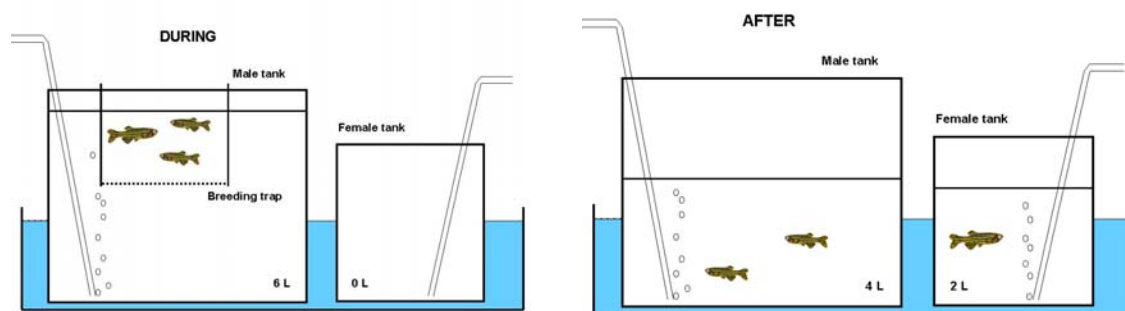
Fig. 2.2.1 - Exposure placement during and after spawning

Environmental conditions Before and after renewal of the media, pH and oxygen were measured in all containers. pH values were considered acceptable between 6.5 and 8.5, and dissolved oxygen concentration in the test solution during the test was considered acceptable at a minimum of 60% saturation. Oxygen supply was by aeration through glass tubes. Hardness was measured once in the stock control medium (upper limit 14 dH°), before use in the PLC. The temperature was monitored daily and maintained at 27 ± 2 ºC by keeping the containers in a water bath (Fig. 2.2.1). Nitrite was measured when increased respiration was observed in the fish. Light / dark regimen was kept at 14-10 hours (see Annex 1).

Experimental media A single stock of test compound was prepared for each PLC test and kept at 4 °C. From these stocks, pre-dilutions were prepared each week, and final test solutions were prepared at room temperature from these pre-delutions at the day of use. If necessary a solvent was used, usually 0.01% DMSO. Ethanol, which was used initially, was abandoned since this facilitated microbial growth in the tanks. Solvent concentration was equalised in all test and control groups. The carrier medium was Dutch Standard Water (DSW, see Annex 1). Contact of the test system with synthetic materials was kept to a minimum to avoid the introduction of endocrine active contaminants, such as plasticisers. Test compound concentration was maintained in a semi-static way, i.e. with medium changes twice a week (3-4 day intervals). Duplicate water samples were taken daily for test compound analysis in such an interval. The concentration of stock solutions was monitored, and in some cases also of the highest test concentration with or without aeration, and with or without test organisms. Medium samples were kept frozen at -70°C until analysis.

Used medium was discarded after charcoal filtration, and containers were cleaned at each medium change (with 96% alcohol, then thoroughly rinsed).

Range finding assays

Initially, a concentration range finding experiment was conducted for each compound, in which adult and fry were exposed for a short period (usually fry 4 days, adults 9 days) to a dilution series with the highest concentration at water solubility (including solvent) or at sublethal concentrations derived from literature data. The concentration exposure range consisted of logarithmic dilutions of the top concentration. Decisive parameters in the range finding assay indicating toxic effects were: reproduction success, mortality, clinical pathology and histopathology.

PLC

Protocol for adults

Spawning units consisting of two males and one female with an age range of 8 –14 months were selected from the batch, on the basis of successful reproduction, as indicated by the number of clutches (at least two clutches in eight days), by fertilisation rate (at least 100 fertilised eggs per clutch), and by hatchability (at least 50% per brood), all under reference conditions. Three spawning units were used per treatment (see Annex 1). The adults were exposed to a range of three concentrations of test compound with a dilution factor of preferably 3.2, or 10 at most; carrier medium (DSW) served as control. The highest test concentration was based on absence of toxic effects and successful reproduction as anticipated from the range finding test. The total exposure period for adults was 21 days (Fig. 2.2.2).

Breeding protocol was as follows: immediately after each medium renewal, spawning units were placed in a breeding trap with a mesh sieve without spawning substrate in a 6L container. The next day eggs were collected and sexes separated until the next spawning episode.

Protocol for eggs

Immediately after separation of the sexes the produced eggs were collected by siphoning from the bottom of the tank. Fertilisation ratio was expressed as the percentage of fertilised eggs (non-fertilised eggs appear opaque). Fertilised eggs were rinsed with temperature controlled DSW to remove debris (remaining feed and excreta). From the spawning brood numbers 2, 4 and 6 produced during the exposure period, four groups of 50 fertilised eggs were used for further incubation. Two of these groups were placed in control medium (with
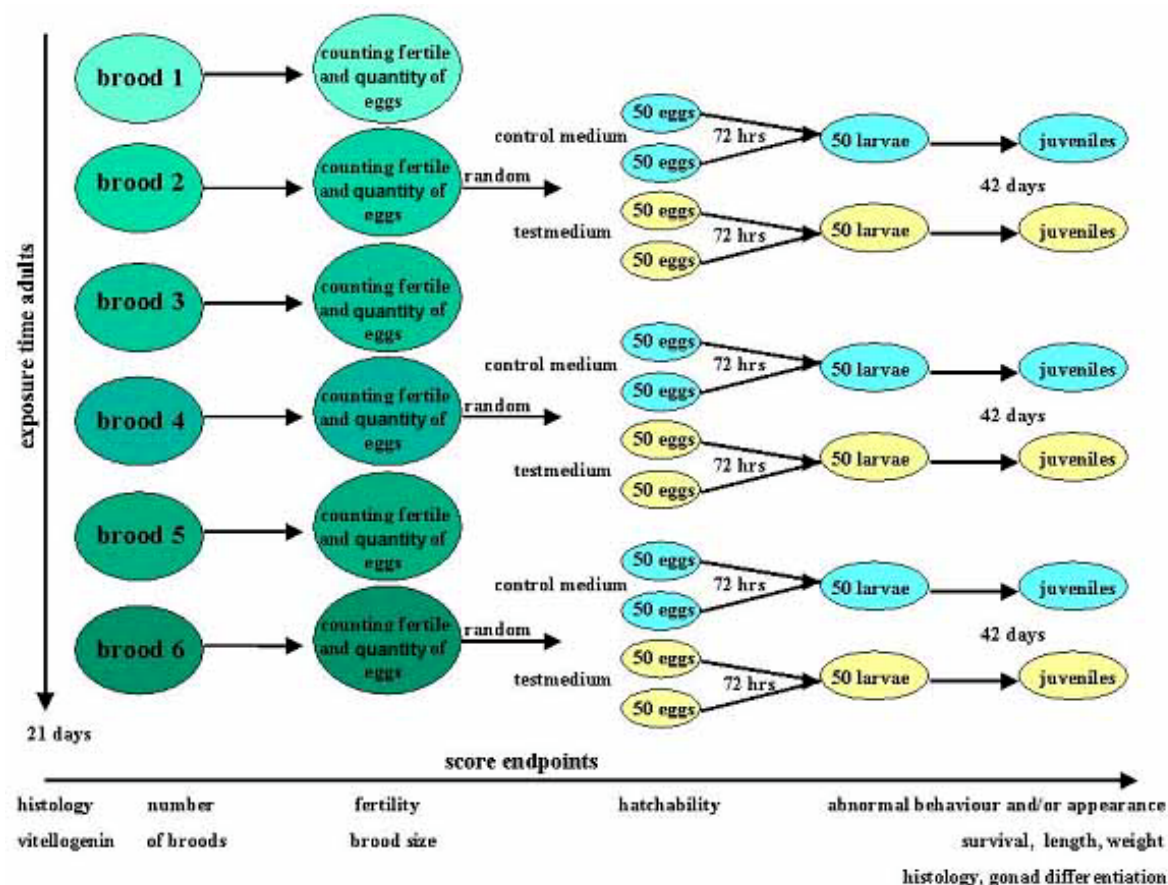
Fig. 2.2.2 - PLC, exposure and assessment of endpoints regimen

solvent if applicable), and the two other groups in the same test concentration as the parents had (Fig. 2.2.2). Two groups of each of the broods 2, 4 and 6 from control parents were also incubated in the highest test concentration. Incubation was performed in 10 cm diameter petridishes with 50 mL test medium. This design allows duplicate observations and distinction between parental and postnatal effects. Eggs were maintained at 28.5 ± 2 ºC and mortality and hatching were scored after 24, 48 and 72 hours.

Protocol for larvae and juveniles

If available, a total of 50 hatchlings obtained from pooled duplicate groups of eggs was transmitted to a 1.5 L glass tank for continued exposure for another 42 days (see Annex 1). During each of the biweekly medium changes, the juveniles were photographed in a small volume of medium to facilitate counting and data storage. The volume of medium was adjusted to the actual number of fish.

Parameters

Both adult and juvenile fish were inspected daily for mortality, abnormal appearance and behaviour. After 21 days of exposure, adult fish are euthanised in an aqueous solution of 100 mg/L tricaine methanesulphonate (MS-222, CAS RN 886-86-2, Sigma-Aldrich), neutralised with sodium bicarbonate (2/1 MS-222, w/w). From two females and four males per concentration group the tail was cut with a pair of scissors and blood obtained from the incision with a heparinised glass capillary. The blood was diluted with an equal volume of aprotinin (6 μg/mL), stored on ice and centrifuged at 600 g for 5 min. to separate and collect plasma, which was stored at –20 ºC for future analysis of e.g. VTG. Specimen were fixed in Bouin's fixative for 24 hours, thereafter kept in 70% ethanol until further histological processing; for details see Annex 2.

Juveniles were euthanised in MS-222 after 42 days, blotted dry, length measured on calibrated paper, weighed and fixed as above. Moribund animals were also sampled for histology, if indicated. Condition factor (K) was calculated for individual fish by the equation: K= weight (g) x 100/(length cm)^3. One day prior to euthanasia, fish were not fed to reduce intestinal content that might interfere with histology.

Data treatment and interpretation

For statistical analyses, the experimental unit of the PLC-tests is the spawning unit (two males, one female) in a single tank. Egg clutches deposited in one tank were defined as repeated measures because these egg clutches, which originate from the same experimental unit, are not independent from each other. The same accounts for the juveniles hatched from the egg clutches originating from the same experimental unit; these are also defined as repeated measures. ANOVAs, t-tests and regressions were executed with means of repeated measures per aquarium. All life history parameters were analysed with One way ANOVA followed by Dunnett's Multiple Comparison to compare treatments with the controls (GraphPad Prism 2.01). Exceptions were hatching of eggs and juvenile parameters coming from the same parents but subjected to different exposure concentrations. These data were analysed by paired t-tests with the adult couples defining the pairs. Linear regression was applied to number of egg clutches, to total number of eggs per aquarium, to fertilisation rate and also to hatching and juvenile parameters from the treatments in which the juveniles were exposed to the same concentration as their parents.

2.3.   Population modelling

The ecological relevance of endocrine disrupting effects in individual fish is in the impact at the population level. As a tool to extrapolate effects measured in the PLC-test to the population level, the zebrafish model of Oertel (1992) was applied. The model is described in detail by Schäfers et al. (1993). In summary, the Individual Based Model (IBModel) is based on long-term experiments in large aquaria with zebrafish or guppy, representing opposite reproduction strategies. Zebrafish are typical r-strategists, with a life history directed to maximize reproductive rate (r); r-strategists typically produce large numbers of offspring and show no brood care behaviour (Nagel, 2002; Halliday, 1993). Populations of r-strategists can recover relatively easy from environmental disturbances.

K-strategists such as the guppy have a life history adapted to maximize competitiveness and survival. They typically produce less offspring than r-strategist, but invest in some form of brood care (e.g. viviparity in guppies). K-strategists mostly live in relative stable environments and the population is relatively sensitive to environmental disturbances. In the laboratory setting of Oertel, population dynamics were monitored and some of the population parameters were estimated with additional experiments.

Variables which were entered in the model were as measured in the PLC-tests: number of clutches, clutch size, fertilisation, hatching, developmental variables (survival, length, weight), and sex differentiation. The Von Bertalanffy growth curve was used to estimate time to develop from hatching to adult animals. It was assumed that maturity was reached when the animals reached 24 mm of length (24.9 mm for females and 23.1 mm for males; Laale, 1977). Sex differentiation was incorporated as proportion of the juveniles that developed into females. The model accounts for predation of progeny by adult zebrafish, varying with life stage; this characteristic was maintained. Mortality of adults was set at 0 % in all cases, also when mortality had occurred among adults. Exposure concentration levels used during the present study were set at non-lethal levels for adults and it was assumed that possible mortality among adults was incidental. Mortality due to exposure within the population is already represented by juvenile mortality.

The modelled system was set at 800 L water and 200 L of refugium, in contrast to 200 L water and 50 L refugium in the laboratory setup of Schäfers et al. (1993); pilot calculations showed that such a larger system better accounts for changes in life history parameters which may have an impact on population size or survival. With the smaller system, population

extinction occurred in a relatively high proportion of simulations, thereby possibly veiling effects of PLC test variables.

Output parameters were extinction risk (survival changes of the population), day of extinction, and average population density. The calculation period was 2004 days (equals 4-5 generations); pilot calculations showed that there were no additional effects after 3000 days (equaling 6-9 generations). To understand the results of the calculations, and to validate the specificity of the outcomes on zebrafish populations (r-strategists), the IBModel was also employed to estimate effects on

K-strategists, using the guppy model defaults and the results from the PLC-tests with zebrafish. For this purpose, results from the other PLC variables (adult reproduction parameters and growth and survival of juveniles) were assumed to affect guppies and zebrafish similarly.

2.4.   Histopathology atlas

Introduction

One of the endpoints of the project was an inventory of the normal histology and effects of endocrine disruptors in small laboratory fish. To facilitate dissemination it was decided to present this in the format of a digital Toxicological Pathology Atlas of Small Laboratory Fish. This atlas is intended as a reference guide, to help investigators and other professionals interested to use histology and pathology of small fish. Although the data are focused on the model species *Danio rerio*, it must be acknowledged that the information will be applicable to other species to a large extent. Material from other sources than the current project is also included; references are included in the concerning sections. Acknowledgement is made to those scientists that have contributed as peer reviewers in their respective expert fields.

Outline of the atlas

The atlas is a html-based product (hypertext markup language), optimised for Microsoft Internet Explorer 5.0+. This format enables easy browsing and the inclusion of advanced techniques to improve understanding, such as image animation or sophisticated detail identification. The possibility of instant updating is also considered as a major advantage.

The atlas contains five main sections, which are available from a top menu (Fig. 2.4.1). These include:

- normal histology, aiming to show overview and detail sections of all organ systems;
- histopathological effects of exposures to endocrine active compounds, including estrogen agonists and antagonist, androgen agonist and antagonist, and thyroid antagonist; this module contains major results from the present project;
- MRI animations for better understanding of the (zebra)fish anatomy;
- a text search module;
- a general information module.

Each section has an index menu on the left-hand side, and a contents area. For the histological modules, this contents area consists of a central image part and a descriptive text, which contains interactive links to the image.
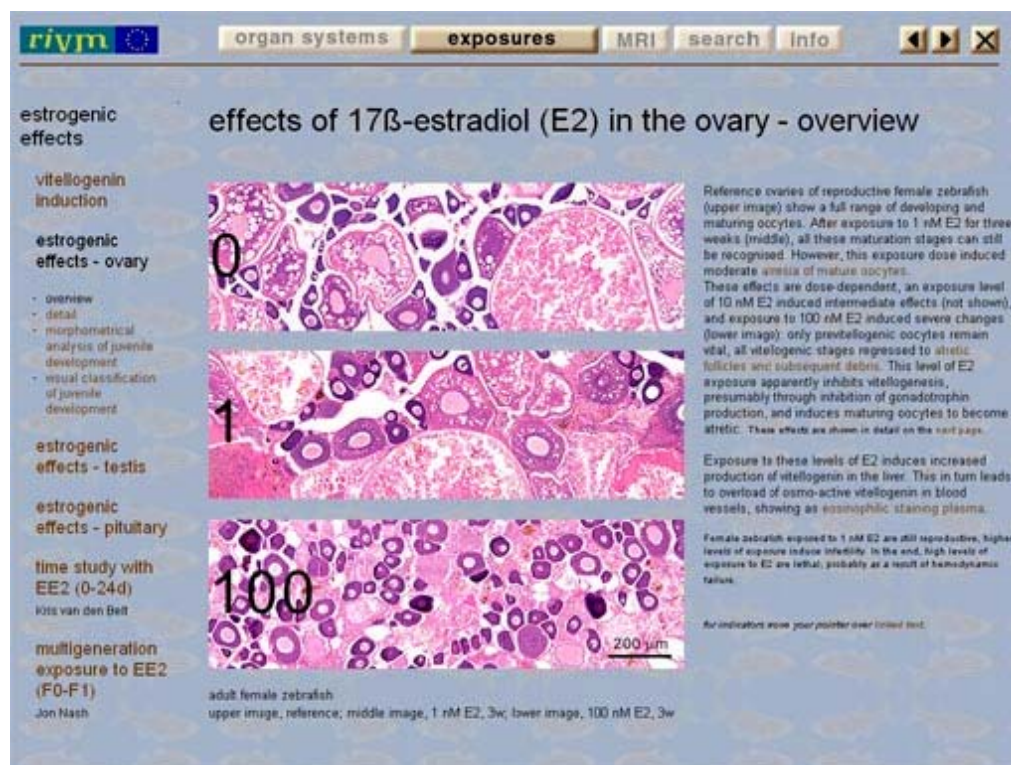
Fig. 2.4.1 – Screenshot of the Toxicological Pathology Atlas of Small Laboratory Fish
(http://www.rivm.nl/fishtoxpat). The top menu indicates the main sections of the atlas and is available
throughout the application. The left-hand menu is activated by selection of specific items and contains links to
pages with detailed information. These contents pages have a title, a single or a composite image, or a
(animated) sequence of related images, and a descriptive text, which contains links which activate indicators to
areas of interest on the image.

The atlas was presented at the annual meetings of the Society of Environmental Toxicology
and Chemistry (SETAC) Europe and America in Brighton and Nashville, TN, respectively,
both in 2000. It was announced to expert organisations, and hyperlinks are now available at
websites of e.g. Society of Toxicological Pathology. A mailing list with approximately 200
addresses of colleagues in this area is used to announce major updates.

The atlas has been used in practice as a basis for an OECD workshop held in September 2002
at the RIVM. Also it is often cited and used in OECD guidelines under development, and it
will be included in a workshop CD ROM to be organised in October 9-10, 2003 (Fraunhofer
Institut, Hannover).

The atlas is available on the institute's website http://www.rivm.nl/fishtoxpat/; for dedicated
users a CD ROM version can be provided.

3.   Partial Life Cycle study, application with reference compounds

3.1.   Introduction

In order to test the practical applicability of the prototype test protocol, a number of reference compounds was tested. The compounds selected were 17β-estradiol (E2), a natural estrogen. Initially, ZM 189,154, a preclinical drug from a pharmaceutical industry R&D program, was tested as anti-estrogen, in accordance with the proposed compound from the OECD panel, but analytical difficulties and potential problems with future supply made us to choose tamoxifen, a therapeutic anticancer drug. For androgen initially methyltestosterone was selected, but appeared to have significant estrogenic properties and thus the non-aromatisable methyldihydrotestosterone was chosen. As anti-androgen the therapeutic drug flutamide was selected and for antithyroid the therapeutic drug propylthiouracil. Finally, a field sample from a suspected hot spot for estrogenic effects was investigated.

3.2.   PLC-test with estrogen: 17β-estradiol

Introduction

As reference estrogen, 17β−estradiol (E2) was chosen. E2 is one of the natural estrogens in vertebrates. It is often used a reference compound, although many researchers prefer ethynylestradiol (EE2), a synthetic pharmaceutical and the active ingredient in oral contraceptive. Both E2 and EE2 are found in environmental surface water samples mainly through sewage treatment effluent, but EE2 is known to be a more potent estrogen.

Materials and methods

E2 (CAS 50-28-2, Fluka) was dissolved in stock medium ethanol 96% and stored at 4 ^{0}C. End solutions were prepared from this stock and contained maximal 0.01% solvent.

The test was performed as described in "General Protocol". Briefly, range finding tests were conducted up to 21 days using concentrations from 1-100 nM with adults and 1-1000 nM with fry. These tests revealed significant effects in histology in both sexes such as accumulation of VTG in circulation, body cavities and interstitial tissues, with dilation of these compartments. In excessive cases this resulted in hydrops, ascites and abduction of scales (see Atlas). This increased vitellogenesis was also observed in 4 dph larvae at 10 nM E2 and higher. In addition, hepatocellular basophilia was seen in conjunction with vitellogenesis, and collapse of the ovaries (extensive atresia and absence of vitellogenic oocytes). The absence of vitellogenic oocytes in the 10 nM-exposed females indicated cessation of reproduction at this concentration. Therefore, 1 nM was chosen as the top concentration for the PLC test, with 0.32 and 0.1 nM as mid- and low concentrations. Analysis of the exposure medium for actual concentrations of E2 revealed a gradual decline of 102.0 - 28.4 - 12.5 - 3.8 (percentage of nominal value of 1 nM) at days 1-4, respectively. A similar decline was found in the 1000 nM medium at days 1-2.

Adults in triplicate spawning units per concentration were exposed for 21 days, and eggs were collected, incubated and juveniles were sampled after 42 days of exposure to the same or complementary medium compared to their parents. To examine effects of high E2 concentrations on histopathology of the gonads in more detail, data from the preceding range-finding assay (range 1 - 10 - 100 nM) were included.

Table 3.2.1 - Reproduction parameters of P generation after exposure with E2

concentration (nM)	number of clutches [1]	clutch size	total number of eggs	fertilisation rate (%)
control	7.0 ± 0 [a]	183 ± 8 [a]	1280 ± 59	61.3 ± 16.8
0.1	6.5 ± 2.1 [a]	243 ± 68 [a]	1649 ± 958	72.8 ± 1.1
0.32	4.3 ± 0.6 [a]	234 ± 56 [a]	1009 ± 226	67.4 ± 24.9
1	3.3 ± 2.5 [a]	448 ± 199 [a]	1159 ± 465	70.3 ± 28.5

All values are average ± sd of three spawning units (two in control and 0.1 nM due to non spawning).
[1] maximum number of clutches is 8.
[a] significant (p<0.05), linear regression; non spawners in control and 0.1 nM are not taken into account.

Animals were monitored daily for general health and clinical effects such as mortality, abnormal behaviour and appearance. Eggs were monitored for fertility and hatching. At termination of the experiment, animals were euthanised, length and weight were measured (juveniles), and blood was collected from adults for VTG determination. Animals were fixed *in toto* for histopathology of target organs (gonads, plasma, liver, etcetera), or for further development of VTG immunohistochemistry. The results reported below are from the PLC, unless specified otherwise.

The experiments were approved by the Institute's Animal Experiment Committee (AAP 199900019, 199900608, 199800376 and 200100203).

Results and discussion

In life observations - adults

In life observations during adults exposure revealed no effect on clinical appearance and behaviour. In the control and 0.1 nM groups only two out of three units were reproductive, and the data in Table 3.2.1 are based on the reproductive units only. There was a concentration-dependent decrease of the number of clutches, whereas the clutch size increased in a concentration-dependent way. These effects apparently compensated each other, as the resulting total number of eggs showed no change. The fertilisation rate was also unaffected.

Table 3.2.2 - Hatching after exposure to E2

treatment P - F1 (nM)	n[1]	hatching (%)
control - control	2	90.3 ± 2.6
0.1 - control	2	93.5 ± 3.5
0.32 - control	2	87.0 ± 18.4
1 - control	2	85.5 ± 4.9
0.1 - 0.1	2	89.5 ± 7.8
0.32 - 0.32	3	93.0 ± 4.0
1 - 1	2	89.3 ± 2.5

Values represent the average ± sd of all replicates.
[1] number of spawning units

In life observations - juveniles

It appeared that parental nor juvenile exposure had any effect on hatching percentage (Table 3.2.2). No abnormal appearance or behaviour was observed in any of the treatment groups. Increased mortality was recorded with the 1 nM exposed juveniles (Table 3.2.3).

Table 3.2.3 - In life observations of F1 zebrafish exposed to E2 for 42 days

treatment P - F1 (nM)	survival (%)	length (mm)	body weight (mg)	condition factor
control - control	96 ± 1.4[a]	12.1 ± 0.2[b]	25.1 ± 0.5[c,d]	1.32 ± 0.01
0.1 - control	97.8	10.9	20.9	1.60
0.32 - control [1]				
1 - control	90.8 ± 6.8	12.8 ± 0.1	29.7 ± 1.3[d]	1.35
0.1 - 0.1	93.2 ± 3.7	12.4 ± 0.7[b]	28.1 ± 5.3[c]	1.42 ± 0.02
0.32 - 0.32	92.4 ± 1.9	13.0 ± 1.4[b]	33.6 ± 8.5[c]	1.44 ± 0.08
1 - 1	73.3 ± 9.9[a]	14.2 ± 1.0[b]	45.8 ± 10.5[c]	1.41 ± 0.03

Values are mean ± sd of two replicates, except for 0.1 - control (single observation) and 0.32 - 0.32 (triplicate observation).
[1] no data for the 0.32 nM-control group due to insufficient offspring in the 0.32 nM
[a] p<0.05, Dunnett's test
[b,c] p<0.05, p<0.01, linear regression for exposed juveniles
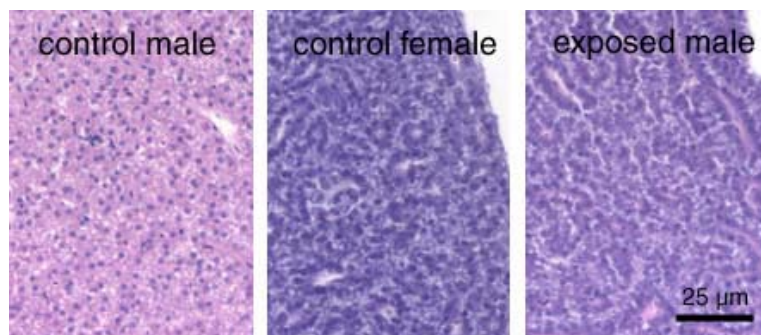[d] p<0.05, T-test

Fig. 3.2.1 - Routine H&E staining of zebrafish liver; control male liver stains acidophilic (eosin), control female liver stains basophilic (haematoxylin) due to high contents of mRNA. Liver of males exposed to E2 stains as control female, due to induction of vitellogenin mRNA expression.

Importantly, there was a significant concentration-related increase in length and body weight in the exposed juveniles (with similarly exposed parents; linear regression), which is possibly related to the anabolic properties of estrogens. There was also an increased juvenile body weigth after exposure of parents only to 1 nM E2.

Histopathology - adults

In the PLC, only moderate effects were observed in the highest exposure group (1 nM): moderate to strong basophilia in male hepatocytes (Fig. 3.2.1), and occasionally eosinophilic (proteinaceous, vitellogenic) intra-/extravascular plasma. Histology in the lowest exposure group (0.1 nM) was comparable to control: no aberrant VTG expression (eosinophilic hepatocytes in males, no colloidal plasma accumulations), no gonadal pathology. There was some variation of the intensity of these effects between this and other studies where E2 was used as a test compound (see Chapters 4.1, VTG analysis; and 3.7, test with field sample). No obvious changes in females (possibly increased atresia in the ovaries) were detected.
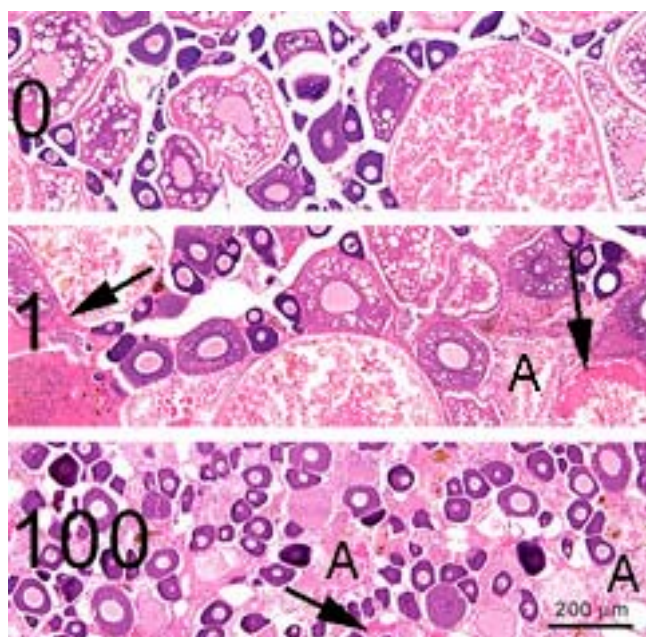

Fig. 3.2.2 - Zebrafish ovaries showing a concentration dependent decrease of vitellogenic oocytes and an increase of atretic follicles (A), compared to control (0). Arrows indicate accumulations of vitellogenin. E2 concentrations (1-100) in nM (range finding test). H&E staining
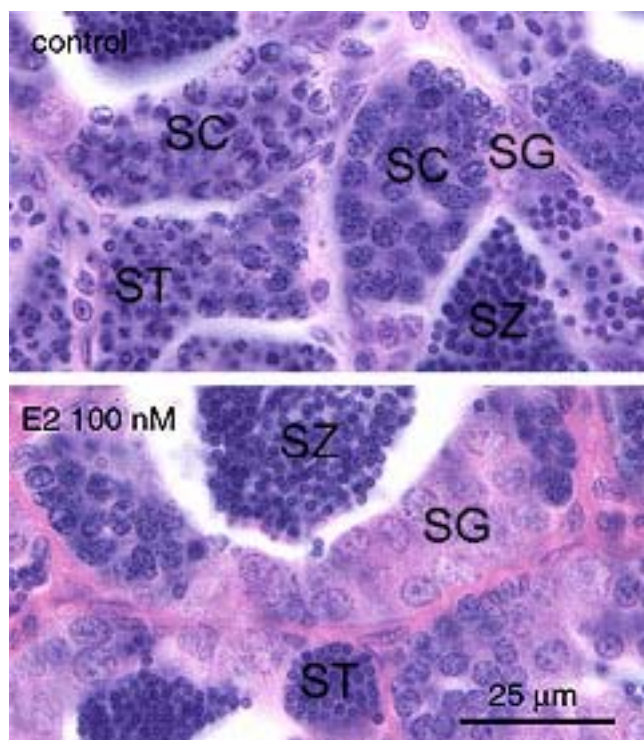
Fig. 3.2.3 - Zebrafish testis showing a decrease of progressed stages of spermatogenesis (SC , spermatocytes and ST, spermatides) and a subsequent relative increase of early stage spermatogonia (SG), after exposure to E2 (bottom), compare to control (top). Note eosinophilic vitellogenin accumulations in the interstitial tissue in the E2-exposed specimen. H&E staining.

At high concentrations (10 nM and up, range finding test), severe intravascular and interstitial VTG accumulations were detected in both males and females (Fig. 3.2.2). No vitellogenic oocytes were present in the ovaries, and a high incidence of atretic follicles, compared to control ovaries, was observed (Fig. 3.2.2).

In the testis, microscopic observation revealed an increase of spermatogonia (Fig. 3.2.3); morphometrically, this increase proved to be relative, since there was a decrease in size of progressed classes of spermatogenic cysts (mainly spermatocytes) in size (Fig. 3.2.4); the
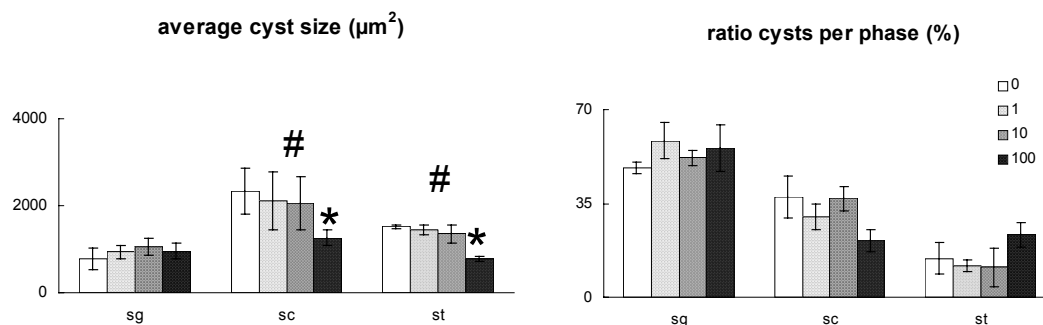

Fig. 3.2.4 -Morphometrical analysis of spermatogenic cysts after exposure to 0-1-10-100 nM E2. There is a concentration dependent decrease in cyst size of spermatocytes (sc) and spermatids (st), linear regression , $p < 0.05$ (#); when compared to the control, sc and st are smaller in the highest concentration ($p<0.05$, t-test). sg, spermatogonia.
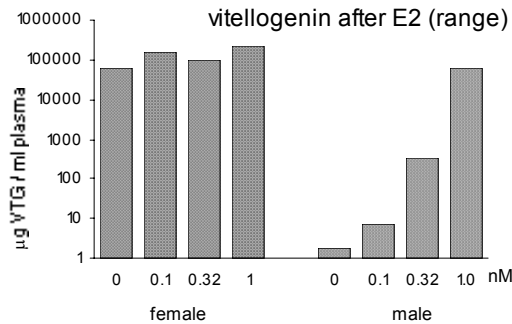
Fig. 3.2.5 – VTG-ELISA of zebrafish plasmas after exposure to a range of E2 concentrations, given as nM. Each bar represents a mixed sample of either 2-3 females or 3-5 males.

suggested decreased proportion of spermatocytes was not statistically significant. This indicates a decreased proliferation of spermatogonia and a decreased progression of differentiation. These conclusions were further supported by labelling experiments with BrdU and immunostaining of PCNA (not shown). There were no aberrations of Leydig or Sertoli cells.

Other observations included occasional chronic inflammation and fibrosis in pancreas or bile ducts, and were considered as background pathology.

Vitellogenin Pooled plasma of 2-3 females or 3-5 males was analysed for VTG contents with ELISA (M. Fenske, UFZ Leipzig; Fig. 3.2.5). These values revealed a concentration dependent increase in males after exposure to E2, up to a similar value as found in females after exposure to 1 nM E2. Although these represent mean values, since they were obtained from pooled plasmas, the increase could not be verified statistically on these singular entries. There was no change in females. Details on methodology and interpretation of VTG analysis are further discussed in the chapter on VTG analysis (see Chapter 2.1).

Histopathology - juveniles

Vitellogenesis in juveniles was not convincing (with 1 nM as highest concentration). On the other hand, there was a marked statistically significant shift in sex ratio, after each of the exposure concentrations. This shift is mainly due to decreased percentage of males and increase of undifferentiated individuals (Fig. 3.2.6 left). This indicates either a mere delay of differentiation, or a real shift in sex ratio, depending on final phenotype of the yet undifferentiated specimen. The results from experiment with the field sample (Chapter 3.7), using only 1.0 nM support the latter option, since the significant shift in sex ratio observed in this case was mainly due to the absence of males and an increase of females after the E2 exposure (Fig. 3.2.6 right panel). This experiment also showed that the induced shift is due to juvenile exposure, since there was no effect of parental exposure only. The different outcome between these two experiments may result from a generalised delayed development in
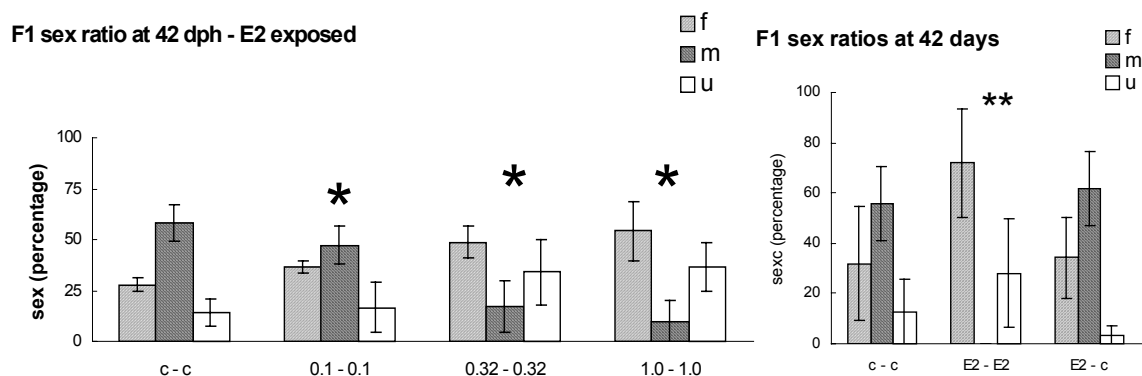
Fig. 3.2.6 - Sex ratios in two separate assays after 21 (P) - 42 (F1) days of exposure. Both graphs show the relative presence of female (f), male (m), and undifferentiated (u) specimen. The left graph shows the effects of a concentration range of E2 (0.1 - 0.32 - 1.0 nM; c, control), adults and offspring exposed, in the left graph, there is only exposure to 1.0 nM, both in adults and offspring (middle set), or in adults only (right set). *, **, p< 0.05, 0.01 respectively, T- test.

juveniles in the first experiment, indicated by the lower overall average length (13.0 ± 1.6 mm) compared to the overall average length in the second experiment (16.5 ± 0.4 mm). In this second experiment, ovary maturation was determined on the presence of the most matured stage of oocytes, defined by size and progression of vitellogenesis (Fig. 3.2.7). This analysis showed that exposure to 1 nM E2 inhibited maturation of oocytes significantly, compared to control.

Population modelling

By means of the zebrafish model no significant effects of the E2 treatments in the PLC-test were found on population survival and population size of zebrafish (Fig. 3.2.8), in spite of skewed sex ratio and decreased juvenile survival. The large CVs of extinction chances and population size of the model simulations have probably prevented the appearance of more

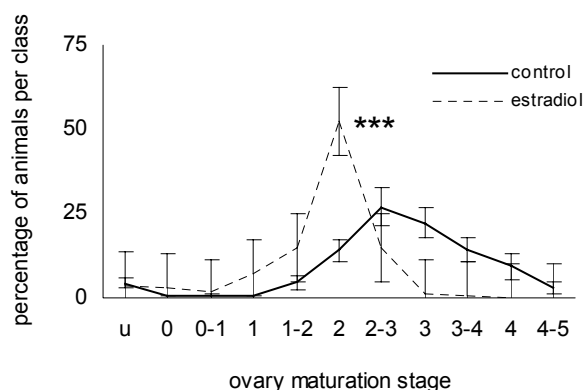*Fig. 3.2.7 - Staging of ovaries according to the most advanced oocytes present in the gonad, in control animals and after exposure to 1 nM E2. u, undifferentiated, further numbers on the horizontal axis indicate arbitrary classes (defined in the atlas).* Exposure to E2 induced a statistically significant shift to the left (***p<0.0001 in a Chi-squared test), indicated a delayed development in females.

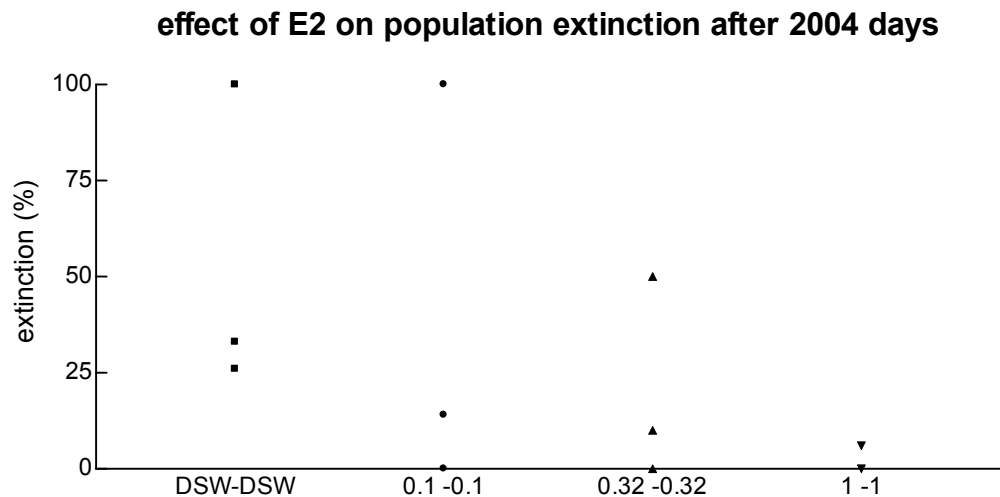**effect of E2 on population extinction after 2004 days**

Fig. 3.2.8 - Population model for E2. No effects of treatment on population extinction. Total extinction in one of the control and 0.1 populations due to non-spawning. Treatments indicate P and F1 exposure, respectively. DSW, control medium; other groups are indicated with nominal values of E2 exposure in nM.

obvious effects of E2.

Again, the 10 nM concentration was not tested because no eggs were found to develop at this concentration (data from range finding). Therefore, this concentration was not included in the PLC-test, but it can be anticipated that at 10 nM E2 the population's reproduction and survival are severely compromised.

Conclusions

- At 10 nM E2 ovaries were completely blocked within a few days. Therefore, this concentration must be considered as incompatible with normal reproduction and survival of the population.

- At 1 nM there was a lower number of egg clutches but an increased number of eggs per clutch. Total number of eggs was unaffected. Survival was decreased in offspring.

- There was a dose dependent increase in length and weight of the exposed juveniles; this could be related to the anabolic properties of estrogenic hormones.

- At 0.32 nM and higher, vitellogenesis was induced in males, and the sex ratio was skewed in offspring with a preference for females to develop. Therefore, this histologically detectable VTG increase could serve as an indicator for adverse other effects. Overall, this was the lowest observed adverse effect level.

- Spermatogenesis was inhibited in adult males (1 nM, concentration dependent), as well as ovary development in juvenile females (1 nM) .

- Up to 1 nM, combined effects had no influence on survival of the population or population size.

- In view of the decline in E2 concentrations during the exposure period, the results are most likely an underestimate when expressed as nominal values.

3.3.   PLC-test with anti-estrogen: tamoxifen

Introduction

Tamoxifen was chosen as anti-estrogen reference compound. Initially, ZM 189,154, a preclinical drug from a pharmaceutical industry R&D program, and propesed by the OECD panel, was tested. However, analytical difficulties and potential problems with future supply made us shift to the alternative tamoxifen, a therapeutic anticancer drug used in breast cancer therapy. Tamoxifen binds directly to the estrogen receptor and acts as an (partial) antagonist, or, depending on the estrogen receptor type, cell or target tissue, as an estrogen agonist (Gallo and Kaufman, 1997; Dhingra, 1999).

Materials and methods

Tamoxifen (CAS RN 10540-29-1, Sigma-Aldrich) was dissolved in stock medium with DMSO as solvent. Stocks were stored at 4 °C. The final concentration of the solvent was 0.01% in all test media.

The test was performed as described in "General Protocol" (4.2). Briefly, a range finding test was conducted using nominal concentrations of 0.01 - 0.1 - 1 - 10 mg/L in a 10 day test with larvae and adults. In that test, toxicity was observed in the 1 and 10 mg/L exposed animals and therefore the test concentrations for the PLC were set at 32, 100 en 320 μg/L. There was insufficient egg production after incubation with 320 μg/L; additional groups of eggs from control parents were incubated with 3.2 and 10 μg/L to obtain comprehensive information on juvenile parameters.

A full chemical analysis (days 1-2-3) of tamoxifen was only performed in the 32 μg/L medium of the PLC. In the adult tanks, there was an immediate decline at day 1 to average 4.5% of nominal values, and values of 1.1-2.5% at days 2-3. The initial day 1 value in juvenile tanks was 95.9%, with decreases to 9.1-5.3% at days 2-3. The difference between adult and juvenile day 1 values may indicate a high consumption in the adult fish tanks, possibly due to a difference of fish load. Higher average day 1 values were recorded from the high concentration exposure tanks with adults, i.e. 33.0% with 100 μg/L and 15.4% with 320 μg/L tamoxifen, higher concentrations are possibly more close to a biodegradation saturation level.

Thus, actual concentrations were considerably lower than nominal values (average 11.3% for adults and 36.8% for juveniles). There also may not have been a substantial difference between the exposure levels in the two highest concentration groups (100 and 320 µg/L). It can, however, not be excluded that differences between nominal and actual concentrations result from high initial intake, and in that case, there would have been high initial exposure levels.

In the PLC, adults were exposed in triplicate spawning units per concentration for 21 days, eggs were collected, incubated and juveniles were sampled after 42 days exposure to test or control medium. Animals were monitored daily for general health and clinical effects such as mortality, abnormal behaviour and appearance. Eggs were monitored for fertilisation and hatching. At termination of the experiment, animals were euthanised, length and weight were measured (juveniles), and blood was collected for future VTG analysis (adults). All animals were fixed *in toto* for histopathology of target organs.

The experiment was approved by the Institute's Animal Experiment Committee (AAP 20000796)

Results and discussion

In life observations - adults

Haemorrhages and locomotion abnormalities were observed in the majority of tamoxifen exposed adult fish. Mortality was recorded in the highest exposure group. These findings may

Table 3.3.1 - reproduction parameters

concentration (µg/L)	number of clutches[1]	clutch size	total number of eggs	fertilisation rate
control	6.3 ± 1.0 [ad]	399 ± 130	2426 ± 309 [ac]	87.5 ± 3.2 [b]
32	6.0 ± 2.0 [d]	292 ± 80	1816 ± 883 [c]	62.4 ± 0.4 [b]
100	4.3 ± 1.0 [d]	337 ± 59	1444 ± 153 [c]	58.3 ± 29.7 [b]
320	1.3 ± 2.0 [ad]	497 ± 434	458 ± 414 [ac]	58.4 ± 20.0 [b]

All results represent average ± sd of three spawning units.
[1] maximum number of clutches is 7.
[a] p<0.01; Dunnets multiple comparison-test
[b] p<0.05 and [c,d] p<0.01; linear regression test; negative correlation

Table 3.3.2 - hatching of eggs exposed to tamoxifen

treatment P - F1 (µg/L)	n	hatching (%)
control – control	3	81.5 ± 9.7 [a]
32 – control	3	57.9 ± 18.4 [a]
100 – control	3	45.7 ± 39.7 [a]
32 – 32	3	66.6 ± 8.9
100 – 100	3	41.4 ± 29.8
control – 32	2	100 ± 0
control – 100	3	78.7 ± 13.8
control – control [1]	4	61.0 ± 2.9
control – 3.2 [1]	5	50.4 ± 5.9
control – 10 [1]	5	52.2 ± 3.8

Data are average ± sd of the number of spawning units shown in column n.
[1] additional groups, tested to compensate for failure of the 320 µg/L group, of which no eggs were obtained.
[a] p=0.0018 (paired t-test with repeated usage of the control group)

be associated with the incidental inflammatory processes observed by histopathology.

The number of egg clutches was reduced in a concentration dependent way (Table 3.3.1), but there were no effects on clutch size, and consequently the total numbers of eggs was also reduced in a concentration dependent way.

The fertilisation rate was reduced in a concentration dependent way.

In life observations - juveniles

Hatching showed considerable variation (Table 3.3.2). For this reason, the suggested decreased hatching rate after parental exposure could only be confirmed statistically when comparing the control - control with groups with only parental exposure to 32 and 100 µg/L (no statistical significant difference when comparing 32 - control and 100 - control with 32 - 32 and 100 - 100).

There was a high intercurrent mortality in the juveniles exposed to 100 µg/L tamoxifen, for which reason these groups were discontinued, but there was no effect on survival at lower concentrations (Table 3.3.3). Length and body weight (but not condition factor) were significantly reduced after exposure of juveniles to 32 µg/L tamoxifen, regardless of parental exposure. Only weight was reduced at 10 µg/L tamoxifen.

Table 3.3.3 - in life observations in F1 zebrafish exposed to tamoxifen for 42 days

treatment P - F1 (µg/L)	n[1]	survival (%)	length (mm)	weight (mg)	condition factor
control – control	3	75.0 ± 8.7 [d]	15.9 ± 0.5 [a]	65 ± 3.9 [a,c]	1.52 ± 0.09
32 – control	3	69.3 ± 20.2	16.6 ± 0.7	72.3 ± 3.1 [b]	1.49 ± 0.06
100 – control	2	85 ± 12.7	15.9 ± 0.3	63.5 ± 2.0	1.50 ± 0.08
32 – 32	3	71.7 ± 17	14.5 ± 0.8	54.8 ± 3.8 [b,c]	1.48 ± 0.11
control – 32	2	52 ± 15.6 [d]	13.8 ± 1.2 [a]	48.9 ± 8.1 [a]	1.58 ± 0.02
control – control[2]	4	72 ± 0.3	16.4 ± 0.3	68.4 ± 2.2 [e]	1.46 ± 0.07
control – 3.2 [2]	5	69.6 ± 4.8	16.2 ± 0.7	65.9 ± 6.2	1.44 ± 0.03
control – 10 [2]	5	72 ± 6.9	16.1 ± 0.2	60.8 ± 0.8 [e]	1.38 ± 0.04

[1] Data values are average ± sd of the number of replicates given in column n
[2] additional groups, tested to compensate for reproductive failure in the high concentration groups (100, 320 µg/L).
[a,b] p <0.05, paired T-test
[c,e] p <0.05, Dunnets multiple comparison test
[d] paired T-test not executable because of absence of variation (both 70% survival) in the control observations that are coupled to the two c - 32 groups. Analysis of the confidence intervals around the c – 32 group indicates that survival in this group is not different from c – c.
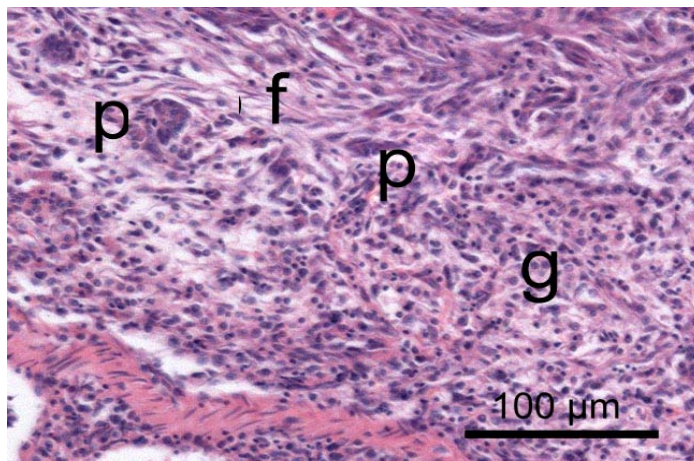
Fig. 3.3.1 - Pancreas with inflammation from a male zebrafish exposed to 100 µg/L tamoxifen for 21 days. Granulomatous (g) and fibrotic (f) areas can be distinguished; only occasional clusters of pancreatic parenchyma (p) remain.
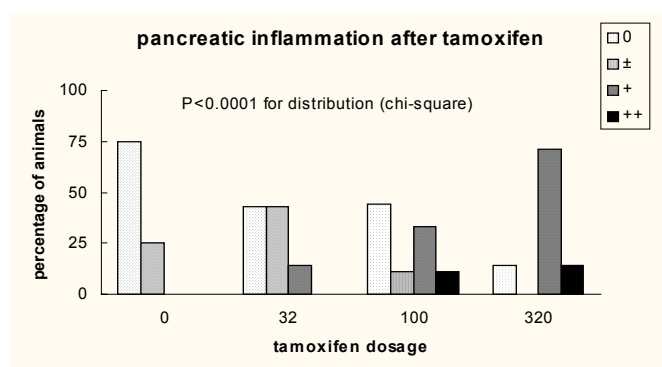
Fig. 3.3.2 - Semi-quantitative assessment of severity of inflammation of the pancreas after exposure to tamoxifen for 21 days. The severity of inflammation shows a concentration dependent increase (p<0.0001, Chi-squared in a contingency table).

Histopathology - general

Animals from this experiment showed visceral (mainly pancreatic) inflammatory lesions with hyperaemia, infiltration of mononuclear cells, and fibrosis (Fig. 3.3.1). The severity, based on semiquantitative assessment of extension of the inflammatory process, was dose-dependent (Fig. 3.3.2).

Other inflammatory processes as mononuclear infiltrations were found in the gills and skin, although these were not concentration-related.

The causative agent for this complex or these combined lesions (both in gills, skin and pancreas) remains unknown. Mycobacteriosis could be excluded by Ziehl-Nielson stain, but non-identified structures, reminiscent of protozoans (Reichenbach-Klinke, 1980) were observed in skin (see atlas). A possible explanation could be that tamoxifen had a direct or indirect immune modulating effect. Immune modulating effects of tamoxifen have been described in humans after breast cancer therapy (Robinson et al., 1993).
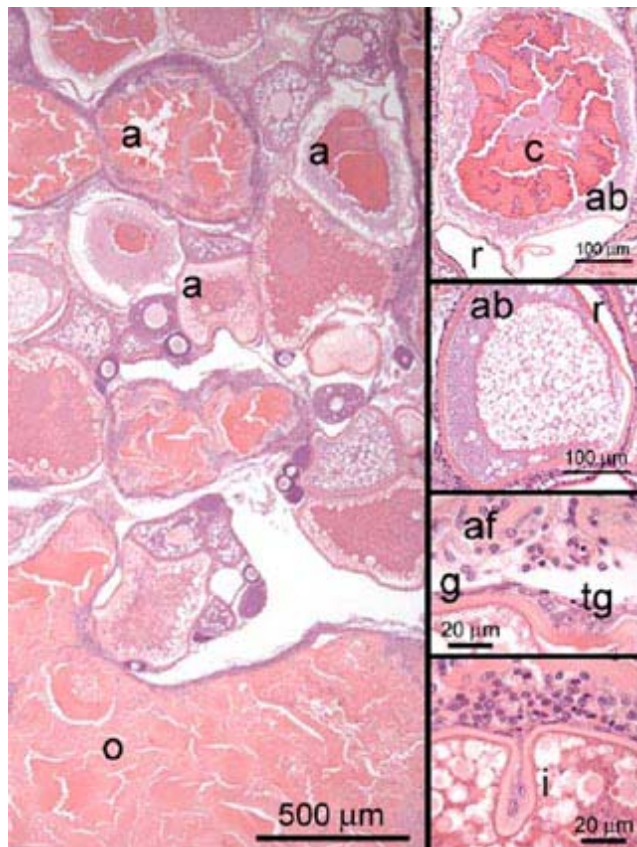
Fig. 3.3.3 - Degeneration of mature oocytes after exposure to tamoxifen (320 µg/L). Mature oocytes are atretic (a), showing condensation (c) of vitellogenin granules, accumulations of basophilic granular material (ab), and retraction (r) of the oocyte body from the zona radiata and/or from the granulosa cell layer. Foci of transformed granulosa cells (tg; compare to granulosa cells with a normal aspect, g) are present, as well as sharp invaginations of the zona radiata (i). The oviduct (o) is filled with degenerated eggs.

Table 3.3.4 - ovary histopathology after exposure to tamoxifen

concentration (µg/L)	condition of <u>mature</u> oocytes					atretic follicles	degenerated eggs in oviduct
	mature oocytes present	membrane invagin.ation	central VTG condensation	fusing of VTG granules	amorphous degeneration		
0	± ·· +	-	-	-	-	±	-
32	- ·· +	-	±	±	-	± ·· +	++
100	+	- ·· +	± ·· +	± ·· +	- ·· +	± ·· +	± ·· ++
320	+	+	± ·· +	+ ·· ++	± ·· +	+	++

Results represent semi-quantitative observations (visual scores): -, not present; ±, +, ++, present to a minor, moderate, strong degree, respectively.

Histopathology – adult females

Ovary - After exposure to tamoxifen, there was a deteriorating vitality of mature oocytes, as indicated by central condensation and fusion of VTG granules and amorphous degeneration (Fig. 3.3.3). The ovaries also contained many atretic follicles. These findings were associated with focal transformed morphology of granulosa cells (increased cell height, enlarged and hypochromatic nuclei, occasionally multilayered), and with sharp invaginations of the oocyte membrane (zona radiata including granulosa cell lining).

Additionally, all tamoxifen-exposed females had oviducts filled with degenerated eggs. These effects appeared to be concentration-dependent (Table 3.3.4), as they were found most severely in the highest dosage group. These changes fit well with the observed decreased egg production.

Table 3.3.5 - plasma vitellogenin immunohistochemistry

activation intensity	**females**[a][b] concentration tamoxifen (mg/L)				**males** concentration tamoxifen (mg/L)			
	0	**32**	**100**	**320**	**0**	**32**	**100**	**320**
-		1		2	6	6	6	6
±		1	3	1				
+		1						
++	3							

Semi-quantitative observations (visual scores) of vitellogenin immunostaining intensities; categories are: -, no staining, ±, +, ++, weak, moderate, strong intensity of immunostaining. Data are numbers of animals in each category.
[a]Vitellogenic oocytes had strong positive immunostaining, irrespective of exposure to tamoxifen
[b]distribution of immunostaining intensity is concentration-dependent, $p=0.0215$, Chi-square test
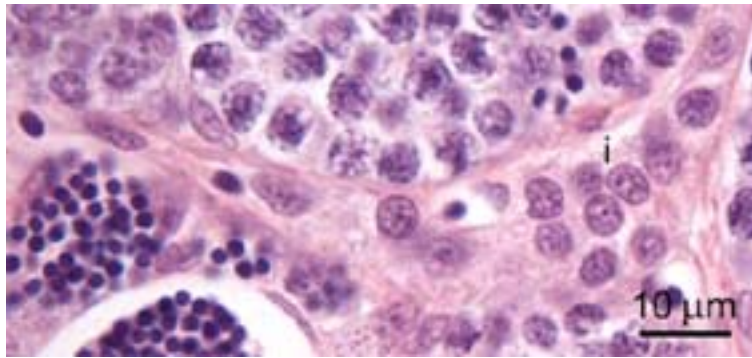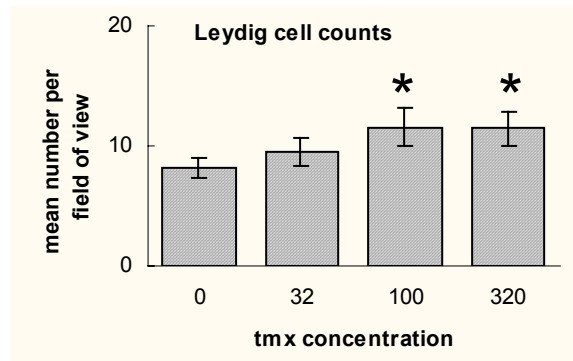
Fig. 3.3.4 - Detail microphotograph of a distended interstitial compartment of the testis after exposure to 320 µg/L tamoxifen. Note the cluster of interstitial cells (i).

Fig. 3.3.5. Number of interstitial Leydig cells per microscopic field (obj. x 40). Each bar represents the average of 4 fields of 5-6 fish. *p<0.05, T-test.

Liver/plasma – On H&E stained sections, there was no effect of tamoxifen exposure on liver basophilia intensity. However, immunohistochemistry for VTG showed reduced staining intensity, significantly decreasing with concentration based on blind semi-quantitative scoring (Table 3.3.5).

Histopathology – adult males

Testis - Tamoxifen exposed males showed several changes in the testis. There was expansion of the interstitial compartment, edema, and proliferation of interstitial Leydig cells, which, in contrast to their solitary occurrence in control specimen, were observed in large clusters (Fig. 3.3.4). The increased presence of Leydig cells was statistically apparent as increased cell numbers per high magnification field of view (Fig. 3.3.5). Another feature was asynchrony of spermatogenesis, *i.e.* spermatogenic cells of subsequent stages occurring within a single spermatogenic cyst (Fig. 3.3.6), as opposed to one single stage per cyst in controls. These changes appear to be concentration dependent, although this was not confirmed statistically. There were no obvious changes in size or ratio of the various spermatogenic stages (confirmed by morphometry, not shown), suggesting that neither induction of meiosis, nor general rate of maturation are altered by tamoxifen. The observed asynchrony, however, indicates a disturbed meiotic maturation, which may have contributed to the tamoxifen-dependent decrease of fertilisation rate.
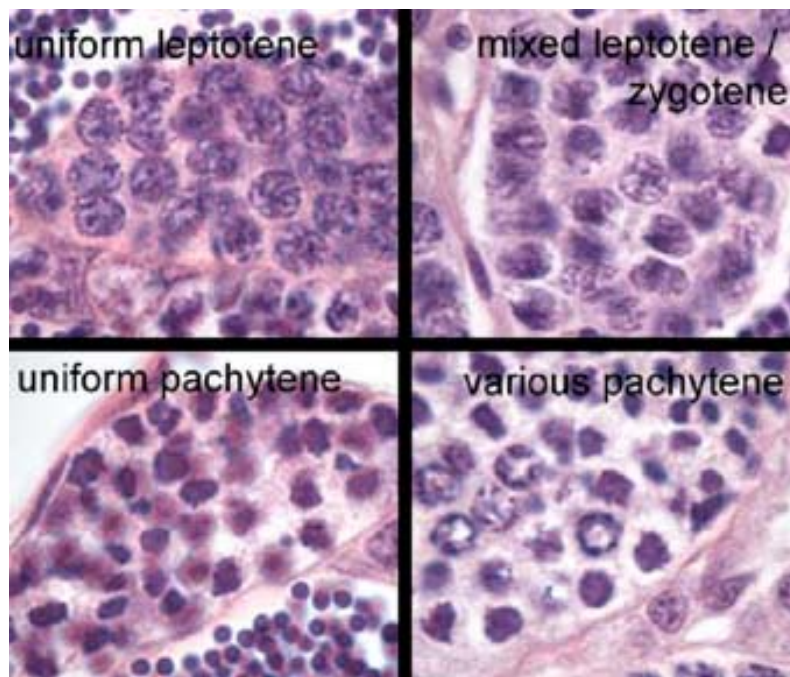
Fig. 3.3.6 - Detail microphotographs of spermatogenic cysts, illustrating asynchronous meiotic maturation after exposure to 320 µg/L tamoxifen (right panel), compared to uniform meiotic stages in controls (left panel).

Liver/plasma - There were no apparent changes in the liver, nor was there a change in VTG immunostaining intensity in plasma.

Histopathology - juveniles

Sex ratios - There was a significant, nearly complete sex reversal towards the masculin phenotype after exposure to the highest available concentration of tamoxifen (32 µg/L, higher concentrations were not available due to reproductive failure; Fig. 3.3.7), as evaluated from gonad histology. This effect was related to exposure of the F1 generation (no effect after
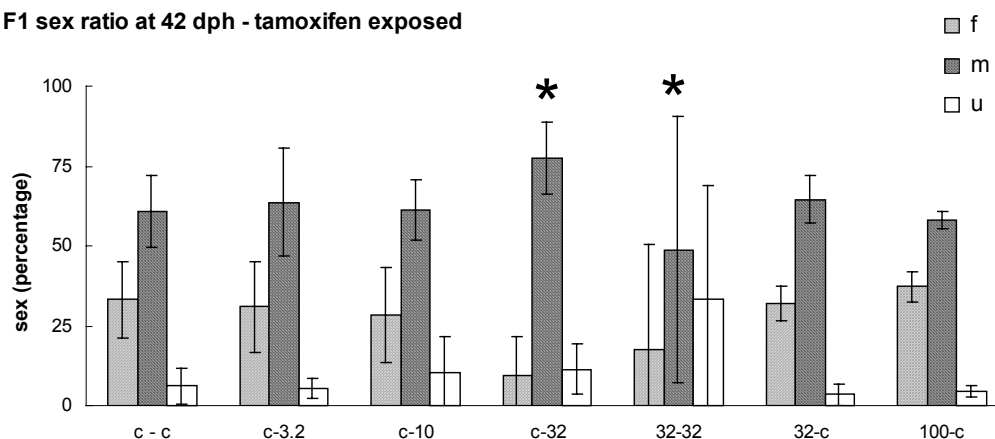
Fig. 3.3.7 - Sex ratios after exposure to tamoxifen. Exposures are indicated as P-F1, respectively; c, control (carrier, no tamoxifen). 79-491 Juveniles were evaluated per group. *: $p < 0.05$, T- test. Differences of statistical outcomes between 32-c and 100-c are due to different number per treatment group. Note that the exposure range of juveniles differs from that of parents (see above); this lower exposure range was chosen because of high mortality after F1 exposure to higher concentrations.

**effect of tamoxifen on population
size after 2004 days**

Fig. 3.3.8 - Population modelling for tamoxifen. Percentage of extinct population of the 100 simulations after 2400 days. Treatments indicate P and F1 exposure, respectively. DSW, control medium; other group is indicated with nominal values of tamoxifen exposure in μg/L.
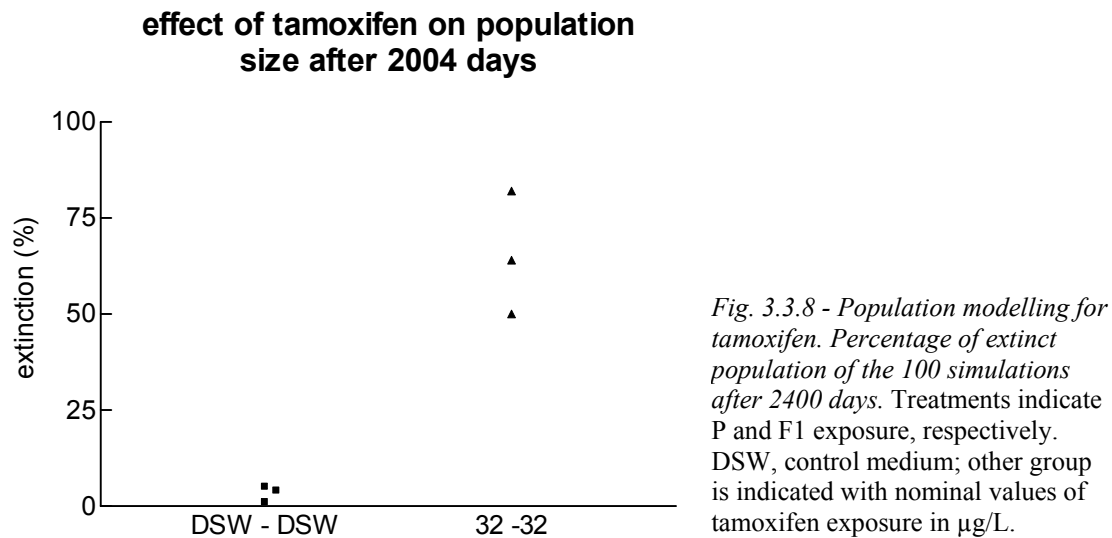
exposure of only the parental generation to the highest concentrations). Exposure of P and F1 generations induced an increase of the ratio of undifferentiated gonads, at the expense of juveniles with both feminin and masculin differentiation.

Other histopathology - Inflammation of primary and secondary gill lamellae (mononuclear infiltrate) was observed in some groups, however, this seemed to be without association with treatment. No other aberrations were found.

Larvae from the range-finding pilot, which were exposed to up to 300-fold higher concentrations compared to this partial life cycle (10,000 μg/L) showed severe dysmorphogenesis (an-/dysencephalia, an-/dysophthalmia, oro-pharyngeal and intestinal malformations), suggesting general toxicity. Most embryos in the 1 μg/L group had a normal appearance, only occasional specimen showed the malformations (data not shown).

Population modelling

Only two concentrations of tamoxifen could be evaluated on population effects with the zebrafish model, due to incomplete data. However, significant adverse effects were already observed at the level of 32 μg/L, on extinction changes (Fig. 3.3.8) and on average number of adults present during 2004 days of population simulation. The decreased population size is due to combined effects of the major shift of sex ratio to males (>90%) and reduced egg production at this exposure concentration.

Conclusions

- In females the number of egg clutches was decreased in a dose dependent fashion; histologically, vitellogenesis was inhibited, and vitality of mature oocytes was not sustained, leading to a concentration dependent increase of typical atresia, at higher dosages associated with extensive phagocytosis. The presence of degenerated eggs in the oviduct may indicate inhibited oviposition, or further degeneration of eggs that were already subvital at ovulation.

- In progeny, reduction in survival, length and weight were seen. Hatching was reduced after parental exposure.

- In males, the most striking effect is asynchronous meiotic maturation of spermatogenesis. This effect might have been responsible for the observed decreased fertilisation, although the relative contribution of effects in males and females to reproductive failure cannot be assessed.

- The animals showed a concentration-related severity in visceral inflammation, as well as inflammations in gills and skin. The pathogenesis of these processes remains unknown, but an immunomodulatory effect of tamoxifen, in combination with infections (possibly with protozoa) was hypothesised.

- All histopathological changes appeared to be concentration dependent. In addition, tamoxifen induced sex reversal, indicated by a nearly 100% male population.

- The skewed sex ratio, combined with reduced egg production, had an adverse impact on population dynamics.

- For most variables the lowest effect concentration was 32 μg tamoxifen/L, namely for reproduction and development parameters, histopathology in the ovary, sex differentiation, inflammatory processes, and for population effects.

3.4.　　PLC-test with androgen: methyldihydrotestosterone

Introduction

As reference androgen in the PLC, 17α-methyldihydrotestosterone (MDHT) was chosen. As initially methyltestosterone appeared to produce predominant estrogenic effects attributed to aromatisation (data not shown), the non-aromatisable MDHT was selected in order to focus on specific androgenic effects.

Materials and methods

MDHT (CAS 521-11-9, Sigma-Aldrich) was dissolved in stock medium with DMSO as a solvent. Stocks were stored at 4 °C. The final solvent concentration in all test media was 0.01%. The test was performed as described in "General Protocol". Briefly, a range finding test was conducted using concentrations of 0.1 - 1 - 10 - 100 - 1000 μg/L in a 4-day test with fry and a 9-day test with adults. Based on the undesired estrogen-like effects in the two highest concentrations (VTG induction, regressed ovaries), 100 μg/L was determined as the highest concentration for a PLC, thus aiming at adequate survival and reproduction at least in the mid en low concentration group. A first PLC was conducted with 1 - 10 - 100 μg/L, but due to experimental failure the test with offspring was discontinued. The PLC was repeated with a lower dose range (0.1 - 1 - 10 μg MDHT/L), because of absence of offspring in 100 μg/L, and results from this test are principally presented here. In addition, histopathological effects in adults from the first PLC are included where applicable.

Analysis of actual MDHT concentrations revealed a rapid decline of the compound in the test medium, to values below 30-50% of nominal within one day, as determined in 10 μg/L. Because of the relatively high detection limit of the analytical method, the actual exposure at lower concentrations could not be determined. At the high concentrations, actual levels were below the detection limit after the first day (1 μg/L) or day 3-4 (10 μg/L).

Adults in triplicate spawning units per concentration were exposed for 21 days, and F1 juveniles were sampled after 42 days exposure in test or control medium. Animals were monitored daily for general health and clinical effects such as mortality, abnormal behaviour and appearance. Eggs were monitored for fertility and hatching. At termination of the

experiment the animals were euthanised, and blood was collected for VTG analysis. Animals were fixed in toto for histopathology of target organs and tissues (plasma, liver, gonads). The experiment was approved by the Institute's Animal Experiment Committee (AAP 200100295).

Results and discussion

In life observations - adults

During the entire 21-day exposure period, the overall health condition appeared good, with exception of a single mortality and increased respiratory activity in most animals in the 10 μg group.

The total number of eggs produced and the clutch size were significantly reduced in the 1 and 10 μg/L groups, compared to control (Table 3.4.1). The total number of eggs was reduced in a concentration-dependent fashion ($p < 0.0001$; $r^2 = 0.8008$). In the 10 μg/L group, egg production ceased completely after a few days, resulting in a total of only two clutches in all spawning units early in the test. These eggs were not used for further testing. Clutch size was decreased in a concentration dependent way, but only when considering the treatment range without the 10 μg/L.This approach, which ignores the absence of an effect of MDHT on the early clutches in the exposure period, suggests that there is an effect of MDHT on clutch size but only after a defined incubation time. There was no change in egg production in the 1 μg/L group over time. Fertilisation rate was not affected.

Table 3.4.1 - Reproduction parameters in F0[1]

treatment (μg/L)	number of clutches [2]	clutch size	total number of eggs	fertilisation rate (%)
control	4.0 ± 3.0	765 ± 316 [c]	2589 ± 827 [d]	77 ± 24
0.1	5.7 ± 2.0	384 ± 3 [c]	2177 ± 590 [d]	64 ± 19
1	4.7 ± 1.0	231 ± 97 [ac]	1040 ± 291[ad]	58 ± 8
10	0.7 ± 0.5	364 ± 135 [a]	364 ± 135 [bd]	79 ± 27

[1] average ± sd of three spawning units (two in 10 μg/L due to non spawning)
[2] maximum number of clutches is 7
[a,b] $p < 0.05$, 0.01, Dunnett's multiple comparison test (compared with control)
[c] $p < 0.05$, regression, ANOVA; [d] $p < 0.0001$, regression

Table 3.4.2 - Hatching

treatment P - F1 (µg/L)	hatching[1] (%)
control – control	90.3 ± 2.6
0.1 – control	82.1 ± 13.7
1 – control	71.5 ± 35.5
0.1 - 0.1	81.6 ± 13.7
1 - 1	67.2 ± 26.6
control - 10	90.6 ± 2.6

[1] average ± sd of three experimental units
No data available for 10 µg/L-control and 10 µg/L-10 µg/L.

In life observations - juveniles

There was no influence of MDHT on hatching (Table 3.4.2).

Abnormal development (curved tails) was observed in a low incidence (maximum 1%) with no apparent relation to exposure and was therefore considered background pathology .

Treatment with MDHT did not affect survival (Table 3.4.3). Length and weight were reduced in the control - 10 µg/L group compared to the control – control and weight reduction alone was observed in the 0.1-0.1 compared to 0.1-control group (note that no offspring was tested

Table 3.4.3 - In life observations of F1 zebrafish exposed to 17 α-MDHT for 42 days

treatment P - F1 (µg/L)	survival (%)	length (mm)	body weight (mg)	condition factor
control – control	89.3 ± 8.1	15.2 ± 0.6[a]	58.4 ± 6.5[c]	1.61 ± 0.03
0.1 – control	86.7 ± 9.3	16.0 ± 0.5	64.0 ± 4.9[b]	1.52 ± 0.02
1 – control	76.8 ± 10.7	15.7 ± 1.0	65.9 ± 13.9	1.64 ± 0.07
0.1 – 0.1	93.2 ± 2.9	15.9 ± 0.4	62.7 ± 5.4[b]	1.52 ± 0.06
1 – 1	88.3 ± 7.1	14.9 ± 1.6	54.1 ± 13[d]	1.50 ± 0.02
control – 10	92.1 ± 6.2	11.9 ± 0.3[a]	26.7 ± 3.6[c]	1.52 ± 0.009

All values are average ± sd of three replicates, except for two replicates in the 1 - 1 group (due too small brood size and insufficient hatching)
No offspring was produced in the 10 µg/L group.
[a,b,c] $p < 0.05$, paired T-test
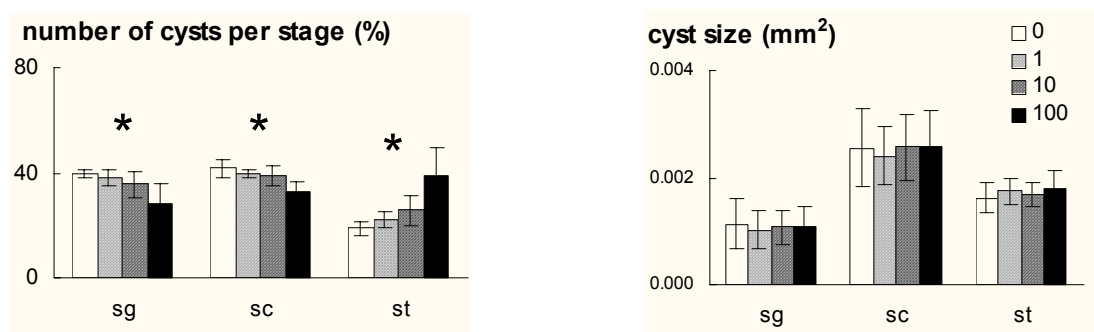[d] significance could not be tested due to insufficient data

Fig. 3.4.1 - Morphometrical analysis of testis after MDHT. Left: relative presence of three consequetive spermatogenic stages (sg, spermatogonia – sc, spermatocytes – st, spermatids). Right: Cyst sizes after in MDHT. *, significant concentration dependent effect, ANOVA. These results are from the first PLC.

from the 10 µg/L adults group). There was no effect of treatment on survival and condition factor; the latter observation indicates that weight and length run parallel in growing animals.

Histopathology - adults

Histological analysis of adults revealed increased vitellogenesis in both males and females in the highest concentration group (10 µg MDHT/L), as deduced from increased hepatocyte basophilia (males) and extravascular acidophilic liquid deposits. These features indicate stimulation of the estrogen receptor (see Chapter 3.2, test with E2), and, assuming that MDTH is not aromatisable, probably result from direct interaction of the androgen with the estrogen receptor, or from directing endogenous E2 towards these receptors. MDHT also induced accelerated spermatogenesis, indicated by the decreased presence of early spermatogenic stages (spermatogonia and spermatocytes) and increased presence of progressed stage (spermatids; Fig. 3.4.1). The size of the spermatogenic cysts did not change.
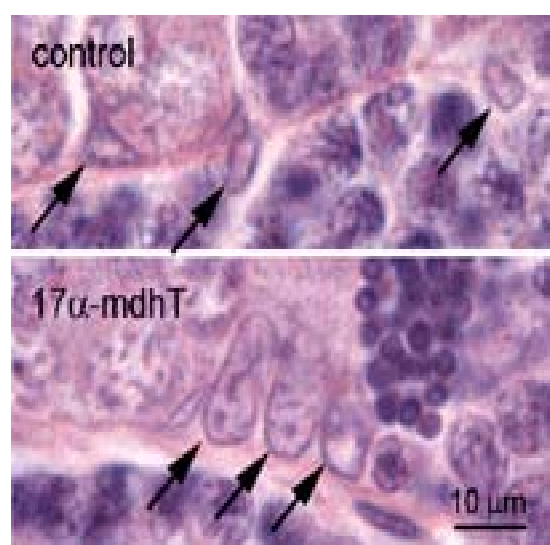

Fig. 3.4.2 - High power micrographs, showing Sertoli cell hypertrophy and hyperplasia after MDHT (10 µg/L), compared to control (arrows).
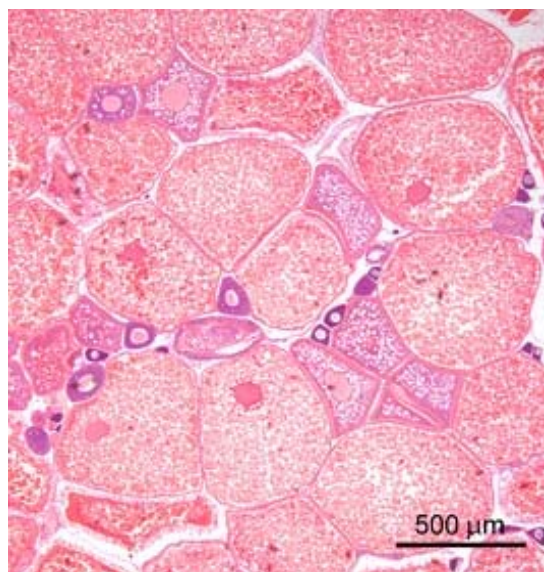
Fig. 3.4.3 - Accumulation of vitellogenic oocytes in the ovary after exposure to 10 µg/L MDHT, resulting in increased abdominal span (see Fig. 4).
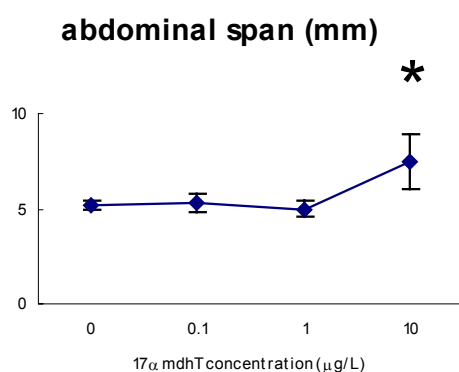
*Fig. 3.4.4 - Morphometry of abdomen of female zebrafish exposed to MDHT. The maximal abdominal span was determined directly on sections. *, statistical significant in a T-test.*

Males of the highest concentration group (10 µg MDHT/L), and to a lesser extent in the lower concentration groups, also showed hypertrophy of Sertoli cells (Fig. 3.4.2), and possibly atrophy of Leydig cells. This is in line with the position of these cells in the steroid axis, namely production of androgens under control of gonadotrophs (Leydig cells) and target cell of androgens (Sertoli cells).

In females, there was a biphasic effect: at 10 µg/L there was accumulation of vitellogenic oocytes (Fig. 3.4.3), indicating inhibited ovulation. This inhibited ovulation was reflected by the increased trunk volume as measured by the maximal span of the abdomen on the sections (Fig. 3.4.4). In contrast, at the high MDHT concentration of 1000 µg/L as applied in a preceding 8 d range finding exposure, there was atresia of vitellogenic follicles, yielding a similar image to atresia after E2, and resulting in a lower abdominal span (data not shown). A further effect at high levels was the reduced size of previtellogenic oocytes (Fig. 3.4.5). Thus, at these high levels, MDHT inhibits both previtellogenic growth as well as vitality of vitellogenic oocytes.

size of previtellogenic oocytes (mm^2)
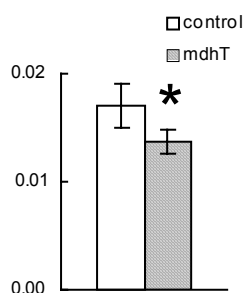
*Fig. 3.4.5 - Morphometry of previtellogenic oocytes (range 0.0015-0.05 mm^2) after exposure to100 µg/L MDHT in a 8 d exposure. *, significant in a T-test*

Histopathology - juveniles

A total of 1693 juvenile fish was analysed for effects of MDHT (Fig. 3.4.6). The mean sex ratio (m/f) in the control groups was 0.56, although with a high variance between experimental units (not between successive breeds of each experimental unit). There was a virtual complete masculinisation in the groups with 0.1 or 1 µg/L MDHT. In the groups with 10µg/L MDHT juvenile exposure there was complete agenesis (%0) or underdevelopment (%u) of the gonads. This skewed sex differentiation is most likely an effect of exposure of the juveniles, in view of the absence of an effect on sex ratio after exposure of parents only. Juveniles in the highest exposure group (10 µg/L) also showed intense vitellogenesis (liver basophilia, extravascular fluid accumulations).

Fig. 3.4.6 - Sex ratio of F1 zebrafish after 42 days of exposure to MDHT.
Average ± sd of three replicates of each treatment, indicated as P - F1 exposures, MDHT in µg/L.
f, female; m, male; u, undifferentiated; 0, no gonad development
***p<0.0001, T-test (masculinisation after juvenile exposure to 0.1 and 1 µg/L MDHT, underdevelopment of gonads after 10 µg/L MDHT)

effect of MDHT on population extinction after 2004 days

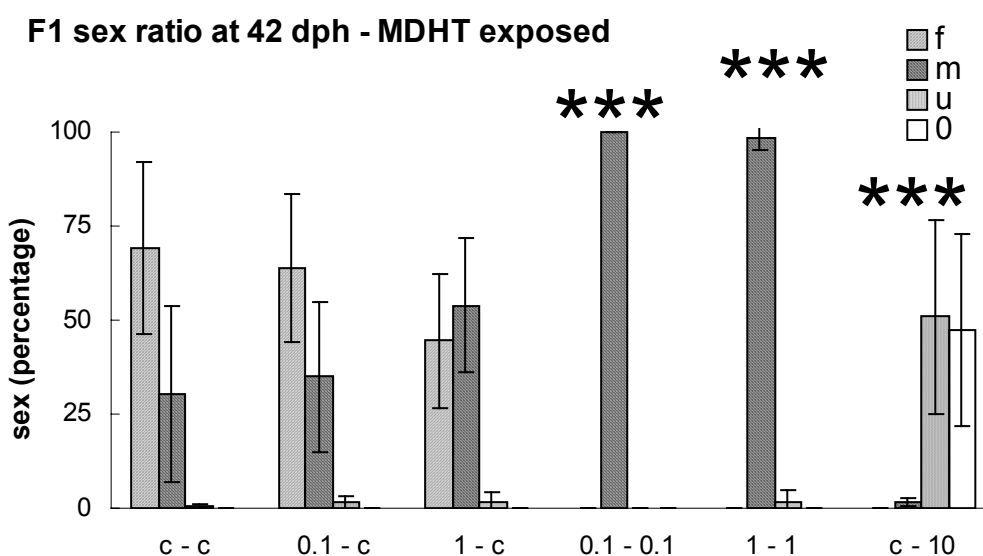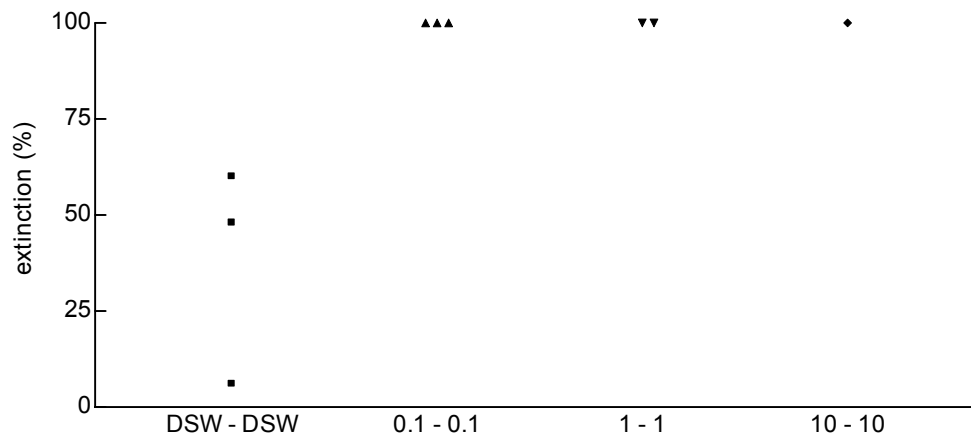*Fig. 3.4.7 - Population modelling for MDHT.* Percentage of extinct population of the 100 simulated zebrafish populations after 2400 days. p=0.0142, F=11.21, R^2=0.8176 in an ANOVA. Treatments indicate P - F1 exposure, respectively. DSW, control medium; other treatments are in nominal values of MDHT (μg/L).

Population modelling

The zebrafish IBmodel indicated complete extinction in the exposed experimental units, even at the lowest concentration tested (Fig. 3.4.7). This was due to complete masculinisation of the offspring, resulting in complete extinction within one life span of zebrafish (± 420 days).

Conclusions

- 10 μg/L MDHT inhibited ovulation within a few days, associated with inhibited spawning. Vitellogenesis was induced in adults and juveniles. Testis morphology indicated stimulation of spermatogenesis and effects on Sertoli / Leydig cells. Respiration rate was increased. In juveniles (from control parents), growth (body weight and length) were reduced. It is anticipated that this concentration level is incompatible with reproduction.

- At 1 μg/L egg production (clutch size) was reduced, as well as body weight gain in juveniles.

- Reduction of egg production showed a dose response.

- No effects were seen on survival, fertilisation rate, hatching and condition factor (juveniles) in any of the groups.

- At low concentrations (0.1 μg/L and higher) complete masculinisation was found in developing juveniles. This is considered the critical effect with respect to projected viability of the population. At higher concentrations gonad development is inhibited.

- MDHT shows a biphasic effect: at lower concentrations the effects are androgen specific, while at higher concentrations the decreased oocyte growth and atresia, as well as the induction of vitellogenesis indicate an estrogenic effect.

- In view of the rapid decline in actual concentration of the test article, the effect observed may be an underestimate when related to the nominal concentrations.

3.5.   PLC-test with anti-androgen: flutamide

Introduction

As reference anti-androgen, the therapeutic drug flutamide was chosen. Flutamide is used as anticancer agent in prostate cancer therapy; it is a non-steroidal anti-androgen that inhibits androgen uptake and/or nuclear binding of androgen in target tissues. It causes a gradual increase in plasma testosterone due to blockage of feedback inhibition of the hypothalamus and pituitary by testosterone (Nagahama, 1994).

Materials and methods

A stock solution of flutamide (CAS RN 13311-84-7, Sigma-Aldrich) was prepared in DMSO and kept at 4 °C. Final concentration of DMSO was adjusted to 0.01% in all groups. The test was performed as described in "General Protocol". Briefly, a range finding test was conducted using concentrations of 1 - 10 - 100 - 1,000 - 10,000 µg/L. Only the highest concentration showed significant toxicity, and therefore the concentration range for the PLC was chosen as solvent control – 10 - 100 - 1,000 µg/L. Chemical analysis of the exposure medium showed mean actual flutamide concentrations of 84.3 - 75.7 - 63.1 - 58.7 - 52.8 percentage of nominal values at days 1-5, respectively, indicating a gradual temporal decline. In the PLC, adults were exposed for 21 days in triplicate spawning units per concentration, eggs were collected, incubated and juveniles were sampled after 42 days exposure to flutamide or control medium. Animals were monitored daily for general health and clinical effects such as mortality, abnormal behaviour and appearance. Eggs were monitored for fertilisation and hatching. At termination of the experiment, animals were euthanised, length and weight were measured, and blood was collected for future VTG analysis. Animals were fixed *in toto* for histopathology of target organs. The experiment was approved by the Institute's Animal Experiment Committee (AAP200100411).

Results and discussion

In life observations - adults
In life observations during exposure of adults revealed no effect on survival, appearance and behaviour. Reproductive performance was affected in the 1000 µg/L group only through a

Table 3.5.1 - Reproduction parameters of P generation after flutamide

concentration (µg/L)	number of clutches [1]	clutch size	total number of eggs	fertilisation rate
control	6.3 ± 1.0	333 ± 65	2094 ± 297	74.7 ± 3.9
10	6.0 ± 0	341 ± 64	2044 ± 387	88.3 ± 4.2
100	6.7 ± 1.0	318 ± 63	2097 ± 218	85.2 ± 6.1
1000	3.3 ± 1.0*	353 ± 94	1141 ± 152*	72.7 ± 16.7

All data represent average ± sd of three spawning units.
[1] maximum number of clutches is 7.
* $p < 0.01$ Dunnets multiple comparison-test

significant reduction of the number of clutches (Table 3.5.1). As the average clutch size was not altered, the total number of eggs produced was also reduced.

In life observations - juveniles

Total hatching per treatment showed no significant differences (Table 3.5.2). Sporadically, anomalies were observed in behaviour and appearance mainly at 1 dph, such as curved tail or other malformations; these were, however, not associated with treatment.

After the 42-day exposure period, condition factor was reduced in the control – 1000 µg/L group (Table 3.5.3); however, as this was not reproduced in the 1000 - 1000 group, this finding is not considered relevant. Survival was significantly reduced after exposure of F1.

Table 3.5.2 - Hatching

treatment P - F1 (µg/L)	hatching (%)
control – control	88.1 ± 1.9
10 – control	81.2 ±15.6
100 – control	85.2 ± 12.3
1000 – control	67.5 ± 23.8
10 - 10	84.8 ± 13.6
100 – 100	84.1 ± 14.4
1000 – 1000	71.0 ±23.0
control – 1000	88.5 ± 2.1

data represent average values ± sd of three replicates (spawning units)

Table 3.5.3 - In life observations in F1 zebrafish exposed to flutamide for 42 days

treatment P - F1 (µg/L)	survival (%)	length (mm)	body weight (mg)	condition factor
control – control	91.1 ± 4.7 [a]	15.7 ± 0.4	62.5 ± 6.1	1.40 ± 0.05 [b]
100 – control	81.3	16.2	66.4	1.51
1000 – control	76 ± 9.2	15.8 ± 0.3	64.7 ± 3.3	1.41 ± 0.11
10 – 10	82 ± 5.5 [a]	15.7 ± 0.6	64.4 ± 4.7	1.49 ± 0.02
100 – 100	79.3 ± 11.6 [a]	16.3 ± 0.1	67.1 ± 2.4	1.45 ± .0.05
1000 – 1000	70.3 ± 9.3 [a]	16.0 ± 1.3	66.1 ± 9.3	1.49 ± 0.08
control – 1000	87.7 ± 5.8	16.0 ± 0.5	63.9 ± 6.3	1.35 ± 0.06 [b]

Values are average ± sd of three replicates (one in 100 - control).
[a] p<0.05, linear regression
[b] p<0.05, paired T-test

Histopathology -males

After exposure of male zebrafish to the flutamide, histological changes in the testis included an increase of interstitial cells (Fig. 3.5.1), nuclear hypertrophy of Sertoli cells, and an increased size of early gonocytes. The latter effect was morphometrically confirmed in the pilot range-finding experiment (0.00019 ± 0.00004 mm^2 in controls vs 0.00026 ± 0.00005 mm^2, exposed to 100 µg/L, p<0.00001). In the pilot experiment, there was also obvious oocyte development in the testis in a single case after 100 µg/L exposure; this was, however not confirmed in the PLC. These occasional testis-ova are in line with the increased size of early spermatogonia (or gonocytes), and could be considered as an enhancement of this latter effect. Inhibition of androgen action with flutamide may thus direct gonocytes / early spermatogonia to oocyte development.

The observed effects of flutamide on Leydig and Sertoli cells and spermatogonia can be
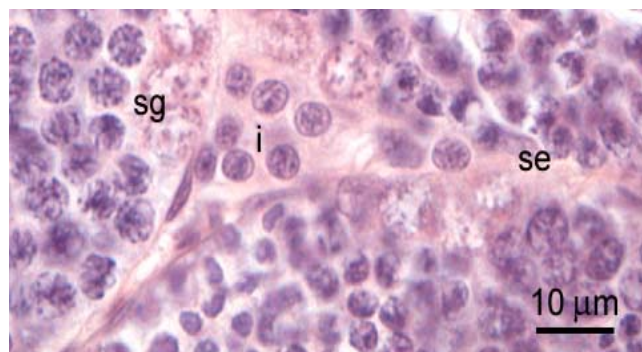

Fig. 3.5.1 - Histological changes in the testis of an adult zebrafish after exposure to 1 mg/L flutamide (21 d). Interstitial Leydig cells (i) are present in large clusters, early gonocytes (sg) are enlarged, Sertoli cells (se) show nucelear hypertrophy. Enhanced illustrations are available in the atlas.
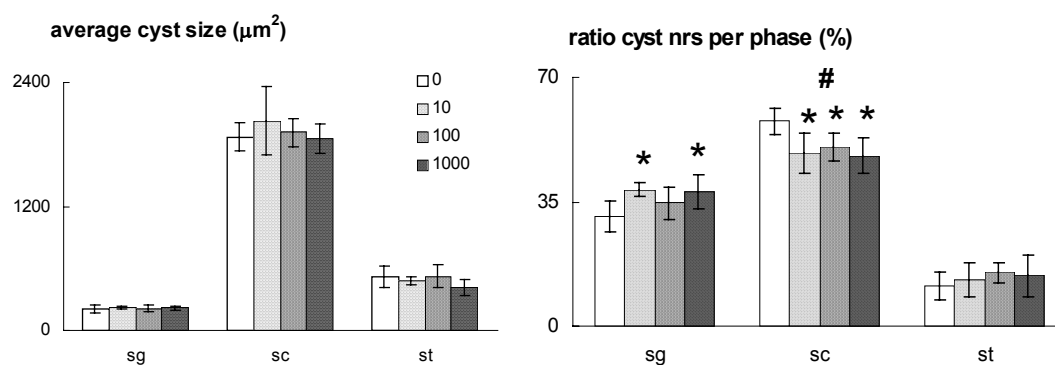
Fig. 3.5.2 - Morphometric analysis of effects of flutamide in the testis of adult zebrafish. Size of the respective stages sg (spermatogonia), sc (spermatocytes), and st (spermatids) do not change. The relative occurrence of these stages is skewed toward the sg stage
#, $p < 0.05$, linear regression. *, $p < 0.05$, T-test, for individual concentrations compared to control

explained from disruption of androgenic stimulation. The androgen-producing Leydig cells, which are subject to autoregulation and feedback from pituitary-derived gonadotropins, may be activated after blocking of (pituitary) androgen receptors (simulation of low androgen levels). Sertoli cells are the primary target cells of androgens in the testis, and they produce mediators after androgenic stimulation, which in turn regulate proliferation and maturation of the spermatogenic epithelium (Nagahama, 1994). Decreased levels of these androgen mediators may be suspected after blocking of the androgen receptors, thus decreasing stimulation of spermatogonia.

Morphometric analysis of the testis (Fig. 3.5.2) revealed relatively more spermatogonia and less spermatocyte cysts, as compared to control animals; the reduction of spermatocyte cysts was also reflected in a small increase in spermatid cysts, which however is not statistically significant. This shift is dose-dependent, and calculated changes were in the range of +12-24% for spermatogonia, and of -13-17% for spermatocytes. It indicates inhibition of transition from spermatogonia to spermatocytes, *i.e.* inhibition of meiosis. This effect is, as expected, contrary to that of the androgen agonist MDHT, which resulted in stimulation of this transition process (see Chapter 3.4, MDHT). Cyst sizes were not altered by flutamide. Overall, flutamide inhibits spermatogenesis in adult zebrafish, although probably confined to early stages. The observed changes are limited, but serious temporal effects of flutamide on spermatogenesis and sperm function cannot be excluded.

Histopathology - females

No histological changes were observed in the ovaries after exposure to the tested concentrations of flutamide. Global inspection revealed also no alterations in other organs.
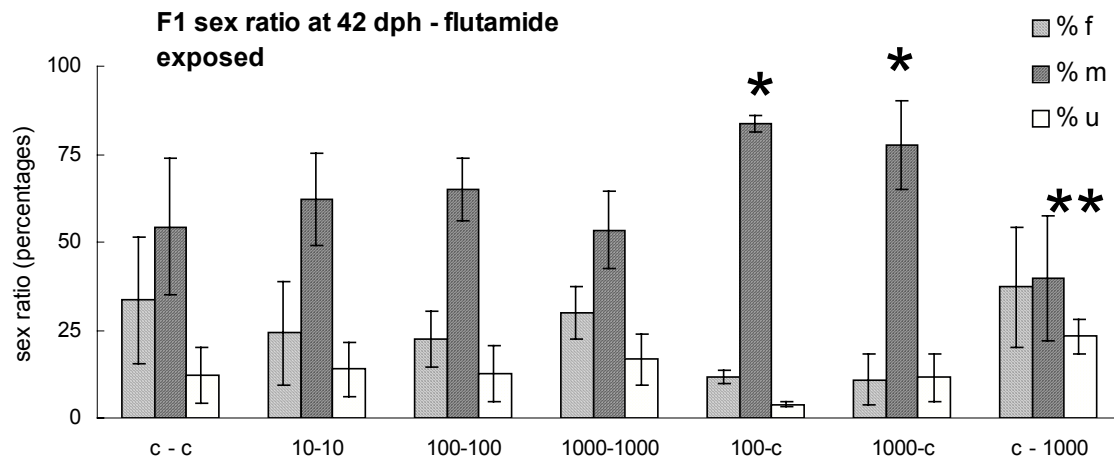
Fig. 3.5.3 - Sex ratio of day 42 juveniles. P - F1 exposures in µg/L flutamide.

*,** p<0.01, 0.0001, respectively, compared to c-c (T-test).

Histopathology - vitellogenin

Immunohistochemical evaluation of VTG in plasma revealed no alterations after flutamide, *i.e.* all females had positive staining with similar intensity, and all males were negative.

Histopathology - juveniles

Exposure to flutamide induced skewed sex ratios, towards the male phenotype after only parental exposure at the highest concentrations, and minor increase of undifferentiated gonad ratio after juvenile exposure to the top concentration (Fig. 3.5.3). The masculinisation in the F1 generation - after only parental exposure - is a paradoxal observation. Speculative explanations are meiotic drive (selective advantage for a male-determining gamete; Ricklefs, 1980), or some imprinting mechanism. However, this effect was not observed in juveniles with subsequent exposure to flutamide (P-F1, no masculinisation in these groups). The increased ratio of undifferentiated gonads in the F1 treated group with the top concentration appears mainly at the expense of the ratio of males in these groups.

As in adult males, these juvenile males showed large clusters of Leydig cells and hypertrophied Sertoli cells.

No other histological changes were observed.

effects of flutamide on population extinction after 2004 days
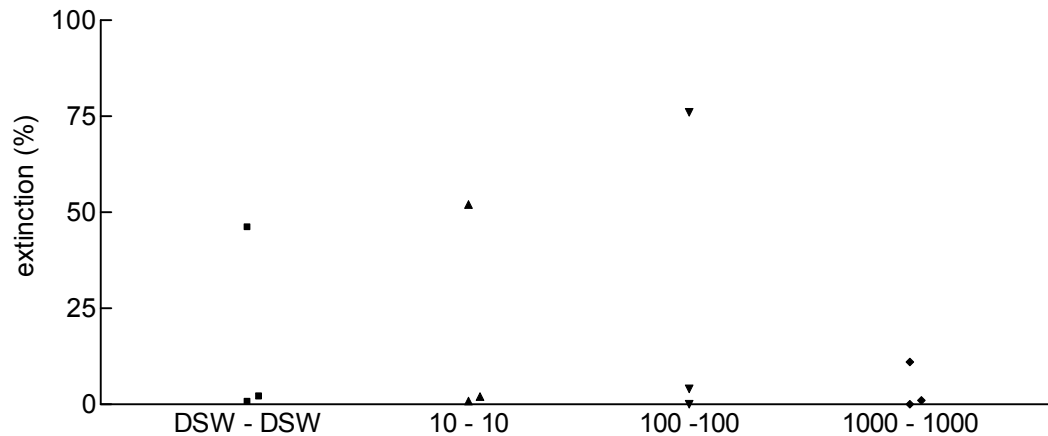
Fig. 3.5.4 - Calculated effect of flutamide in a population model of 2400 days. There is no significant effect of the exposure on the number of adults in the population. Treatments indicate P and F1 exposure, respectively. DSW, control medium; other groups are indicated with nominal values of flutamide exposure in μg/L.

Population modelling

Exposure to flutamide showed no significant effects on populations of zebrafish (ANOVA; Fig. 5.5.4), although differences between average number of adults were almost significant (p=0.06). Egg production and juvenile survival were reduced but these effects were negligible at population level.

Conclusions

- Flutamide at 10 mg/L is clearly toxic to zebrafish.

- At 1 mg/L egg production was reduced, and juvenile survival is reduced in a concentration dependent way.

- Histologically evidence was seen for hormone disturbance even in the lowest concentration tested, consistent with an anti-androgen action.

- The masculinisation in juveniles was paradoxal as it was seen after parental exposure only; no explanation can be given yet.

- No effects were calculated at the level of the population.

- The effects detected with this PLC were not alarming, although it cannot be excluded that prolonged exposure would induce more severe effects, notably on spermatogenesis.

3.6.   PLC-test with antithyroid agent: propylthiouracil

Introduction

As reference antithyroid agent, propylthiouracil (PTU) was selected. PTU is a well-known and powerful inhibitor of thyroid hormone synthesis, and is used as a therapeutic drug in humans. Due to feedback from reduced levels of circulating hormone, the pituitary excretion of TSH will increase, and in turn induce stimulation and hypertrophy / hyperplasia of thyroid epithelium. Thyroid hormones are known for their role in development (in particular metamorphosis in amphibians; Brown, 1997) and metabolism. In the context of endocrine disrupters the thyroid axis is often mentioned as a target system but studies focused at the thyroid in the toxicology of EDCs are limited. This study was aimed at studying the role of thyroid inhibition on general aspects, reproduction and histopathology of zebrafish.

Materials and methods

PTU (propylthiouracil, CAS RN 51-52-5, Sigma-Aldrich) was dissolved in DMSO and stored at 4^0C. Due to poor solubility at 100 mg/L, ultrasone-assisted solution was used. Final concentration of DMSO in test media was adjusted to 0.01% in all groups. Actual concentrations of PTU were 106-125% of nominal values during a 96 hour period, and remained constant in time.

The test was performed as described in Chapter 2.2, Protocol design. Briefly, a range finding test was conducted using concentrations up to 1000 µg/L PTU in a 10 day test with juveniles and adults. In the absence of significant effects a second range finding study was performed at an exposure range of 32-1000 mg/L PTU. This showed 100% acute mortality in the 1000 mg/L group and thyroid activation in 100 mg/L onwards. Based on these effects the concentrations of 1, 10 and 100 mg PTU/L were selected for the PLC, thus aiming at adequate survival and reproduction at least in the mid en low concentration group.

In the PLC, adults in triplicate spawning units per concentration were exposed for 21 days, eggs were collected, incubated and juveniles were sampled after 42 days exposure to PTU or control medium.

Animals were monitored daily for general health and clinical effects such as mortality, abnormal behaviour and appearance. Eggs were monitored for fertility and hatching.

Table 3.6.1 - Reproduction parameters in F0

concentration (mg/L)	number of clutches [1]	clutch size	cumulative number of eggs	fertilisation rate
control	2.3 ± 1.5	412 ± 119	961 ± 394	61 ± 19
1	4.3 ± 2.3	326 ± 146	1414 ± 424 [a]	80 ± 16
10	4.7 ± 1.2	299 ± 202	1394 ± 335 [a]	75 ± 20
100	4.7 ± 2.3	368 ± 197	1717 ± 335 [a]	88 ± 11

data are average ± sd of three spawning units.
[1] maximum number of clutches is 7.
[a] positive correlation (not transformed, p=0.0487, r^2=0.3348, Spearman)

At termination of the experiment, animals were euthanised, length and weight were measured (juveniles), and from adults, blood was collected for thyroid hormone analysis (total T3 and T4 by radio-immunoassay using commercially available reagents), which was generously performed by dr. D.M. Power, Porto University, Portugal. Animals were fixed *in toto* for histopathology of target organs, in particular thyroid and endpoints for metamorphosis (scale thickness).

The experiment was approved by the Institute's Animal Experiment Committee (AAP 20000795).

Results and discussion

In life observations - adults

In life observations during exposure of adults revealed no effect on survival, normal appearance and behaviour.

Total number of eggs per female revealed a positive correlation with treatment PTU (linear regression analysis, no transformation; Table 3.6.1). No significant effect between the groups was seen with ANOVA. It should be noted that egg production was highly variable per individual. No effects were found on number of clutches, mean clutch size, or fertilisation.

In life observations - juveniles

Hatching was monitored and analysed as total hatching per treatment. No significant differences were observed (Table 3.6.2). Sporadic anomalies (significant in the control-100 group) were seen in the exposed animals in behaviour and appearance, such as malformations and immobility shortly after hatching.

Table 3.6.2 - Hatching and early clinical pathology in F1 zebrafish (until 3 dph)

treatment P - F1 (mg/L)	hatching (%) [a]	clinical pathology until 16 dph	
		malformation (%)	abnormal behaviour (%)
control – control	56.3 ± 25.9	0	0
1 – control	60.7 ±24.9	0.8	0
10 – control	74.9 ± 24.8	2.8	0
100 – control [b]		2.6	0.6
1 – 1	61.1 ± 22.7	0.5	0
10 – 10	78.4 ± 15.8	3.4	0
100 – 100	70.0 ±18.8	2.4	0.9
control – 100	58.1 ± 25.7	5.7[c]	0

[a] on average 9.9 clutches per treatment examined (range 6-14)
[b] no data for hatching
[c] $p=0.0145$, Fisher's exact test

Table 3.6.3 - Developmental parameters of F1 zebrafish exposed to PTU for 42 days

treatment P - F1 (mg/L)	survival (%)	length (mm)	body weight (mg)	condition factor
control – control	55.6 ± 10.7	16.5 ± 1.0 [d e]	67.4 ± 3.6 [c f g]	1.45 ± 0.14
1 – control	67.4 ± 16.1	16.2 ± 0.6 [d]	62.7 ± 5.2 [f]	1.41 ± 0.09
10 – control	64.9 ± 21.7	15.6 ± 0.3 [d]	61.5 ± 1.4 [f]	1.59 ± 0.05
100– control	59.7 ± 4.5	15.6 ± 0.4 [d]	57.4 ± 6.4 [b f]	1.44 ± 0.02
1 – 1	72.2 ± 22.5	16.4 ± 0.5 [e]	66.2 ± 10.5 [g]	1.42 ± 0.10
10 – 10	55.5 ± 18.3	16.0 ± 0.5 [e]	62.7 ± 6.5 [g]	1.48 ± 0.06
100 – 100	55.6 ± 11.8	14.3 ± 0.1 [e a]	42.4 ± 3.0 [b g]	1.39 ± 0.07
control – 100	54.3 ± 4.6	13.3 ± 0.3 [a]	38.7 ± 0.3 [c]	1.48 ± 0.01

Values are average ± sd of three replicates (two for control P)
[a] $p<0.05$, paired T-test
[b] $p<0.001$, paired T-test
[c] $p<0.05$, paired T-test
[d-g] significant concentration dependent linear regression (Spearman) for length and weight with only parental exposure and with consecutive P-F1 exposure ([d] $p=0.05$, $r^2=0.3579$; [e] $p<0.001$, $r^2= 0.7414$; [f] $p<0.05$, $r^2=0.4250$; [g] $p< 0.01$, $r^2=0.6887$)
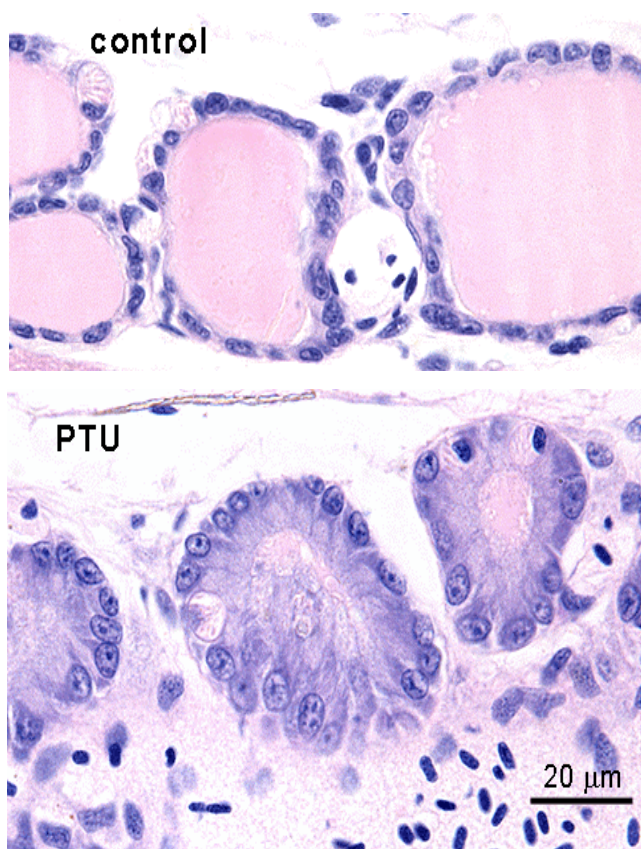
Fig. 3.6.1 - thyroid follicles in control adult female zebrafish (top), and after exposure to 320 mg/L PTU for 10 days (bottom).

Juvenile survival was relatively low in all groups, including controls, compared to other experiments (Table 3.6.3). Survival was not related to treatment, but probably due to the quality of the used batch of live feed. After 28 days, several animals in de high dose group showed swelling and hyperaemia of the thyroid area, and less pronounced pigmentation. Length of juveniles was significantly reduced by exposure to 100 mg PTU, irrespective of parental treatment (c-c against c-100 and 100-c against100-100). There was also a significant concentration-dependent reduction of both length and weight after exposure of both parents and offspring. Weight of the juveniles followed the same pattern as length; consequently there is no effect of PTU treatment on condition factor.

Histopathology - adults

In control animals, thyroid follicles were found dispersed in the loose connective tissue adjacent to the ventral aorta and its final rostral branching. Most follicles were small-sized, well-filled with colloid, and had low-cuboid or flat epithelium (Fig. 3.6.1, top); one relatively large follicle was invariably present directly rostral to the aortic branching.
After exposure of adult zebrafish, PTU had caused activation of the thyroid follicular cells (Fig. 3.6.1, bottom), as is shown by the hypochromasy and increase of size of the nuclei, and by basophilic cytoplasm. This is likely the result of hypertrophy of the synthetic apparatus

Table 3.6.4 – Activation of the thyroid after PTU in adult zebrafish

activation intensity	concentration PTU (mg/L)			
	0	1	10	100
-	9	8		
+		1	6	
++			3	3
+++				6

Semi-quantitative observation (visual scoring of follicular epithelium height,
basophilia, and nucleus hypertrophy
n=9, exposure 21 days

(RER, Golgi complex and mitochondria). This was accompanied by a morphological change of these cells to a columnar appearance (compare with the reference thyroid follicles in the upper image). The follicles were almost completely depleted of colloid (thyroglobulin). This activation was concentration-dependent (Table 3.6.4) and time-dependent, since the effects appeared more intense in the 21day test compared to the 10 day exposure pilot. Furthermore, this thyroid pathology appeared less severe in adults than in the F1 juveniles (42 day exposure; see below). These observations are explained by the interference of PTU with the synthesis of thyroid hormone, thus inducing an enhanced secretion of thyrotropic hormone (TSH) by the pituitary.

There were no other PTU-related effects. Other pathological observations (peritonitis, bile duct hyperplasia) were recorded in a relatively high incidence, but were considered unrelated to treatment.

Thyroid hormone analysis

Analysis of thyroid hormone in blood plasma of these adult fish showed reduced levels of both T_3 and T_4 at 10 (only T4) and 100 mg/L PTU (both hormones; Fig. 3.6.2). There was also a significant concentration-dependent decrease of both hormones.

Histopathology - juveniles

At six weeks of age, surviving juveniles exposed to 10 –100 mg/L PTU showed a struma (goitre) which was manifest as a hyperplasia and hypertropy of the scattered thyroid follicles, comparable to the picture in adults (see Fig. 3.6.1). Usually the follicles were microfollicular, containing little or no thyroglobulin although occasionally macrofollicular struma was seen.
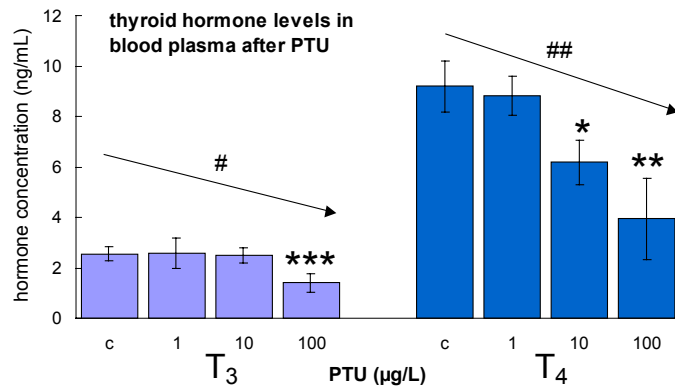
Fig. 3.6.2 - thyroid hormone analysis following exposure to PTU. T_3 is decreased at 100 mg/L PTU (T-test, ***, $p<0.0001$). Regression analysis revealed a concentration dependent decrease (#, $p<0.01$). T_4 is decreased at 10 and 100 mg/L PTU (*, $p<0.05$ and **, $p<0.01$, respectively). There is also a concentration dependent decrease (regression analysis, ##, $p<0.0001$).

The increased volume of the thyroid tissue caused expansion of the interbranchial tissue compartment with extension into the branchi and along the jugular vein. Remarkably there was also a striking hyperaemia in this region, as seen by marked and tortuous dilatation of the jugular vein, the interfollicular capillaries and the branchial vessels (Fig. 3.6.3). This could be the result of an active hyperaemia from increased circulatory demand, and / or passive hyperaemia due to obstruction. Occasionally edema was seen in the secondary gill lamellae, the latter probably due to physical circulatory insufficiency resulting from the struma. Both the thyroid hyperplasia and the vascular dilatation are thought to have caused the swollen and red bulging mandibular area seen grossly. The effect was dose dependent (Table 3.6.5).
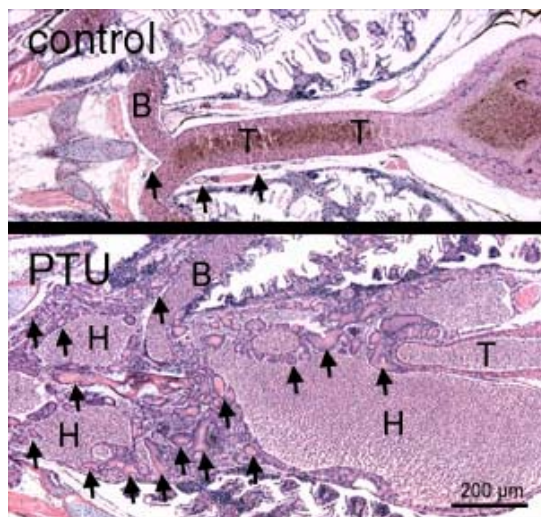

Fig. 3.6.3a - jugular area of juvenile zebrafish (42 dph), control (top) and after exposure to 100 mg/L PTU, showing numerous activated thyroid follicles (arrows) and extensive hyperemia (H) after exposure to PTU. T, truncus arteriosus, B, branchial arteries.
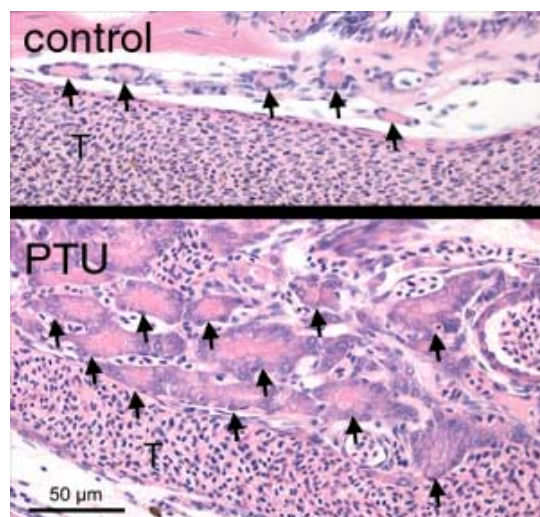
Fig. 3.6.3b - as in Fig. 3.6.3a, but higher magnification

Table 3.6.5 – Activation of the thyroid after PTU in juveniles

activation intensity	concentration PTU (mg/L)					
	0-0	**100-0**	**1-1**	**10-10**	**0-100**	**100-100**
-	18 [a]	10 [a]	16			
+	3 [a]	13 [a]	3	5		
++				11	6 [b]	
+++					14 [b]	19 [b]

Thyroids of 16-23 animals per group were analysed. Exposure was 42 days. There is a significant association between activation intensity and PTU concentration ($p<0.0001$, Chi-square=103.92), and a concentration-dependent effect ($p<0.0001$, Chi-square=96.672) after combination of exposure and outcome categories (parental exposure ignored). There is a increased activation after parental exposure ([a] 0-0 versus 100-0, $p<0.005$ and [b] 0-100 versus 100-100, $p<0.05$; Fisher's exact test)

A concomitant effect was depletion of liver glycogen (Fig. 3.6.4), which was dose-dependent in severity, from 1 mg/L onwards (Table 3.6.6). This is in line with the stimulating effect of thyroid hormone on glycogen synthesis.

There were indications for an effect of parental exposure for both the struma and liver glycogen depletion (observed effects after parental exposure alone, or more severe effects after consecutive parental and juvenile exposure compared to juvenile exposure alone (see Tables 3.6.5 and 3.6.6).

In this PLC, the thickness of the scale plates was selected as a histologically evaluable parameter, possibly representative of metamorphosis (Fig. 3.6.5). Six scales per fish at a comparable level were measured in each treatment group; each bar represents the average of six fish, which were preferentially taken from two replicate groups (Fig. 3.6.6). The animals were matched for length, since development is obviously correlated to growth. Exposure to 10 mg PTU/L and higher in the 12 mm groups, and to 1 mg PTU/L and higher in the 16 mm
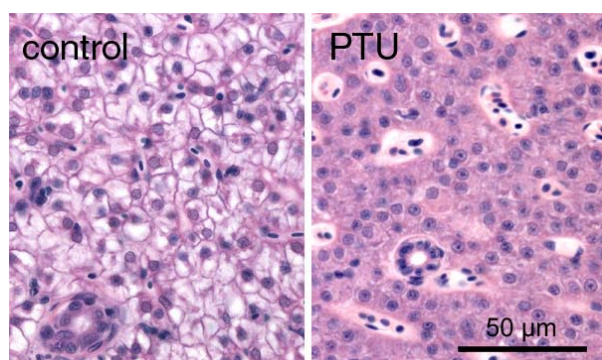

Fig. 3.6.4: liver of juvenile zebrafish (42 days), control (left) showing high glycogen contents (open intracellular areas) and after exposure to 100 mg/L PTU (right); this specimen is c virtually depleted of glycogen.

Table 3.6.6 – Liver glycogen storage after PTU in F1 zebrafish

storage	concentration PTU (mg/L)					
	0-0	100-0	1-1	10-10	0-100	100-100
+					11	15
++		13 [a]	17	14	9	4
+++	22 [a]	10 [a]	3	3		

Livers of 16-23 animals per group were analysed. Exposure was 42 days. There is a significant association between liver glycogen storage and PTU concentration (p<0.0001, Chi-square=107.56) after combination of exposure categories (parental exposure ignored).; there is also a significant concentration-dependent effect (p<0.0001, Chi-square=36.900) after limiting outcome scores to two categories. There is increased glycogen depletion after parental exposure without consecutive juvenile exposure ([a] 0-0 versus 100-0, p<0.0001; Fisher's exact test)

groups yielded significant inhibition of scale development, and the severity of the effect was concentration-dependent. Exposure of only F1 (c-100) induced a similar effect to continuous P and F1 exposure (100-100), in the case of 100 mg PTU/L (no lower dosage groups only F1 exposed were available). This inhibitory effect of PTU may reflect just a delay of morphogenesis with no functional implications. As other lower vertebrates, teleosts pass
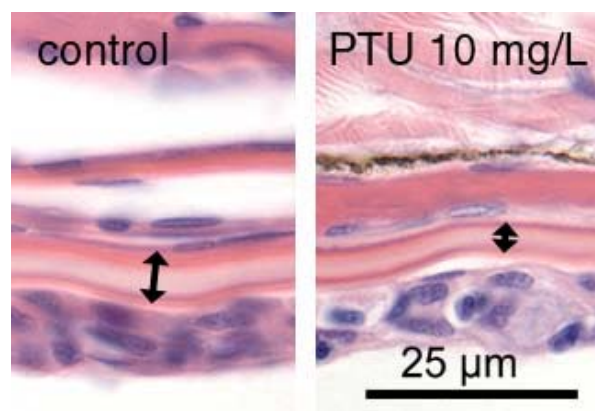
Fig. 3.6.5 - Integument of juvenile zebrafish (42 dph), control (left) and after exposure to 10 mg/L PTU (right). Scale thickness is decreased after PTU exposure (compare arrows).
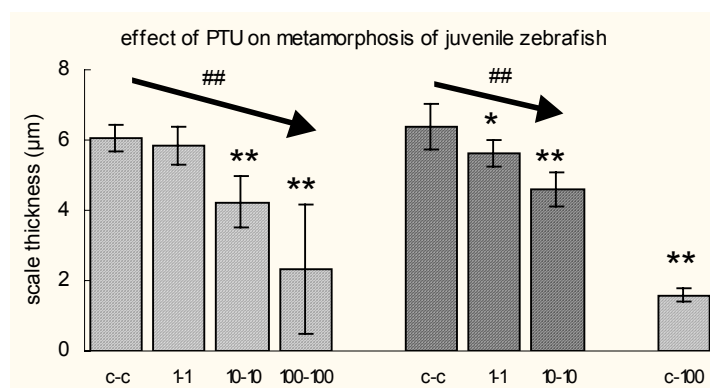
*Fig. 3.6.6 - decreased scale thickness after exposure to PTU in 12 and 16 mm juvenile zebrafish (42 dph, light and dark shading, respectively). */ **, statistical different from respective control in a Student T-test; ##, p<0.0001 concentration dependent effect (regression analysis). Group designations represent P-F1 exposures, respectively.*

effect of PTU on population extinction after 2004 days

Fig. 3.6.7 - Population modelling for PTU. Percentage of extinct population of the 100 simulations after 2400 days. Treatments indicate P and F1 exposure, respectively. DSW, control medium; other groups are indicated with nominal values of PTU exposure in mg/L.

through a stage of metamorphosis, which marks the transition of the larval stage to the juvenile stage. In zebrafish, some characteristics of this metamorphosis are the outgrowth and development of paired fins (pectoral and pelvic), the appearance of the adult pigmentation pattern (stripes), the development of scales. Metamorphosis is controlled by thyroid hormones (Brown, 1997), and therefore exposure to goitrogens may be expected to affect the mentioned features.

There were no further changes, notably no effect on sex ratio and morphology of thymus and adrenal cells.

Population modelling

The population simulations of zebrafish populations showed no effect of PTU on chances on extinction and on population size (Fig. 3.6.7). Number or eggs produced per female increased with PTU-exposure but an increase of egg production does not necessarily lead to higher survival rates, especially if survival rates are already high, as they are in the controls. Also sex ratio remained unchanged under PTU exposure.

Conclusions

- Exposure of adult zebrafish and offspring had no significant effect on reproduction parameters at concentrations as high as 100 mg/L. There were even indications for a higher egg production after treatment.

- Exposure of juveniles caused concentration related retardation in growth (length and weight).

- Exposure of juvenile zebrafish decreased scale thickness at 1 mg/L and pigmentation was reduced. These findings may be interpreted as an effect on metamorphosis.

- In both adult and juvenile zebrafish struma was observed in histopathology from 1 mg/L onwards. In plasma of adults, indeed a dose dependent decrease of thyroid hormones was measured. Also liver glycogen was reduced, attributed to the known glyconeogenetic activity of thyroid hormones.

- No significant effect on reproductive performance is induced by the thyroid inhibitor PTU. Developmental effects were limited to reduced growth and metamorphosis; the functional impact at the population level remains unclear.

3.7.   PLC-test with a field sample: the LOES survey

This study will be published in detail by Bulder et al, in preparation

Introduction

As a part of a large national field study (LOES) where hot spots for estrogenic activity in surface waters were identified, a partial life cycle test was conducted with effluent from the sewage treatment works (STW) from the city of Eindhoven (Eindhoven effluent), as (xeno-)estrogens had been detected earlier in this effluent (Belfroid et al., 2000; Vethaak et al., 2002), as well as a high incidence of intersex and high plasma VTG levels in male fish in the receiving Dommel river. Also a synthetic analogue containing (xeno-)estrogens identified in the effluent was tested in the PLC.

Materials and methods

Exposure protocol was essentially as described described in Chapter 2.2, Protocol design. Specifically, exposure media were as follows:

positive control - 1 nM 17β-Estradiol (E2, Fluka, >97%, CAS RN 50-28-2) in DSW prepared from a concentrated stock solution in ethanol;

Eindhoven effluent – municipal effluent from the Eindhoven STP. Effluent samples were collected twice weekly in the period of September to November 1999 (LOES Period 3, Vethaak et al., 2002), aerated and kept at 27 ºC after arrival until used for media renewal later that day;

synthetic effluent – (xeno-)estrogens in DSW prepared from a concentrated stock solution in ethanol. The levels of a number of (xeno-)estrogens in the Eindhoven effluent were analysed during the pilot study in the autumn of 1997 (Belfroid et al., 2000) and in LOES period 1 (March-April 1999, Vethaak et al., 2002). Based on these levels, a synthetic effluent analogue was prepared, consisting of the following compounds: (synthetic) hormones Estrone (E1, 5 ng/L) and ethynylestradiol (EE2, 2.8 ng/L), bisphenol-A (BPA, 4 μg/L), alkylphenol(ethoxylate)s nonylphenol (NP, 2 μg/L), -ethoxylate (NP-4-EO, 9.3 μg/L), octylphenolethoxylate (OP-8/9-EO, 0.5 μg/L) and the phtalate diethylhexylphthalate (DEHP, 2.7 μL).

Exposure media were analysed for actual compound levels by chemical methods and for activity with bioassays. For details, see Bulder et al., 2002). Results for the E2-medium were as described in the chapter on E2 (Chapter 3.2). All experimental media displayed estrogenic activity in bioassays.

Plasma VTG was measured in adult zebrafish at the end of the exposure period. Per adult exposure group, blood samples were collected from two females and four males by tail incision and blood withdrawal from the tail vessels. Blood samples were pooled per sex. VTG in pooled blood plasma was determined using ELISA analysis (see Fenske et al., 2001). The PLC assay was approved and performed according to the guidelines of the Dutch Institutional Animal Experimentation Committee (AAP 199900608).

Results and discussion

In life observation - adults

The exposures induced no mortality, nor effects on behaviour or clinical appearance. There was no significant effect of exposure on egg production (Table 3.7.1), compared to control. On the other hand, there was a significant difference between the E2 positive control and the effluent (E2 lower fertilisation rate), indicating a disparity among these two treatments.

Table 3.7.1 - Reproduction parameters in F0 after 21 days of exposure to effluent

treatment	number of clutches [1]	clutch size	cumulative number of eggs	fertilisation rate
control	5.3 ± 1.7	268 ± 26	1455 ± 670	71.7 ± 22.6
E2	5.0 ± 0.8	223 ± 63	1072 ± 99	49.4 ± 21 [a]
synthetic effluent	3.3 ± 1.9	309 ± 108	934 ± 459	52.1 ± 11.3
effluent	1.7 ± 0.5	324 ± 244	620 ± 530	93.3 ± 10.9 [a]

All data are average ± sd of three spawning units, two in control and E2 due to non spawning
[1] maximum number of clutches is 7.
[a] $p < 0.05$ (ANOVA and Tukey's test)

Table 3.7.2 - Hatching after exposure to effluent

treatment P-F1	n[1]	hatching (%)
control – control	3	49.8 ± 34.4
E2- control	3	56.3 ± 22
synthetic effluent – control	3	34.4 ± 27.4
effluent – control	2	70.0 ± 22.6
E2 - E2	3	56.4 ± 35.5
synthetic effluent – synthetic effluent	3	32.4 ± 3.4
effluent – effluent	2	51.6 ± 7.9
control – effluent	2	43.0 ± 20.3

[1] number of sampled spawning units.
data are average ± sd

In live observations - juveniles

There were no significant effects of treatment on hatching (Table 3.7.2).

There was no aberrant behaviour nor were there clinical abnormalities. No effects on

survival, length or weight were observed (Table 3.7.3). Juveniles exposed to Eindhoven

Table 3.7.3 - In life observation of F1 zebrafish exposed to effluent for 42 days

treatment P - F1	n[1]	survival (%)	length (mm)	body weight (mg)	condition factor
control – control	3	94.8 ± 2.8	16.5 ± 0.7	70 ± 7.9	1.51 ± 0.02
E2 – control	2	93.6 ± 5.1	16.1 ± 0.1	64.6 ± 1.9	1.47 ± 0.01
synthetic effluent – control	3	91.3 ± 8.1	16.6 ± 0.4	72.8 ± 0.7	1.56 ± 0.11
effluent – control	2	97 ± 4.2	15.8 ± 0.1 [a]	62.9 ± 1.4	1.56 ± 0.02 [a]
E2 - E2	3	84.4 ± 11.8	16.5 ± 0.3	70.8 ± 5.0	1.49 ± 0.01
synthetic effluent – synthetic effluent	3	91.3 ± 9.0	16.2 ± 0.2	67.0 ± 3.6	1.53 ± 0.09
effluent – effluent	2	89 ± 7.1	16.1 ± 0.1 [a]	61.1 ± 1.6	1.42 ± 0.02 [a]
control – effluent	2	82.3 ± 20.3	16.2 ± 0.3	63.5 ± 2.2	1.43 ± 0.01

values are average ± sd
[1] number of replicates
Less than three replicates were available in the case of non spawners, too small brood sizes and/or insufficient hatched juveniles
The initial average number of juveniles ranged between 35.0-50.5.
[a] $p < 0.05$, paired t-test

Fig. 3.7.1a - Testis of adult zebrafish after exposure to Eindhoven effluent (medium power magnification). Testis showed an image similar to that of control animals. There is a normal ratio of various spermatogenic maturation stages. sg, spermatogonia; sc, spermatocytes; st, spermatids and sz, spermatozoa.

Fig. 3.7.1b - Testis of adult zebrafish after exposure to synthetic effluent. Note mixed appearance of maturation stages within single cysts (arrows). l, leptotene spermatocyte; z, zygotene spermatocyte and p, pachytene spermatocyte

effluent showed a minor though statistically significant increase of length and a reduced condition index compared to the control group, but only when parents were exposed to the effluent.

Histopathology - adult males

After exposure to the positive control substance E2 (1 nM), testes showed intensely stained plasma (VTG) in blood vessels. Furthermore, there was eosinophilic material (droplets / aggregates) within and associated with spermatogenic cysts. The spermatogenic cysts appeared smaller than in control animals, although they seemed to occur in a normal ratio (see Chapter 3.2, test with E2 for details). In males exposed to the Eindhoven effluent, there was an apparently normal ratio of spermatogonia/ spermatogenic cysts/ spermatids, and the tubular lumen was filled with spermatozoa. No VTG-filled vessels wer seen, and Sertoli and Leydig cells had a normal appearance (Fig. 3.7.1a).
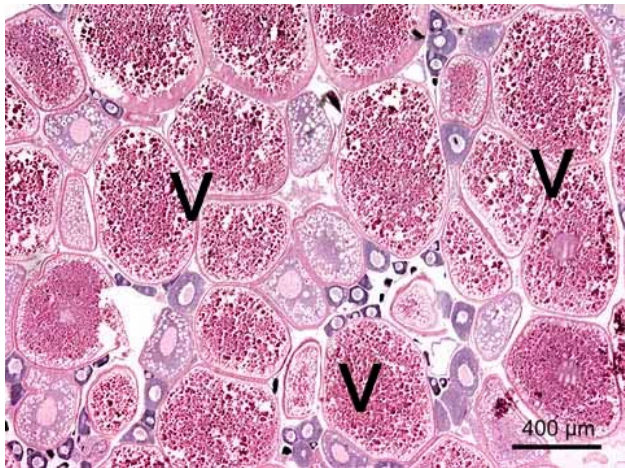
Fig. 3.7.2a - Ovary of adult zebrafish after exposure to synthetic effluent (at low power view). There is accumulation of mature vitellogenic oocytes (v).
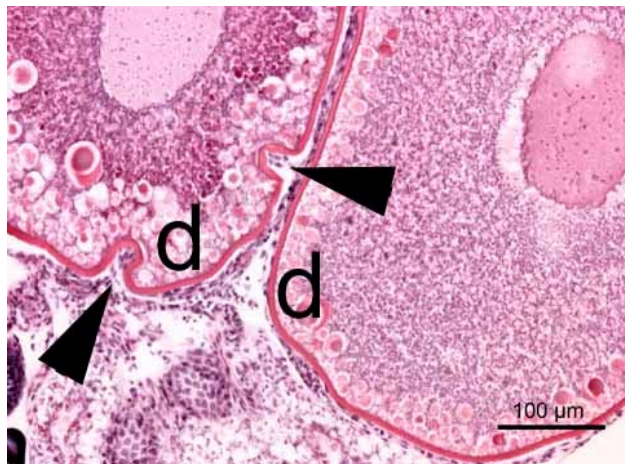

Fig. 3.7.2b - Medium power magnification of oocytes after exposure to synthetic mix. Oocytes show peripheral disintegration (d), and there is typical folding of the oocyte membrane (arrowheads).

Fig. 3.7.2c - High power view of oocytes after exposure to synthetic mix. Associated with folding oocyte membrane, granolosa cells are hypertrophic (h); compare to normal appearing granulosa cells (n).

After exposure to the synthetic effluent, there was a normal appearing ratio of spermatogenic cysts, however, there was an indication of asynchronous maturation within spermatogenic cysts (Fig. 3.7.1b). No other abnormal features were found after exposure of adults to E2, Eindhoven effluent or synthetic effluent.

Histopathology - females

Exposure to E2 resulted in typical estrogenic effects on ovaries such as a high ratio of atretic follicles (>25 per 10x field), combined with decreased numbers of vitellogenic oocytes

compared to control females (see Chapter 3.2, test with E2 for details). This was apparent in three out of eight exposed females.

The appearance of the ovary in females exposed to the effluent was comparable to control females: vital oocytes in all stages with a normal appearing granulosa cell layer, occasional fields with exclusively early stages oocytes (perinucleolus stage), some postovulatory follicles were present (±5 per 10x field).

After exposure to the synthetic effluent, the ovaries of most females showed accumulation of vitellogenic (mature) oocytes (Fig. 3.7.2a); atresia (peripheral desintegration); folding of oocyte membranes (Fig. 3.7.2b), and activation (hypertrophy) of granulosa cells (Fig. 3.7.2c).

Further histological observations

Exposure to E2, but not to effluent or synthetic effluent, induced liver basophilia and intravascular acidophilic fluid accumulation identified previously as VTG-rich plasma, in males. There were no further histological changes in females. Some animals showed fibrosis of bile ducts and in the pancreas, unrelated to exposure. No other organ tissue changes were noted in the total body sections.

Vitellogenin ELISA

Analysis of VTG of pooled plasma samples showed a lower level of VTG in control males compared to control females. VTG increased after exposure to E2 in both males and females, compared to controls (Table 3.7.3). Exposure to Eindhoven effluent and synthetic effluent resulted in increased VTG levels, but only in females.

Table 3.7.4 - Vitellogenin concentration in pooled plasma samples of adult zebrafish

treatment	male	female
control	240	2242
E2	25982	165361
Eindhoven effluent	11	34710
synthetic effluent	325	27530

Data are results from pooled plasma samples (3-5 zebrafish); concentrations are in µg/mL.
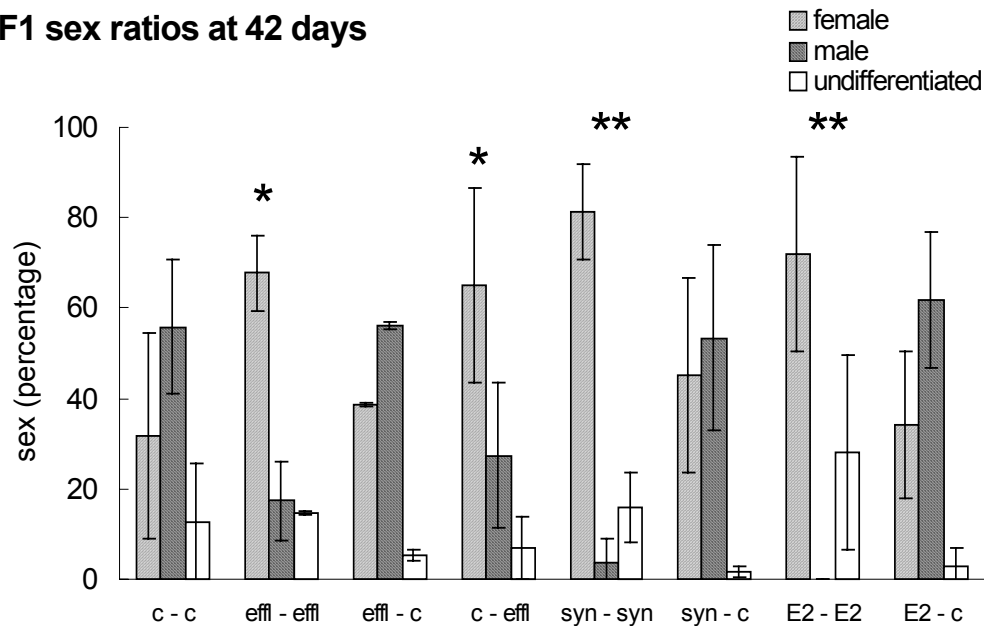
F1 sex ratios at 42 days

Fig. 3.7.3 - Juvenile sex ratios at 42 days after exposure of P-F1; c, control - effl, Eindhoven sewage treatment works effluent - syn, synthetic effluent - E2, 17β-estradiol.
*, ** p<0.5 , 0.1, respectively, T-test

histopathology - juveniles

Statistically significant skewed sex ratios, towards the female phenotype, were observed in juveniles exposed to each of the test media (Fig. 3.7.3). The control juvenile populations showed an average sex distribution of approximately 60% of males to 30% of females, with the remaining animals showing undifferentiated gonads. No phenotypic males were seen after E2 exposure. Sex ratios were not significantly affected in offspring after parental exposure only.

This study confirmed the effects observed with the reference estrogen as described in Chapter 3.2, such as VTG induction (VTG filled blood vessels, liver basophilia, oocyte atresia). Also a skewed sex ratio was seen in the offspring.

In the effluent and synthetic mix study, however, this was not reproduced at the histological level. The induction of VTG in females as measured with ELISA with both the Eindhoven and the synthetic effluents indicates some estrogenic activity of these media. On the other hand, since this induction of VTG was only detected in female samples (while males are normally equally sensitive), a differential effect of these media may be suspected in both sexes. This indicates that complex mixtures of estrogen active compounds may elicit effects that differ from those evoked by the individual compounds when tested in isolation. This concept is further supported by the specific histological findings in the gonads after exposure

to the synthetic effluent, which were identical to those induced by the anti-estrogen tamoxifen (chapter 3.3); furthermore, this inconsistency indicates that the synthetic effluent does not model the STW effluent without further consideration. These findings emphasise the importance of *in vivo* studies including histopathology in the studies of (mixtures) of endocrine disrupting compounds. The absence of obvious effects with the Eindhoven effluent may be the result from a sub-active total level of the individual compounds, or from complicating factors, such as the presence of non-identified interacting chemicals or biota. Juvenile exposure to Eindhoven and synthetic effluent resulted in feminisation similar to that achieved with E2. This is a well-known effect of exposure to compounds with estrogenic activity (reviewed by Piferrer, 2001).

The inconsistency for the various endpoints (VTG induction in females and feminisation in juveniles on one hand, absence of male VTG induction and tamoxifen like effects in adult gonads on the other) indicate that for different target tissues diverging mechanisms and sensitivities may exist, resulting in a diverging net effect.

As in the E2 PLC, feminisation is the critical endpoint, determining the hazard of exposure to the studied effluents in the individual fish, and moreover, for the population. Comparison with effects in wild species (bream), in which vitellogenesis and testis-ova (although no sex reversal), and the possibly decreased population size (Vethaak et al., 2002) were recorded, indicates that the zebrafish PLC is predictive for possible effects in the field.

Conclusions

- The effects observed in the positive controls are in accordance with those observed in the previous E2 study.
- The effluent exposed animals displayed some estrogenic effects but also tamoxifen-like (anti-estrogen) effects.
- Mixtures of chemicals may exert effects which differ from (and even contradict) expected effects from individual compounds.
- The PLC study reflects the hazards identified in the field.

4. Discussion and evaluation

4.1. Species

The zebrafish is an established laboratory species and recommended by the OECD, as are medaka and fathead minnow. This species was selected for this project for its availability, ease of breeding, continuous (season-independent) breeding, the short life cycle and rapid development, the widespread application in science, and the small size that allows whole body sectioning for histology. However, some disadvantages also exist: visual differences between sexes are limited and require skillful animal technicians, and there may be strain-dependent biology differences, e.g. with respect to prevalence of testis-ova. Another point of consideration relates to extrapolation of effects to wild fish species in the field (Schäfers et al., 1993): exotic fish versus endemic species; species relations (order / family); reproduction strategy and behaviour, feeding habits, etcetera. Such considerations, however, will apply to any model.

In this project, the anticipated advantages of the zebrafish were confirmed. An unforeseen drawback was the high variation in reproduction parameters, which can, however, be overcome by appropriate changes in the test protocol (see below). The overall experience with the zebrafish was positive, and it was valued as a highly useful species in the laboratory.

4.2.   Assessment of individual parameters

Life cycle parameters

Parameters employed in the PLC tests of this project are evaluated individually hereunder to identify the power and weakness of the applied PLC test protocol. The results of this evaluation can be used to further improve the design of the PLC-test protocol.

For the analysis of parameters of reproduction, coefficients of variance (CVs: standard deviation / average * 100%) among experimental units to which the same treatment is applied, give an indication of the variability of a parameter. If the CV of a variable is large, significant differences between treatments will only be found when a large number of replicates is used. The employed parameters are discussed viewing the CVs among the untreated controls. Sensitivity of the parameters to detect effects of endocrine active compounds are discussed by evaluating significant differences between controls and exposed experimental units tested with ANOVA.

Egg production

Control experimental units produced between 0 - 7 egg clutches during the exposure period of 21 days (Fig. 4.2.1). CVs calculated from the three experimental units ranged between 9-87% for the number of egg clutches within the PLC-tests with the five reference compounds. The relatively high CV of 87% was produced during the PLC-test with 17β-estradiol; in two of the experimental units 7 egg clutches were produced and none in one of the experimental units. In the present setup, non-producing control experimental units will generally not be identified as outliers by appropriate tests, due to the low number of replicates per treatment. Furthermore, there is no obvious replacement value for a possible outlier, and removing an outlier leaves only two replicates, making statistical differences highly improbable. It should be noted that the overall variation in number of eggs is mainly derived from variation between individuals; the variation between clutch sizes from a single individual was relatively small. In our PLC-tests, egg production did not show a regular pattern in time, in line with reported variability of egg release in zebrafish (Bresch, 1982; Van den Belt, 2002).

In three of the five PLC-tests, significant differences were found between number of egg clutches as well as total number of eggs produced in the control and exposed experimental units (MDHT, flutamide, tamoxifen). In this context it should be noted that in the study with
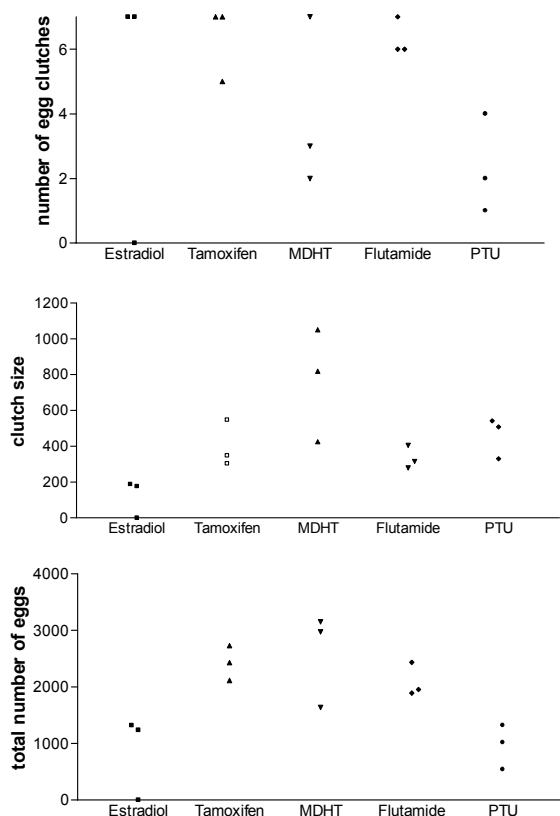
Fig. 4.2.1 – Egg production parameters in untreated controls

estrogen the highest concentration (1 nM) was chosen because of complete regression of the ovary at 10nM in the range finding study, which concentration therefore was not deemed useful in the PLC-test (no egg production expected). Evaluation of the egg production parameters logically depends on the selection of concentrations, more obvious effects can be expected at higher concentrations of most tested compounds. The number of egg clutches and total number of eggs produced during the 21-day exposure period have revealed some effect of EDCs in the PLC-tests when the effects are major, e.g. complete cessation of egg production. However, statistical analysis of variables with this low number of replicates is highly susceptible to (incidental) reproduction failure in the control experimental units; increasing the number of adult fish per experimental unit will result in more evenly distributed data among experimental units with the same treatment because of two reasons: i) an occasional non-spawning female will not immediately result in the experimental unit to be an outlier, and ii) egg production per fish will probably show a more regular pattern. This was confirmed by Bresch et al.(1986) and Roex et al.(2000), who both observed less fluctuation in egg production with more than five female zebrafish in a group, because fluctuations in egg production between individuals were leveled out. In the present experimental setup, the number of control replicates should be increased to at least 16 to detect a difference of 50% in egg production parameters with a certainty of 80% (calculated according to Sokal and
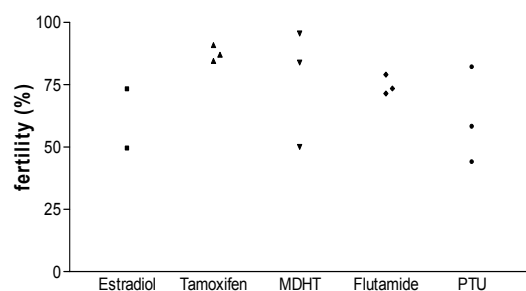
Fig. 4.2.2 - Fertilisation in untreated controls

Rohlf, 1981). Such a large number of replicates is not practicable. In addition, it may be considered to limit the evaluation of clutches to the end of the exposure period, to avoid bias from an initial absence of effects. Another possible modification of this parameter is semiquantitative evaluation of the number of eggs per clutch, which will not affect the statistical results.

From the previous paragraph it is clear that egg production may be a useful parameter for major effects at relatively high concentrations. As variations in controls are significant, egg production may be assessed semi-quantitatively. For statistical considerations more adults per experimental unit are required if small differences are to be detected. Assessment of only the final clutches could be considered.

Fertilisation

For the controls, percentages of fertilisation between 0 and 100% were found (Fig. 4.2.2) with CVs ranging between 8 and 141% (for those experimental units for which CV could be calculated).

For most of the compounds tested in this study, no differences were found between controls and treatments for percentage of fertilisation (except for tamoxifen, showing significantly reduced fertilisation rate with exposure concentration). This reproduction parameter showed high CVs within experimental units of the same exposure concentration, and did not show to be sensitive for effects of the tested compounds.

Fertilisation was considered as one of the most sensitive parameters after exposure to toxicants in an analysis of 176 fish studies (Suter et al., 1987), although zebrafish were not included in this study. However, the high variation in percentages of fertilisation, and the insensitive response to the reference compounds makes it ineffective as parameter to detect response of zebrafish to endocrine active compounds, in the present setup of the PLC-test,. A highly increased number of replicates may result in a more sensitive test system for this variable, but the number of replicates necessary to detect significant differences cannot be
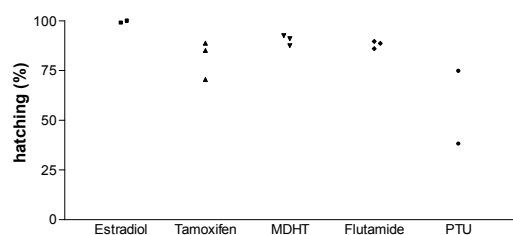
Fig. 4.2.3 - Hatching in untreated controls

calculated with the available information; it is expected to be large (more than 20 replicates). Therefore, the informative value of this parameter in the present PLC is very limited.

Juvenile development - hatching

Percentages of fertilised eggs that hatched varied between 36 and 100% per egg clutch in the control units of the five PLC-tests (Fig. 4.2.3). CVs of hatching per experimental unit were small, ranging between 0 and 28% (for the experimental units for which CV could be calculated) but CVs of exposed experimental units were large in many cases.

Sensitivity - For none of the compounds tested within the present study, differences between controls and treatments were found for hatching, with a possible exception of reduced hatching after parental exposure to tamoxifen. This reproduction parameter showed high CVs within experimental units of the same exposure concentration, and did not show a sensitive reaction to EDCs.

Hatching was considered to be one of the most sensitive parameter after exposure of fish to toxicants in a review of 176 studies (Suter et al., 1987), which, however, did not include studies with zebrafish. In contrast, in the PLC-test described here, the high variation in percentages of hatching, and the insensitive response to the tested reference compounds makes it ineffective as parameter to detect endocrine disruption in zebrafish. Therefore, in the present setup of the PLC-test the informative value of the parameter hatching rate is limited. A highly increased number of replicates may result in a more sensitive test system for this variable, but the number of replicates necessary to detect significant differences cannot be calculated with the available information; it is expected to be large (more than 20 replicates).

Juvenile development - survival

The PLC-tests showed juvenile survival in the controls between 70 and 97%, with the exception of low survival rates in the PTU test (48-63%; Fig. 4.2.4). Juvenile survival differed between PLC-tests but within a PLC-test juvenile survival in controls showed only
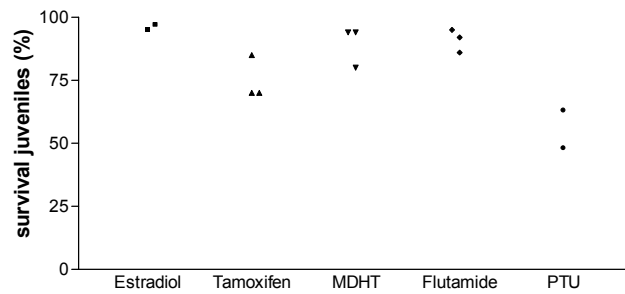
Fig. 4.2.4 – Juvenile survival in untreated controls

small variation (CVs 3 – 19%). In two of the PLC-tests, survival of juveniles in the treated experimental units differed significantly from controls (17β-estradiol, PTU). Survival of juveniles as PLC-test parameter has detected some effects of the reference compounds in the PLC-tests. However, a higher number of replicates is recommended to increase the sensitivity of the test, i.e. to 4-5 replicates with the present setup to be able to detect differences of 25-50% with 80% certainty.

Juvenile development - length, weight and condition factor
CVs were very low for length, weight, and condition factor of juveniles in the controls with a
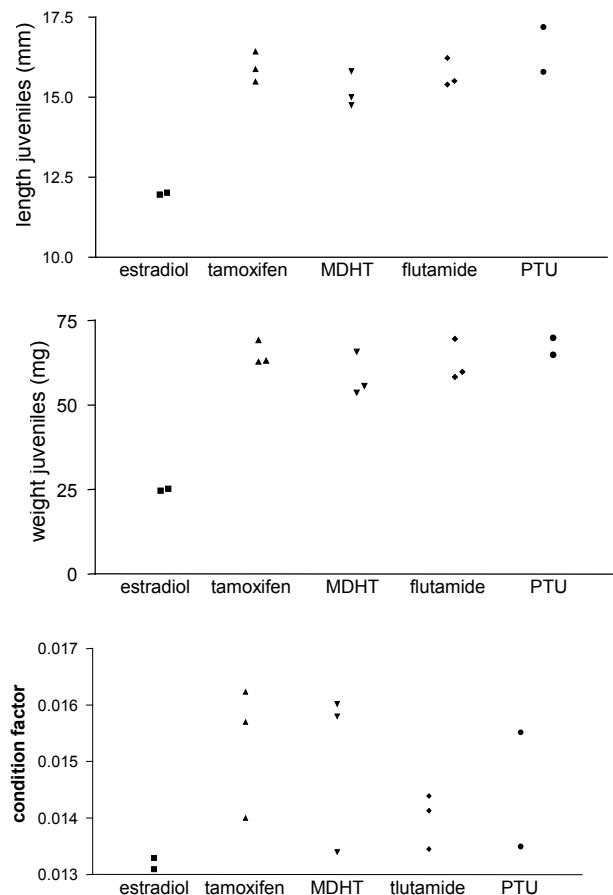

Fig. 4.2.5 – in life parameters, length, weight and condition factor of F1 zebrafish in untreated controls

maximum of 11% (Fig. 4.2.5). In four PLC-tests, significant differences between length / weight of juveniles in controls and in treated experimental units were found (negative with tamoxifen, MDHT, PTU, and positive with 17β-estradiol). The tests with tamoxifen, flutamide, and PTU gave significant differences between condition factors of juveniles in controls and in treated experimental units.

The parameters length, weight, and condition factor reflected some effects of endocrine active compounds. Importantly, length and weight of juveniles even increased after exposure to E2, suggesting anabolic activity, and this might, when assessed in isolation, mask the adverse effect of this compound. Also, the current protocol detects only gross effects in length and weight; for more subtle effects, e.g. a difference of 10% between treatments, a number of at least 16 replicates should be tested for each treatment with the present experimental setup (Sokal and Rohlf, 1981).

VTG

VTG is an egg yolk precursor protein produced under control of estrogens. Thus, this endpoint is particularly relevant for test compounds that directly or indirectly activate or block estrogen receptors. In particular in males this is a sensitive parameter as background levels are negligible, and VTG tends to accumulate due to the lack of a natural outlet (spawning of eggs; Van den Belt et al., 2003). As most of the concern for EDCs is targeted at ER binding compounds, VTG can be considered as an extremely useful biomarker indicating endocrine disruption. It is therefore widely used in field and laboratory studies. In the proposal of the project it was envisaged to develop a (semi)quantitative assessment of VTG levels specifically for zebrafish, preferably by an ELISA. However, to prevent duplication of efforts of other laboratories in this field, and as histology was one of the principal techniques in this project, we aimed at developing an immunohistochemical method of VTG determination employing anti-zebrafish VTG antibodies. The reaction was measured by morphometry, and the results were compared with plasma VTG levels as measured by ELISA. The method appeared sensitive and the results were largely comparable with ELISA; additional advantages were the possibility to study very small samples (histological sections) and archived material (Van der Ven et al., 2002), and the reduction of laboratory animal use. It should be noted, however, that the basic standard histology assessment of VTG, based on liver basophilia and plasma / body fluid intensity, appeared more or less equally sensitive, less complicated and thus more practical.

With these (immuno)histological methods we managed to identify changes in VTG in the studies with E2, MDHT (increase in males and females), and tamoxifen (decrease in females).

In view of the wide application of VTG as parameter for estrogenic activity in fish, the possibility exists that estrogen mimics might be over-represented as EDCs. Furthermore, question has raised about the functional relevance in terms of the predictive value for reproductive and developmental disturbance. Indeed high levels of VTG may lead to hydropic changes in various organs due to the osmotic activity (Wester et al., 1985). However, in the present study such effects were noted in the range finding study using excessively high doses of estrogen, but hardly at the more environmentally realistic concentrations in the PLC. Thus extremely sensitive methods may indeed detect compounds with estrogenic potential at very low levels, but in view of a probably negligible functional impact, classification as an endocrine disrupter may be doubtful for such compounds. Androgens can also give rise to VTG production although at much higher concentrations, beyond a level where other relevant changes had occurred.

Histopathology

The need for studying histopathology was imminent as one became interested in endocrine disrupting pollutants. Classical parameters such as growth and mortality are non-specific and inadequate for this purpose, and analysis of VTG, although invaluable, has limited application as is only a biomarker for hazard from (anti)estrogenic activity. For more physiological and mechanistic relevant parameters histopathology is the method of choice in the context of hazard identification and dose/concentration-response assessment, as is the case in classical rodent toxicology for human risk assessment. The purpose of this project was to investigate the spectrum and sensitivity of histopathological responses under practical conditions of laboratory testing.

Using various hormonal active agents it appeared that in all cases histopathology was the most sensitive parameter under the conditions of the study. Importantly, also the observed pattern of responses was specific for and could be explained from the different hormonal actions studied. A brief summary of these responses is given in Table 4.2.1.

Table 4.2.1 - Summary of main histological effect in target tissues

	estrogen	anti-estrogen	androgen	anti-androgen	anti-thyroid
liver	VTG ↑		H[1]: VTG ↑		glycogen ↓
ovary	collapse	degeneration of eggs	L: ovulation ↓ H: collapse		
testis	regression	asynchrony Leydig cells ↑	spermatogenesis↑ Sertoli cells ↑	spermatogenesis ↓ Sertoli cells ↑ Leydig cells ↑	
thyroid					stimulation
offspring	feminisation	masculinisation	masculinisation	masculinisaton	

[1]: H / L: effect at high or low concentration

Sex ratios

Sex differentiation in (zebra)fish is poorly understood and not merely genetically determined; environmental factors may influence the outcome of the gender phenotype (Yamamoto, 1969). In addition, it has been claimed that zebrafish are undifferentiated gonochorists: after initial development of an ovary-like gonad, and only later during development, i.e. from week 8 onwards, differentiation towards males would occur in a fraction of the juveniles (Takahashi, 1977; Maack and Segner, 2003). However, we have not succeeded in confirming this phenomenon, and by contrast, we have observed early sex differentiation in our juvenile zebrafish directly towards male or female phenotype as early as 4-5 weeks after hatching. The sensitivity to environmental conditions and, more specifically, hormone active agents, renders sex differentiation in this species as a useful and specific endpoint, which requires histological assessment. In our PLC tests we found skewed sex ratios after exposure of juveniles to estrogen (shift towards females), and androgen, anti-estrogen and anti-androgen (shift towards males in the latter three cases). Such findings are consistent with those described elsewhere (Petersen et al., 2001). It appeared that this sex ratio was equally sensitive as VTG changes in estrogen exposure, and even the most sensitive endpoint in androgen exposure. In addition, it is likely that major shifts in sex ratios is of relevance to population dynamics and thus ecologically important as an EDC effect.

Gonad histology

Changes in the gonads appeared to be sensitive and specific. Sexually active gonads are under dynamic endocrine control and therefore likely subject to endocrine disruption. Indeed in our studies we observed changes in the ovaries that indicated down-regulation (estrogen), degeneration (anti-estrogen), and inhibited spawning (androgen), effects that were generally easy to detect. In the testis the changes were significant indeed, but required more detailed analysis such as morphometry and therefore may be less suitable for routine screening. These changes were quantitative shifts in spermatogenic stages (estrogen, androgen, anti-androgen), morphological changes in Sertoli cells (androgen, anti-androgen) or numerical changes in (interstitial) Leydig cells (anti-estrogen, anti-androgen). Combination of these changes in males and females appeared rather specific in the sense that they may be considered indicative for the mode of action. Importantly, in the study where a synthetic mixture of environmentally relevant compounds with estrogenic activity was applied, the histological changes in the gonads were indicative of anti-estrogen rather than estrogen activity. This observation may indicate that *in vivo* effects may differ from what is expected from chemical and *in vitro* data, and underlines the importance of *in vivo* studies with histological endpoints.

Other organs

Other organs relevant for endocrine functioning and disruption would be those of the endocrine system such as pituitary, interrenal cells (equivalent to adrenals in mammals), ultimobranchial body (equivalent to parafollicular cells in mammals), Stannius' corpuscle (calcium-regulating hormone), pancreatic islands and the thyroid (see Atlas for examples and details). The only practical methods for analysis in small fish would be determination of circulating hormones or histology, in some cases (pituitary) with the help of special staining techniques (Wester et al., 1985; Wester and Canton, 1986). The former is normally not feasible due to the limited availability of analytical reagents, and histology is routinely of limited value as these organs are composed of only a limited number of cells and therefore not routinely present in a reproducible way in histology sections. The thyroid and pancreas, however, are generally readily available and therefore candidates for histopathology. In addition, thyroid hormones can be determined in plasma samples, as was done in the study with PTU (Chapter 3.6). The thyroid has been shown to be an interesting target in that case, as well as in previous studies (Wester and Vos, 1994).

Another important organ in the context of endocrine functioning in fish is the liver. The liver is the source of the yolk precursor VTG in egg laying species, and the production is under

control of estrogens. This VTG synthetic activity is reflected by a clear morphology (basophilia due to the high density of ribosomes) and thus the liver morphology is an (additional) indicator of estrogen balance. This feature has been further studied in Chapter 2.1, and has been applied in various studies in this project; it was shown to be a reliable indicator for estrogenicity, together with intense staining of plasma containing unusually high levels of VTG.

Other organ changes directly related to endocrine effects have not been unequivocally established in this project, possibly with the exception of the study with tamoxifen, where a clear effect was seen on the severity of (abdominal) inflammations, possibly related to a compromised immune system. In any case, this illustrates the importance of general histology for other effects that might have a direct or indirect relevance for the test compound under study, but also to identify possible pathological conditions in the test animals that might bias the outcome of the study.

Conclusion on the assessment of individual parameters

Life cycle parameters

Woltering (1984) argued that growth response in fish toxicity tests is an inconsistent endpoint and is not as sensitive as other parameters, such as reproduction. Indeed, in our PLC-tests we noted differences in length and weight of juveniles after 42 days between the controls of the PLC-tests; the reason for this variation remains unknown. Nevertheless, juvenile length and weight were sensitive life cycle parameters in the PLC-tests, although not necessarily typical for endocrine disruption. Sensitivity of the life cycle parameters produced in the present PLC-test is low compared to endpoints determined by histopathology (VTG, sex ratio and gonad pathology). Generally, sensitivity of the life cycle parameters may be increased by increasing the number of experimental units per treatment and/or increasing the number of individuals per experimental unit. However, life cycle parameters are essential in view of the sustainability of the species.

Vitellogenin

VTG is an important biomarker for disruption by compounds acting on the estrogen receptor. It is the most widely applied endpoint in the study for endocrine disrupting substances in fish, and therefore estrogen mimics may be over-represented as EDCs. When comparing the various endpoints used in this project we conclude that VTG increase is the earliest indicator and most sensitive endpoint for estrogenicity, in particular in males, next to changes in sex

ratio in offspring and reproductive performance parameters. Impact on reproductive performance and development, predicted from the zebrafish model, can only be reasonably anticipated when total impairment of gonadal function, i.e. cessation of egg production, is induced. This occurs at an estrogenic potency equivalent of nominal 10 nM E2, and it should be noted that these high concentrations are beyond field levels. The practical implication of very sensitive detection of VTG induction is therefore arguable, in view of the aim of ecological risk assessment.

Histopathology

Histopathology is an important and sensitive tool to identify effects of endocrine compounds on several levels. It is the only method to assess sex differentiation, which can be disrupted by endocrine active compounds. It can determine changes in male and female gonads, specific for effects of compounds with different, specific mechanisms of action. Finally, specific changes in other organs and tissues can be assessed, such as effects on the thyroid or on VTG expression, or possible concomitant pathology (e.g. inflammation).

4.3.   Evaluation of experimental setup

Initially a protocol was designed to cover al the potential relevant endpoints for general and reproductive health in zebrafish. A two-generation study or a mesocosm study would be preferred, but these are not applicable on a routine basis. A compromise was sought to cover the most relevant endpoints within a limited experimental timeframe. This was found in a partial life cycle study design that covered three weeks of a parental (P) generation, and subsequent exposure of eggs and juveniles (F1) during the period of sexual differentiation that appeared to last approximately 6 weeks in the zebrafish. Endpoints were parameters for general health, (mortality, behaviour) and reproduction (egg production, fertilisation) and development of offspring (hatching, mortality, growth, condition). The PLC-test was enhanced by introducing other endpoints such as histopathology and VTG. The general protocol is presented in Chapter 2.2.

The exposure period of parents was apparently sufficiently long. If an effect occurred in females, it was usually detectable within a few days or weeks (E2, MDHT, tamoxifen) due to vulnerability of the mature oocytes and the short reproductive cycle. Effects in males seemed to concern the proliferation and maturation of spermatogenic cycle. It was initially questioned whether the exposure period was sufficiently long to cover the total developmental (spermatogenic) cycle. This was examined in an additional experiment, studying the duration of the spermatogenetic cycle by using BrdU as a marker for mitotic spermatogonia. This experiment demonstrated that labeled spermatogonia are released as sperm in a period of about 12 days (Van der Ven et al., 2003a). Thus, the 21 days exposure period is sufficient to induce effects in the full spermatogenic cycle.

However, a two-generation study with zebrafish exposed to ethynylestradiol has demonstrated that fertilisation rate (male dependent) and mortality were significantly affected in F2 (Nash et al., 2003). This is probably due to the long exposure period, and therefore functional effects, notably on spermatogenesis, may be detectable after prolonged exposure, and therefore be underestimated in a 21 days exposure period.

Reciprocal exposure of juveniles - i.e. exposure of offspring from control parents to test compound, and offspring from exposed parents to control medium - was performed to evaluate the parental influence of effects. Effects in juveniles were almost exclusively associated with juvenile exposure, similar to observations by McKim (1985). Occasionally,

there seemed to be an (additional) effect of parental exposure in the case of PTU (struma, glycogen), of flutamide (sex differentiation), and possibly of tamoxifen (hatching). Nevertheless, for hazard identification with the test compounds used this reciprocal exposure protocol appeared to be of limited added value in view of the additional workload.

The current protocol was used as a template to which, depending on specific requirements, modifications can be introduced as was done during this project in finding a workable protocol. Particularly, the statistical power of the protocol appeared critical in view of cases where outliers occur (non-spawning in controls) and in view of the limited number of replicates, which was the consequence of the compromise that was sought to keep the protocol within practical limits. Thus, if quantitative information is desirable, more animals per replicate, more replicates or more treatments (e.g. for benchmark dose assessment) should be considered. However, for histological assessment, these limited numbers seemed adequate, since most histological assessable effects occurred unambiguously, i.e. all individuals at a given concentration of a test compound were affected in a similar way.

4.4.   Assessment of population impact

Relevance of parameters

Egg production (number of egg clutches, clutch size and total number of eggs per experimental unit) showed some significant effects of endocrine disruption but was not a very sensitive parameter in the PLC-tests. Egg production completely ceased in some of the highest dosed experimental units in two PLC-tests (MDHT, tamoxifen), logically leading to a fast extinction of the population. However, incomplete reduction of egg production did not have an effect in the population model. This is in line with the findings of Nagel et al. (1991), who calculated that a reduction in egg production does not affect the population size.
In the laboratory population of Oertel (1992), it was observed that a reduced egg production resulted in a lower number of larvae and juveniles, and this was accompanied by a lower predation rate: a certain number of juveniles was always retained from predation because they were able to hide in refugia in the aquaria. Thus, at the population level, effects on egg production, and also on other life history parameters, can trigger compensating mechanisms. These are contained in the population model, which simulates the complex and dynamical balances of disturbing and supportive factors.
In conclusion, egg production is not a determining parameter of population dynamics in our system.

Fertilisation and *hatching* were hardly affected by the endocrine active reference compounds used in the PLC-tests of this study (see 6.2). Similar to egg production, these parameters had no impact on population variables in the model.

Growth (length and weight) and *survival of juveniles* were the most sensitive life cycle parameters in the PLC-test, although the observed reductions were without consequence in the population model. Previous studies showed that in life cycle assays, early life stages are the most vulnerable to toxicants in various fish species (reviewed by McKim, 1985). Specifically for zebrafish, juvenile survival was critical for the maintenance of zebrafish populations (Nagel et al., 1991; Oertel, 1992), although only with more severe reductions than observed in our tests.

The impact of *skewed sex ratios* on fish populations depends on the reproduction strategy of the species. In *r*-strategists such as zebrafish, few males can successfully fertilise the eggs released by many females, thus not limiting the reproductive potential of the population (Ricklefs, 1980; Halliday, 1993). On the other hand, with a decreasing number of females, the total egg production and hence the reproductive potential of the population will decrease concomitantly. However, the size of the population will only decrease when this reduced egg production is not compensated by increased juvenile survival, or otherwise. Consequently, a population will only be at risk at a low ratio of females and an even lower ratio of males; calculations with the IBmodel produced increasing extinctions of large populations (200 adult fish) at ratios of <10% females and <5% males. Smaller populations are more susceptible, as are K-strategists, that produce smaller numbers of offspring, and where in some species males do not mate with multiple females (monogamy, brood care by males). Evidently, shifts of sex ratio to 100% male or female individuals will lead to a fast extinction of a population, regardless of reproduction strategy.

The risk of decreased genetic variation and consequent inbreeding depression in case of skewed sex ratios is very limited, since with still large numbers of offspring, healthy individuals will have a selective advantage (Halliday, 1993). Furthermore, the number of recessive lethal alleles is low in zebrafish (McCune et al., 2002). Another factor of concern might be effects on reproductive behaviour, or fertilisation success, which however, in zebrafish is not affected by sex ratios (Nash et al., 2003).

A common parameter used in field and laboratory studies is the analysis of *VTG levels* in plasma or whole body homogenate. Although this is generally seen as a powerful biomarker for exposure to (anti)estrogen compounds, it is not clear what an altered level implies for functioning of the individual or population in terms of reproductive fitness. It seems likely that VTG as such (without impairment of other endpoints) is of limited importance for the (population) fitness. Indeed excessive VTG levels may lead to cardiovascular dilatation and failure, ascites, hydrops and protein leakage and accumulation in the kidney as was seen in the range finding study with E2 and with the xenoestrogen β-HCH (Wester et al., 1985; Van der Ven and Wester, 2002), thus compromising the individual fitness, but the levels at which this occurs are unlikely to occur under field conditions. In the present study, we have shown that appreciable changes in VTG levels, i.e. detectable with histological methods, occur at exposure levels which also induce changes in sex ratios, which is relevant for population dynamics. However, this is only true in the case of E2 (increased VTG in P males associated

with feminisation in F1) and tamoxifen (decreased VTG in P females associated with masculinisation in F1). In the case of MDHT and flutamide, sex ratio is more sensitive than VTG, and effects at the population level will be missed when only VTG is evaluated. Thus, the value of VTG as a biomarker for effects on the level of populations is relative, and limited to specified effectors and at specified levels.

Population modelling

Predicting consequences on complex population dynamics from simplified laboratory conditions is not without pitfalls, even with specifically designed population / ecosystem models (Seitz and Ratte, 1991). For instance, we used a single compound in a limited time window in the water phase under standardised and optimised conditions, while in the field multiple stressors and chemicals are involved in varying concentrations, and other routes of exposure (e.g feed) will occur in addition to exposure via the water phase. Another aspect that calls for caution in the extrapolation is that not all factors determining the representativity of the species used in the laboratory for species in the field are known or can be taken into account.

Calculated with the zebrafish model, zebrafish populations were mainly affected by strongly changed sex ratios. Other effects on single PLC-variables did not reduce population survival chances or population size, with exception of complete inhibition of egg production, which reduced zebrafish populations. As was already postulated by Oertel (1992), effects on a variable can trigger compensating mechanisms in the population dynamics. For instance, reduced numbers of juveniles due to toxic pressure can be compensated by reduced predation. The zebrafish PLC results were also used to simulate K-strategist population dynamics (see Introduction Population Modelling, Chapter 2.3), in order to identify specificity of effects in the zebrafish (r-strategist) model. K-strategist populations showed a different response compared to zebrafish populations in the case of PTU (decreased extinction chances with increasing concentration), flutamide (decreasing population size with increasing concentration), and 17β-estradiol (no effects at the population level). Tamoxifen and MDHT had similar effects on K- and r-strategist populations.

The major differences between determining parameters in the two species were in size of progeny and juvenile survival, both only affecting K-strategist populations. Sex ratio on the other hand was an important determinant for both species.

As mentioned before, egg production ceased completely in a few PLC tets units, with obvious subsequent extinction of populations. However, a significant but incomplete reduction of egg production was compensated by for instance increased juvenile survival in case of zebrafish populations. K-strategists produce smaller numbers of juveniles, which, however, are larger at birth and therefore less susceptible to predation. This strategy limits the potential of compensatory mechanisms for decreased juvenile survival.

Our findings in zebrafish are in line with the conclusion of Nagel et al. (1991) that reproduction (size of F1 progeny) is not critical for the maintenance of zebrafish populations (in contrast to juvenile survival). This concept is further supported by the high variation of clutch sizes (number of eggs per clutch) between individuals (see 6.2, assessment of egg production). Apparently and within limits, there is no reproductive advantage in either a high or low egg production. In other words, zebrafish maintain a relative overproduction of eggs. In summary, the outcomes of the IBModel for zebrafish populations are specific for a species with this reproduction strategy. Sex ratio predominantly determines changes in population survival chance and population size. Other factors are of none or less importance, unless changes are excessive (e.g. complete inhibition of egg production), since changes in these factors can be counteracted. Changes in VTG levels are associated with skewed sex ratios, but effects on the level of the population will be missed in cases where the sex ratio parameter is more sensitive than VTG, as seen in the PLC tests with androgen and anti-androgen.

For species with a different reproduction strategy, the impact of similar changes in reproduction parameters may differ considerably at the level of the population. This should be taken into account when extrapolating effects detected in a PLC test to the population level.

5. Conclusions

Based on the results of this project the following conclusions are drawn:

- The partial life cycle test in zebrafish appears to be a feasible test system encompassing crucial windows of the life cycle within a reasonable experimental time.

- In the present setup, the sensitivity of life cycle parameters is low compared to assessment of histological paramters and vitellogenin, i.e. less replicates are needed to detect effects for the latter.

- Histology is a powerful tool with a high sensitivity and specificity for the detecion of endocrine disruption by (anti)estrogen, (anti)androgen, and thyroid inhibitor; and it may indicate the mode of action.

- Histopathology can provide an alternative for VTG determination by ELISA for the detection of (anti)estrogen action.

- Moderate increased VTG production as measured in males will as such not have a major impact on (reproductive) fitness; however, concomitant effects on gonad morphology and function, and sex differentiation in juveniles may be a concern for population dynamics.

- VTG appears as a specific and practical indicator of estrogenic effects. For other EDCs, other endpoints are more sensitive and relevant.

- For impact at the population level, sex differentiation (skewed sex ratio) is the critical parameter for endocrine disruption according to the applied model (beyond the extreme case of ceased egg production).

Acknowledgements

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Annex 1 - Test conditions for the zebrafish screening assay

Adults

1.	test type	semi-static
2.	water temperature	$27\pm2°C$
3.	illumination quality	fluorescent bulbs (wide spectrum)
4.	light intensity	10-20 $\mu E/M^2/s$, 540-1080 lux
5.	photoperiod	14 h light, 10 h dark
6.	test chamber size	6 L (22x17x24 cm), 3 L (18x13x19 cm)
7.	test solution volume	4 L (2 males), 2 L (1 female)
8.	volume exchanges of test solutions	twice a week
9.	age of test organisms	reproducing adults (8-month minimum)
10.	number of fish per test chamber	1 females and 2 males
11.	number of replicate test chambers per treatment	3 minimum
12.	number of treatments	3 minimum (plus appropriate controls)
13.	number of fish per test concentration	minimum of 3 females and 6 males
14.	feeding regime	frozen adult brine shrimp twice daily
15.	aeration	through glass tubes to prevent O_2 concentration to falls below 6 mg/l
16.	dilution water	or reconstituted water (see next Table, "DSW")
17.	dilution factor	3.2-10
18.	chemical exposure duration	21days
19.	primary endpoints	adult survival and behaviour, number of spawns, number of eggs per spawn, fertility, length and weight, secondary sexual characteristics and vitellogenin, gonadal histology
20.	optional endpoints	morphology
21.	test acceptability	dissolved oxygen $\geq60\%$ of saturation; pH between 6.5 and 8.5; mean temperature $27\pm 2°C$; $NO_2 \leq 1$ mg/L; total hardness is ≤ 14 dH°; 90% survival of adults in the controls; successful egg production in controls

Eggs-juveniles

1.	test type	semi-static
2.	water temperature	$27 \pm 2°C$
3.	hatching temperature	$28.5 \pm 2°C$
4.	illumination quality	fluorescent bulbs (wide spectrum)
5.	light intensity	10-20 $\mu E/M2/s$, 540-1080 lux
6.	photoperiod	14 h light, 10 h dark
7.	test chamber size	(15x10x15 cm) week 1, 2 and 3, (18x13x19 cm) week 4, 5 and 6
8.	test solution volume	150 ml week 1 and 3; 300 ml week 4, 5 and 6
9.	volume exchanges of test solutions	twice a week
10.	age of test organisms	larvae 72 hours after spawning
11.	number eggs to measure hatchability	30-50 eggs (in duplo) in 50 mL water
12.	test hatch chamber size	10 cm Ø petridish
13.	number of fish per test chamber	50
14.	number of treatments	3 minimum (plus appropriate controls)
15.	number replicate test chambers per treatment	1-2
16.	feeding regime	first two week twice daily rotifera (*Brachionus rubens*, own bred) *ad lib* during the first two weeks of life, and from week 2 onwards artemias *(A. salina)* To prevent food deficiency every 5 days from week 2 the amount of artemia solution per fish (starting at 10 μl/fish) is doubled. Artemia solution is prepared from fresh hatched cysts by weighting 5 grams w/w per 30 ml
17.	aeration	through glass tubes to prevent O_2 concentration to falls below 6 mg/L
18.	dilution water	reconstituted water
19.	dilution factor	3.2-10
20.	chemical exposure duration	21 days
21.	primary endpoints	juvenile survival and behaviour, secondary sexual characteristics, gonad histology
22.	optional endpoints	larval development and morphology
23.	test acceptability	overall survival in the controls should be greater than or equal to 50%, dissolved oxygen ≥60% of saturation; pH should be in the range of 6.5-8.5; mean temperature of $27 \pm 2°C$ (juveniles); mean hatch temperature eggs of $28,5 \pm 2°C$. $NO_2 \leq 1$ mg/L; total hardness is ≤ 14 dH°.

Composition synthetic medium DSW (Dutch Standard Water)

1. dilute per liter demi-water
   NaHCO$_3$ - 100 mg
   KHCO$_3$ - 20 mg
   CaCL$_2$ .2H$_2$O   - 200 mg
   MgSO$_4$ .7H$_2$O   - 180 mg
2. aeration for 24 hours, pH should be around 8.3

Based on NNI-prescription NPR 6503 (Netherlands Standardization Institute, 1980).

Annex 2 - Histology protocol

Fixation, embedding, sectioning, routine staining

After euthanasia fish were fixed in Bouin's fixative for an average time of 24 h (shorter for smaller specimen, longer for larger fish, up to 48 h). After fixation, animals were transferred to a 70% ethanol solution and kept until embedding in paraffin (single or three fish per tray for large adults up to ten fish per tray for small juveniles). Coronal sections (thickness 4 μm) were prepared through the regions of interest. In selected cases, these sections were prepared serially; every 250 μm for adults, every 50 μm for small juveniles. These were routinely stained with hematoxylin and eosin (HE). Additional selected sections were stained with periodic acid - Schiff's reagent (PAS).

Histochemical staining of vitellogenin

Histochemical staining of vitellogenin was performed in a two-step protocol as briefly described earlier (Wester et al., 1985), making use of the typical high concentration of phosphate groups in vitellogenin. In the first step, the phosphoproteins in the section were complexed with Fe(III) by a modified method, originally used on isolated phosphoproteins (Donella et al., 1976; Muszynska et al., 1992). For this purpose, sections were deparaffinised in a graded xylene/ethanol series, rinsed in ad, and incubated with a 10 mM/L solution of ferric chloride hexahydrate for 1 h at room temperature, and subsequently rinsed in ad (twice). In the second step, the complexed Fe(III) (as well as endogenous iron) was stained with the standard Perl's Prussian blue method, yielding a characteristic blue colour.

Annex 3 - Collateral products

In the course of the project, several products have been delivered that ran parallel, such as published scientific papers in collaboration with other scientists no directly involved in this project, or poster presentations at scientific meetings.

Peer reviewed papers

Rasmussen TH, Andreassen TK, Pedersen SN, Van der Ven LTM, Bjerregaard P, Korsgaard B (2002) Effects of waterborne exposure of octylphenol and oestrogen on pregnant viviparous eelpout (Zoarces viviparus) and her embryos in ovario. J.Exp.Biol. 205:3857-3876

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Nash JP, Kime DE, Van der Ven LTM, Wester PW, Brion F, Maack G, Stahlschmidt-Allner P, Tyler CR (2003) Environmental concentrations of the pharmaceutical ethynylestradiol impact fish populations. submitted

Miscellaneous

OECD workshop on Histopathology of Small fish gonads for Endocerine disruption. Bilthoven , the Netherlands, September 5-6, 2002

Various OECD expert meetings and Validation / Management group (VMG-ECO) meetings in relation to guideline development

Poster presentations / abstracts

Van der Ven LTM, Van den Belt K, Van Beusekom SAM, Van den Brandhof EJ, Bulder AS, Folkerts AJ, Vos JG and Wester PW. Histopathology of small fish - a tool for the identification of endocrine active compounds in the aquatic environment. 21st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Brighton, UK, May 21-25, 2000.

Bulder AS, Van den Brandhof EJ, Folkerts AJ, Van der Ven LTM, Wester PW andCanton JH. A partial life cycle (PLC) test in zebrafish for measuring reproduction effects of (xeno-) estrogens.21st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Brighton, UK, May 21-25, 2000.

Van der Ven LTM, Wester PW and Vos JG. A Digital Atlas of Histology and Toxicological Pathology of Small Laboratory Fish in Endocrine Disruption Research.

21st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Nashville, 2000

Wester PW and Van der Ven LTM. Histopathology of Small Fish in the Context of Endocrine Disrupting Chemicals. 19th Annual Symposium of the Society of Toxicologic Pathology. Phoenix, Arizona. June 25-29, 2000.

Van der Ven LTM., Wester PW, Van den Brandhof EJ, Folkerts AJ, Bulder AS, Drüke J and.Beekhof P. Histological determinants in the evaluation of endocrine disruption on reproductive fitness in fish. 22st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Madrid, Spain, May 10-14, 2001

Van den Brandhof EJ, Vos JH, Drüke JM, Beekhof PK, Berrag S, Van der Ven LTM and Wester PW. Effects of estrogen 17ß-Estradiol and anti-thyroid Propylthiouracil in a Partial Life Cycle test with zebrafish.23st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Vienna, Austria, May 12-16, 2002

Van der Ven LTM, Van den Brandhof EJ, Loendersloot H, de Waal S, Vos JH, Vos JG and Wester PW. Comparative histopathology of zebrafish gonads after disruption of the sex hormone system. 23st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Vienna, Austria, May 12-16, 2002

Wester PW, Van der Ven LTM, Gielis F and Robinson J. Histological Evaluation of Endocrine Disruption in Fish. 21st Annual Symposium of the Society of Toxicologic Pathology; Denver, Colorado; June 2-6 2002

Van der Ven LTM, Van den Brandhof EJ, Vos JH, Power DM, Wester PW. Effects of propylthiouracil in zebrafish as a reference for the identification of antithyroid effects. 24st Annual Symposium of the Society of Environmental Toxicology and Chemistry (SETAC), Hamburg, Germany, April 27-30, 2003