Estrogen related alterations of gonad development and of reproduction in the zebrafish, *Danio rerio*, Ham. Buc.

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Contents

Zusammenfassung

In der vorliegenden Arbeit wurden die Östrogen bedingten Veränderungen der Reproduktionsparameter und der Histopathologie der Gonaden beim Zebrabärbling *(Danio rerio)* untersucht. Basierend auf 406 Individuen aller Altersgruppen (von 10 Tagen alten Tieren bis zu Adulten) wurde die Gonadenentwicklung detailliert beschrieben. Besondere Berücksichtigung fand die Phase der Sexualdifferenzierung. Der Zebrafisch ist ein juveniler protogyner Hermaphrodit. Unabhängig vom genetischen Geschlecht differenzieren sich alle Gonaden zuerst zu Ovarien. Bei der Hälfte der Fische wird die Entwicklung zur reifen weiblichen Gonade fortgesetzt. Bei den Gonaden der anderen Fische dagegen ist ein Umformungsprozeß zu beobachten. Diese Ovarien werden zu Testes umgebildet. Die strukturellen Veränderungen beginnen mit einer teilweisen Zersetzung der Gonadenwand und einer atretischen Auflösung der Oocyten. Im weiteren Verlauf sind nur einzelne atretische Oocyten innerhalb der Gonade vorhanden. Das Auftreten männlicher Keimzellen markiert den Beginn der Reorganisation der Gonade und den letzten Schritt der Umformung. In dem benutzten Stamm beginnt dieser Prozeß in Woche 5 nach Befruchtung und ist in Woche 11 beendet. Diese Umwandlung findet vor der sexuellen Reife statt. Die Ergebnisse können als Basis für histologische Beobachtungen beim Einsatz des Zebrabärblings als eine Modellspezies zur Einschätzung toxischer Effekte durch endokrin wirksame Chemikalien dienen. Die Berücksichtigung des oben beschriebenen Phänomens des juvenilen Hermaphroditismus ist essentiell bei der Einschätzung toxischer Effekte auf den Zebrafisch, da sonst die normale Ontogenie als Reaktion auf endokrin wirksame Chemikalien missinterpretiert werden kann.

Auf Basis dieser Beschreibung der Gonadenentwicklung wurden im zweiten thematischen Block dieser Arbeit Östrogen bedingte Veränderungen der Gonadenmorphologie histologisch ausgewertet und mit Veränderungen im Reproduktionsverhalten verglichen. Zudem wurden qualitative Unterschiede in der Reaktion der Fische auf Belastung durch eine hoch potentes synthetisches Östrogen (Ethynylestradiol) im Vergleich zu einer schwach östrogen wirksamen Chemikalie (Bisphenol A), von der mehrere 100.000 Tonnen im Jahr hergestellt werden, dargestellt. Zebrabärblinge wurden in Life-Cycle Tests jeweils für 14, 21, 42 Tage sowie permanent mit 3 ng/L 17α-Ethynylestradiol (EE2) belastet. Parallel dazu wurden Zebrabärblinge permanent mit den Nominalkonzentrationen 94, 188, 375, 750 und 1500 µg/L Bisphenol A (BPA) belastet, beginnend jeweils mit den befruchteten Eiern.

In keinem Ansatz war eine Veränderung des somatischen Wachstums zu finden. Eine Verringerung der Befruchtung wurde bei Fischen gefunden, die permanent BPA in Konzentrationen 375, 750 und 1500 µg/L BPA exponiert waren. Histopathologische Effekte

wurden in reifen Ovarien gefunden bei Weibchen, die 750 und 1500 µg/L BPA ausgesetzt waren. Männchen, die diesen Konzentrationen ausgesetzt waren, zeigten keine morphologische Veränderungen der Testes.

Dauerhafte Exposition in 3 ng/L EE2 führte zu einer kompletten Inhibition der Reproduktion. Der Grund hierfür ist eine EE2 bedingte Verhinderung der Transition der Männchen. Erst nach Beendigung der Exposition waren die genetischen Männchen in der Lage sich auch zu phänotypischen, fertilen Männchen zu entwickeln. Aber auch nach 3 Wochen Regeneration unter Kontrollbedingungen waren im Vergleich zu den Kontrolltieren sowohl die Befruchtung als auch die Anzahl der gelegten Eier pro Weibchen reduziert. Eine Beendigung der Exposition vor dem Beginn der sexuellen Differenzierung führte nicht zu irreversiblen Effekten der Reproduktionsparametern. Einzig die Befruchtung, als sensitivster Parameter, war bei den Fischen, die für 43 Tage belastet waren, reduziert. Dieser Effekt konnte nach 3 weiteren Wochen Regeneration nicht mehr festgestellt werden.

Eine histologische Untersuchung der Gonaden zeigte, daß in allen Belastungsbecken ein signifikanter Anteil der Weibchen nicht an der Reproduktion teilgenommen hatte, dies auch nach zusätzlichen 50 Tagen Hälterung unter Kontrollbedingungen.

Populationsrelevante Effekte beim Zebrabärbling lassen sich mit Bisphenol A nur in Konzentrationen erzielen die mindestens eine Größenordnung über denen liegen, die in der Umwelt nachgewiesen werden. Dagegen führt eine Belastung mit umweltrelevanten Konzentrationen von Ethynylestradiol zu einem Totalausfall der Reproduktion und zum Aussterben der Population.

Das Ziel des dritten thematischen Blocks dieser Arbeit war das Finden einer Phase in der Entwicklung des Zebrabärblings, die besonders sensitiv auf die Exposition mit Östrogen reagiert. Aus diesem Grunde wurden die Fische an 10 ng/L Ethynylestradiol (EE2) während der protogynen Entwicklungsphase (Tag 15 bis Tag 42), der Phase der Sexualdifferenzierung (Tag 43 bis Tag 71) bzw. der Wachstumsphase (Tag 72 bis Tag 99) exponiert. Das Wachstum, der Beginn des Laichens, die Anzahl der Eier pro Weibchen, die Befruchtung sowie das Geschlechterverhältnis am Ende des Experiments wurden bestimmt. Belastung mit EE2 führt zu einer Reduzierung des Längenwachstums, unabhängig von der Entwicklungsphase bzw. dem Alter der Fische. Nach einer entsprechenden Regenerationsphase konnte dieser Effekt allerdings kompensiert werden.

Die Reduktion des Wachstums während der Belastung in der Phase der Sexualdifferenzierung konnte allerdings nicht kompensiert werden. Zusätzlich war bei den Fischen, die in diesem Alter belastet waren der Beginn des Laichens verzögert und sowohl

die Anzahl der Eier pro Weibchen als auch die Befruchtung reduziert. Dieser Effekt war nicht zu beobachten, wenn zu anderen Zeitpunkten exponiert wurde. Nach einer weiteren Regenerationsphase von 4 Wochen war die Befruchtung weiterhin reduziert, während in der Anzahl der Eier pro Weibchen kein Unterschied zur Kontrollgruppe mehr zu erkennen war.

In einem zweiten Experiment wurden die Fische während der Phase der Sexualdifferenzierung mit 1.67, 3 bzw. 10 ng/L EE2 belastet. Zusätzlich zu den Reproduktionsparametern wurde die Gonadenmorphologie direkt am Ende der Exposition, unmittelbar vor Beginn des Laichens und am Ende des Experiments ausgewertet.

Am Tag 71, direkt am Ende der Belastungsphase, zeigten sich alle exponierten Fische im Längenwachstum reduziert, was bis zum Ende des Versuches nicht kompensiert werden konnte, allerdings war dieser Unterschied nur in der höchsten Konzentration statistisch signifikant. Die Befruchtung war signifikant reduziert in Fischen, die mit 3 und 10 ng/L EE2 belastet waren. Der Beginn des Laichens war verzögert in der Gruppe, die mit 10 ng/L belastet war. Eine Reduzierung in der Anzahl der Eier konnte nicht gefunden werden. Eine Veränderung im Geschlechtsverhältnis am Ende des Versuches war in keiner Gruppe zu finden. Eine Analyse der Gonadenmorphologie zeigte eine Verlängerung der protogynen Phase nach Belastung von Konzentrationen von 3 ng/L und 10 ng/L. Direkt am Ende der Belastungsperiode waren in den Fischen, die in diesen Konzentrationen belastet waren, alle Gonaden zu Ovarien differenziert, während in der Kontrollgruppe mehr Männchen als Weibchen zu finden waren. Nach einer entsprechenden Regenerationszeit sind dagegen Männchen in allen Gruppen gefunden worden.

Diese Östrogen sensitive Phase korrespondiert mit der Phase der Sexualdifferenzierung. Exposition in dieser Phase führt zu vergleichbaren populationsrelevanten Effekten wie eine dauerhafte Exposition, wenn auch erst bei höheren Konzentrationen. Belastung während anderer Phasen der Entwicklung hatte keinen populationsrelevanten Effekt.

Summary

The present thesis investigated the estrogen related alterations of reproductive parameter and of gonadal histopathology in the zebrafish (*Danio rerio*). The gonadal development was described in detail, based on 406 individuals, beginning at day 10 post fertilisation (pf) up to adult fish. Special attention was given to the period of sexual differentiation. The zebrafish is a juvenile protogynous hermaphrodite. All gonads start differentiating as ovaries irrespective of their genetic sex. In about half of the fish, the ovaries continue to grow, where as in the other half, ovaries begin to transform into testes. The structural change from ovaries into testes starts with a partly disintegration of the gonad wall and an atretic disintegration of the oocytes. In a later single atretic oocytes only are visible inside the gonad. The onset of male germ cells marks the reorganisation of the gonad and the final stage of the transformation. In the studied strain, this process starts, at week 5 post fertilisation (pf) and is completed at week 11 pf. This transition took place in the premature stage of development. These results can serve as a basis for histological observation using the zebrafish as a model fish for assessing toxic effects caused by endocrine alteration chemicals (EACs). It is essential to pay attention to the phenomena of juvenile hermaphroditism when using zebrafish as a model species in aquatic toxicology. Otherwise the normal ontogeny could be misinterpreted as a reaction to EACs.

Using the description of the gonadal development as a basis, the aims of second thematic block were to analyse the estrogen related alterations of the gonadal development and to compare these alterations with the reproductive findings. Additionally possible qualitative differences of the fish response of exposure to a highly potent synthetic estrogen (ethynylestradiol) in comparison to a weak estrogenic chemical (bisphenol A), which is produced in the range of several 100.000 metric tons per year, were addressed. Fish full life cycle was conducted with the zebrafish exposed to 3 ng 17α-ethynylestradiol (EE2) for 14, 21, 42 days and permanent exposure and bisphenol A (BPA) at nominal concentrations of 94, 188, 375, 750 and 1500 µg/L, starting with fertilised eggs. Neither in the fish early life stage toxicity test nor in somatic growth any difference to the controls were visible. An inhibitory effect in fertilisation occurred in fish exposed to 375, 750 and 1500 µg/L BPA. Histopathological effects in mature ovaries were found at concentrations of 750 and 1500 µg/L BPA, but not in of testes. Permanent exposure to 3 ng EE2 completely inhibited reproduction due to the absence of males. Only after stopping the exposure the transition from female to male gonads were possible, however after a recovery period of 3 weeks in tab water the fertilisation and number of eggs laid per female were still reduced. Stopping the exposure before the period of sexual differentiation did not cause irreversible effects. The

fertilisation, as the most sensitive parameter in zebrafish reproduction, only was reduced at fish exposed for 43 days. These reduction were not visible anymore after determination the reproductive parameter a second time after additionally 2 weeks recovery in tab water. Histological observation of the gonads demonstrated in all exposure periods a significant proportion of the female did not take part at the reproduction, even after an additionally 50 days recovery in tab water. As in zebrafish exposed to BPA histopathological lesion in mature ovaries were found but not in males.

Despite the huge amount of bisphenol A (BPA) produced, the concentrations found in aquatic environment did not caused effects at the zebrafish. Only in concentrations more than a magnitude higher effects on fertilisation were found. On the other hand permanent exposure to environmental realistic concentrations of ethynylestradiol (EE2) caused a total failure of reproduction and would let to an extinction of the population.

The aim of the third thematic line of this thesis was to find a period in the development of the zebrafish, which reacts particularly sensitive to estrogen exposure. Therefore zebrafish were exposed to 10 ng/L ethynylestradiol (EE2) during the juvenile stage (from day 15 to 42 pf), during the stage of sexual differentiation (from day 43 to day 71 pf), and during the premature stage (day 72 to 99 pf). Somatic growth, the onset of spawning, number of eggs per female, fertilisation and sex ratio of the adult, after termination of the experiment, were determined. Exposure to EE2 led to a reduction in total length, whatever the life stage was exposed, but the effect was reversible after recovery. However while exposed during the stage of sexual differentiation this effect could not be compensated. Additionally the onset of spawning was delayed and both the number of laid eggs as well as the fertilisation was reduced significant, after exposure during day 43 to day 71 pf. In a second counting, after an additionally period of recovery the fertilisation was still significant reduced, whereas in the number of laid eggs no difference to the control were found. These reproductive effects were not found if exposed before or after the sexual differentiation. In a second experiment zebrafish were exposed during the phase of sexual differentiation to 1.67, 3 and 10 ng/L ethylnyestradiol to examine the concentration dependent sensitivity. Additionally to the reproductive parameter the gonads were examined histologically direct after the exposure, at the begin of spawning and after termination of the experiment. At the end of the exposure period a significant reduction in the somatic growth was found in all exposed fish, which could not be compensated although this was found significant only in the highest exposed group. Fertilisation was significant reduced in fish exposed to 3 and to 10 ng/L EE2. The onset of spawning was delayed in the group exposed to 10 ng/L only, whereas in the number

of laid eggs no difference were found anymore. A screw in the sex ratio of adult fish was not found. Histological observation showed a prolongation of the protogynous stage in zebrafish development after exposure of EE2 during sexual differentiation to a concentration of 3 ng/l and more. Directly after exposure all dissected fish of the group, exposed to 3 and 10 ng/L hold ovaries, whereas in the control group more male than females were found. After a respective recovery period males were found in all groups.

The estrogen sensitive period corresponded to the period of sexual differentiation. Exposure during that period let to the comparable population relevant effects as a permanent exposure, although these effects were not as long lasting and visible only at higher concentrations. Exposure to other developmental stages than the period of sexual differentiation did not have population relevant effects.

General Introduction

Water pollution and endocrine-active chemicals

Water pollution is a problem that is linked to the development of the industrial society and the rising population density. Evidence of the state of many major British rivers during the reign of King George III (1760-1820) was made apparent by a letter reportedly by an member of Parliament to the Prime Minister. The letter complained about the odour and the appearance of the River Thames. It was written not in ink, but with water from the Thames itself (Strandberg 1971). Historically, the Thames had been a major salmon river but towards the end of the 18th century, fish species had declined dramatically and large areas of the river become devoid of fish altogether (Wood, 1982 cited in (Jones and Reynolds 1997). This was typical of many rivers in heavily polluted areas of Europe. For instance Lyon, (1966, cited in Strandberg 1971) reported that the salmon industry had disappeared in 1930 from the River Rhine, because of the extinction of the salmon in the Rhine.

In 1962 the publication of Rachel Carson's "Silent Spring" (Carson, 1962) alerted the public to the environmental degradation resulting from widespread use of pesticides in the "eradication programmes" initiated after World War 2. The effects of such extensive use of pesticides first became evident in the appearance of dead fish and birds after spraying and later in the ill health of exposed human populations. Only later was it realized that both harmful and beneficial insects were equally efficiently destroyed and that the indiscriminate use of pesticides could actually make the problem worse. Carson's book well summarises the known effects of the pesticides in use at the time, but it concentrates on the short-term effects and, there was little evidence at the time of possible long term effects of very low concentrations (Kime, 1998).

An issue of possibly similar dimension as the pesticide problem is the presence of endocrine-active chemicals in the environment, which attracted public awareness since 1996 as Theo Colborn, Dianne Dumanowski and John Peterson Myers published their book "Our Stolen Future" (Colborn et al. 1996). These authors well documented how certain chemicals, even in very low concentrations, are able to reduce fertility of several species, including man and how these chemicals are able to influence the labile endocrine system.

Endocrine-active chemicals

The endocrine system is the chemical communication system of the body which regulates such activities as body fluid homeostasis, management of stress, and perhaps most importantly, reproduction and fertility which are necessary for propagation of the species. It comprises glands such as the testes, ovaries, pituitary and hypothalamus, thyroid and liver. It

has become increasingly apparent that the endocrine system is particularly sensitive to very low levels of pollutants (Kime, 1998). Any anthropogenic chemical which alters normal activity of the endocrine glands, and the production of the hormones which they secrete, can cause profound disruption of the exposed animal. This is known since the 1930's (Legler et al., 2000). Industrial chemicals which may mimic natural estrogens has drawn attention to the possible effects of those "environmental estrogens" on human male fertility as well as to the sensitivity of the vertebrate reproduction system to alterations by environmental pollutants and their ultimate effects on wildlife populations. Manmade chemicals that have been reported to act as hormone mimics include: organochlorine pesticides (DDT and its metabolites, enodsulfan, toxaphene, â-HCH, and dieldrin), polychlorinated biphenyls (PCBs; both mixtures and individual congeners) and their hydroxylated metabolites, dioxin-like chemicals (PCDDs and PCDFs), bisphenol A, alkylphenolic chemicals, vinclozolin, tributyltin (TBT) and a few phthalates (Colborn and Clement, 1992, Colborn et al. 1996, Kime 1995, Kime 1998, Tyler et al., 1998). Some of these chemicals have been demonstated to mimic estrogen, whereas others have masculinizing effects (i.e. TBT), while vinclozolin and p,p' DDE (a breakdown product of DDT) act as anti-androgens. Although most of the endocrine-active chemicals identified to date have a weak activity compared with their endogenous counterparts, (i.e. Sumpter and Jobling 1995), they can be present in significant concentrations in the environment. In addition, most of these chemicals have a strong tendency to bioaccumulate in different organisms. Exposure to chemicals that can act as hormones is not confined to those of industrial origin: some natural chemicals can mimic the effects of estrogens. Fungi can produce estrogenic substances (mycoestrogens), as can can some plants (phytoestrogens) (Tyler et al., 1998). The natural estrogens, estradiol-17â, estrone, and estriol and the synthetic hormone ethynylestradiol (EE2) have also been shown to contaminate the aquatic environment.

Approaches to detect endocrine activity of chemicals

The hypothesis that wildlife may have been negatively affected as a consequence of exposure to chemicals that interact with the endocrine system has led to a surge in scientific effort to evaluate chemical pollutants for hormonal activity (Tyler et al., 1998). A wide range of *in vitro* assays are now available for screening endocrine disruption (Zacharewski 1997).

However, in vitro systems have little relevance in wildlife toxicology as *in vitro* effects are not necessarily predictive of its in vivo effects which may be influenced by biodegradation and metabolism (Laws et al., 1996); furthermore *in vitro* systems are not suitable for population-relevant endpoints like reproduction, fertilisation and hatching success or for

effects on the F1- or the F2-generation. And particularly hormone related effects are not measurable in an *in vitro* assay, because hormones effect not primary on the cellular level but on the physiological level, which is not detectable in an *in vitro* assay.

Due to the complexity of the system the toxic relevance of single substances in the wildlife are very difficult to determine. One integrative approach to assess ecologically relevant effects of chemicals on growth and reproduction can be full-life-cycle or multi-generation tests. However, in order to identify an endocrine activity of the test compound as the cause of the developmental and reproductive alterations, modifications or enhancements of the existing test protocols will be necessary.

Aims and outlines of this thesis

Much of the evidence for endocrine disruption in wildlife populations has been derived from studies on aquatic organisms, and, therefore, fish have been recommended for the development of tests for endocrine-active chemicals (EACs) (Arcand-Hoy et al., 1998, Tyler et al., 1998, OECD 1999, Fenner-Crisp et al., 2000, Huet 2000, Harries et al., 2000).

The objectives of the thesis were to evaluate how (xeno)estrogenic substances affect the gonadal development and reproduction of the zebrafish. One core endpoint was gonad histopathology. This parameter was selected since a number of reports have demonstrated that gonad histology of fish responds sensitively to exposure to endocrine active chemicals (EACs). The observed structural alterations range from the appearance of ovo-testes to malformations of gametes and stromal tissue or the increased frequency of atretic oocytes. Therefore, histopathological examinations may provide insight into the nature of reproductive impairment (Gimeno et al. 1996, Jobling et al., 1996, Miles-Richardson et al., 1999, Metcalfe et al., 2000). During the 4th meeting of an OECD Task Force on Endocrine Disrupter Testing and Assessment (EDTA) in Paris at May, 12th, 2001, it was generally agreed that gonad histopathology, in combination with gross morphology and vitellogenin should be adopted as a core endpoint in the assessment of estrogen-active compounds.

The aim of the present work was to identify developmental gonadal disturbances related to chronic exposure of zebrafish to compounds with estrogenic activity. These results were compared to the reproductive alterations in order to validate and to establish gonad histopathology as an endpoint estrogen related effects in fish full and partial life cycle tests.

If gonad histopathology is used as an endpoint in the evaluation of endocrine effects, it is necessary that for the normal gonadal development of the test species to be well characterised. Therefore **Chapter 1** provides a detailed description of the morphology of gonad differentiation in developing zebrafish. The morphological differentiation status of the gonads of individuals from early life stages up to adults was examined histologically. These results are intended to serve as a basis for histological observation using the zebrafish as a model fish for assessing toxic effects caused by EACs.

In **Chapter 2** histopathological analyses of gonad morphology were carried out as part of Fish Full-Life-Cycle tests which was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology in Schmallenberg, Germany. The synthetic estrogen ethynylestradiol (EE2) was used as a positive reference substance, and the effects induced by EE2 were compared with the response pattern induced by exposure to a weak estrogenic chemical bisphenol A (BPA). The effects of long-term estrogen exposure on zebrafish were assessed by measuring growth, survival, sex ratio, fecundity (number of eggs spawned per

female), fertilisation, and time of first spawn. The reproductive assessment endpoints were compared with the analysis of gonad morphology to find a possible correlation between the reproductive findings and gonad histopathology.

The aim of the work in **Chapter 3** was to clarify whether there exists a period in zebrafish development that is particularly sensitive to the impact of exogenous estrogens and whether possible effects were of the same quality as during permanent exposure. Therefore three different life stages were selected: the protogynous phase, the period of sexual differentiation, and the premature stage. Histopathological data on gonad morphology were assessed at the end of the respective exposure period. The reproduction endpoints of this exposure regime were measured in combination with potential reversibility of these effects.

Models used in this thesis

Model organism

The zebrafish, *Danio rerio*, (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae) is a widely used laboratory model species, especially in developmental biology (i.e. Metscher and

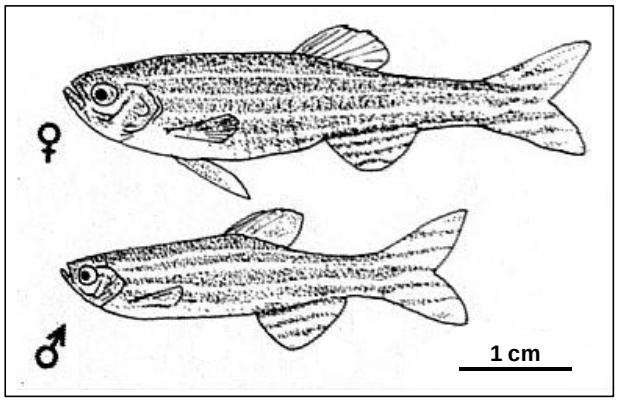

Figure 1: Drawings of female (top) and male (bottom) zebrafish (adapted from Aqua Technologies Group, Inc).

Ahlberg 1999). In OECD guidelines for the testing of chemicals the zebrafish was recommended as a model species, representing aquatic vertebrates (OECD 1984, OECD 1992a, OECD 1992b, OECD 1998, OECD 2000).

The zebrafish has a relatively short life cycle of about 4 months, and, in the laboratory, can be stimulated to breed throughout the year. (Creaser 1934, Hisaoka and Firlit, 1962, Laale, 1975, Eaton and Farley, 1974, Eaton and Farley 1975). For this reason, zebrafish are suitable for assessing toxic effects of chemicals on development and reproduction (Nagel 1993, Nagel and Isberner 1998, Andersen et al., 2000).

Older literature referred to zebrafish as *Brachydanio rerio*. The work of Meyer et al. (1993) and a consensus vote at the 1993 Zebrafish Meeting at the Cold Spring Harbor Laboratory suggested that *Danio rerio* should be used (Westerfield, 1995).

Model compounds

Ethynylestradiol:

Ethynylestradiol (EE2) was chosen due to its very potent estrogenic activity demonstrated in mammals (Brown-Grant et al.1975, reviewed in Tyler et al., 1998) and in various aquatic model species (i.e. Melard 1995, Arcand-Hoy and Benson, 1998, Blazquez et al., 1998, Allen et al., 1999). EE2 is a synthetic estrogen and is contained in the contraceptive pill. It is structurally similar to 17â-estradiol, the active endogenous estrogen, with the exception of an ethynyl substitution at carbon 17 (Figure 2). This substitution renders the compound less susceptible to first-pass hepatic metabolism. Ethynylestradiol has a higher affinity for the estrogen receptor than 17â-estradiol (Arcand-Hoy et al., 1998). These authors calculated the expected concentration to the aquatic environment for the United States as 2.16 ng/L. This value agrees very well to the concentrations found in sewage treatment works in Europe, where it was detected in the low ng/L range (Desbrow et al., 1998, Routledge et al., 1998, Ternes et al., 1999).

Bisphenol-A

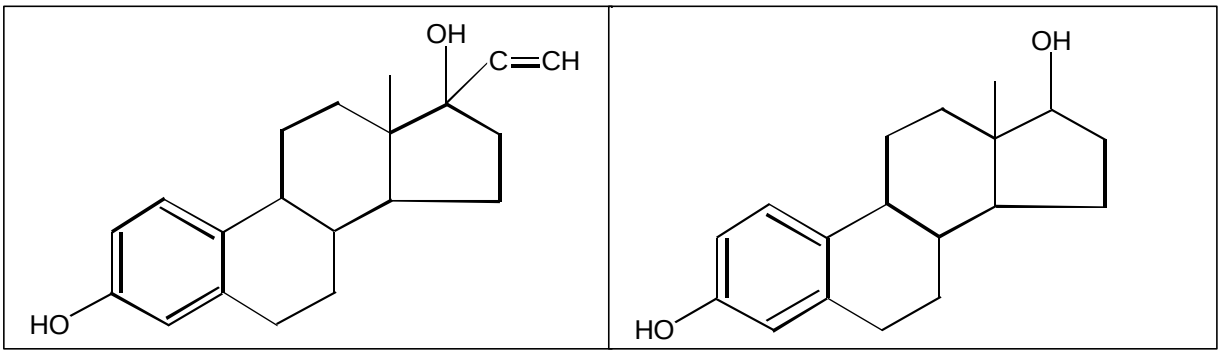

Figure 2: Chemical structure of 17α- ethynylestradiol (left) and 17â-estradiol (right)

Bisphenol-A (BPA) is a commonly used name for 2,2-(4,4-dihydroxydiphenyl) propane (Figure 3). It is a synthetic chemical used in the production of polycarbonate for the manufacture of a wide variety of plastic products with new applications continuously being developed. It leaches out of food packaging material and from lacquer of the coatings of food and drink cans (Brotons et al., 1995). In 1993 an estimated of 640.000 metric tons BPA were

produced (Staples et al., 1998). BPA binds to the estrogen receptor with an affinity 2000 times less than 17â-estradiol, and is one of the more potent known anthropogenic estrogenic mimics (Routledge and Sumpter, 1996). It is known as a weak estrogen, as indicated by in vivo studies in mammals (Bond et al., 1980). Endocrine activity was found in vivo at very high concentrations (355µg/L and higher) only (Yokota et al., 2000, Sohoni et al., 2001). BPA is widely distributed in surface water in Europe and the United States in concentrations up to 25 µm/L as reviewed by Staples et al. (1998). This was at least one order of magnitude lower than the concentrations, where endocrine activity were found.

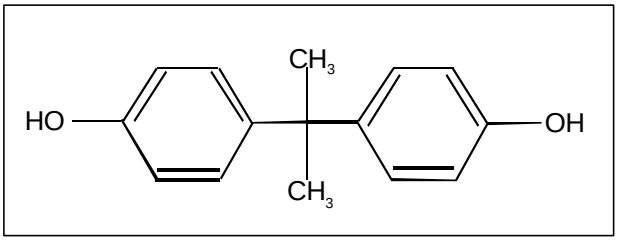

Figure 3: Chemical structure of bisphenol-A

References

Allen, Y., Scott, A. P., Matthiessen, P., Haworth, S., Thain, J. E., and Feist, S., (1999). "Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of the flounder *Platichthys flesus*", *Environmental Toxicology and Chemistry* **18** (8), 1791-1800.

Andersen, L., Bengtsson, B.-E., Björk, M., Gessbo, A., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K. L., Lundgren, A., Petersen, G. I., Steinholz, A., and Örn, S., (2000). "Zebrafish for Testing Endocrine Disrupting Chemicals", Chemical Nordic council, Copenhagen. TemaNord **555**.

Arcand-Hoy, L. D. and Benson, W. H., (1998). "Fish reproduction: an ecologically relevant indicator of endocrine disruption", *Environmental Toxicology and Chemistry* **17** (1), 49-57.

Arcand-Hoy, L. D., Nimrod, A. C., and Benson, W. H., (1998). "Endocrine-modulating substances in the environment estrogenic effects of pharmaceutical products", *International Journal of Toxicology* **17** (2), 139-158.

Blazquez, M., Zanuy, S., Carrillo, M., and Piferrer, F., (1998). "Structural and functional effects of early exposure to estradiol-17 beta and 17 alpha-ethynylestradiol on the gonads of the gonochoristic teleost *Dicentrarchus labrax*", *Fish Physiology and Biochemistry* **18** (1), 37-47.

Bond, G.P.; McGinnis, P.M.; Cheever, K.L.; Harris, S.J.; Platnik H.B. and Neimier, R.W. (1980): Proceedings from the 19th Annual Meeting of the Society of Toxicology, Washington, D.C.

Brotons, J. A., Olea-Serrano, M. F., Villalobos, M., Pedraza, V., and Olea, N., (1995). "Xenoestrogens released from Laquer Coatings in Food Cans", *Environ Health Perspect* **103** (6), 608-612.

Carson, R. (1962). “The Silent Spring”, Houghton Mifflin, USA (1965 paperback edition, Penguin, Middlesex, U.K.).

Colborn, T. and Clement, C. (eds.) (1992). “Chemically induced alterations in sexual and functional development: the wildlife/human connection.”, Priceton Scientific Publishing, Princeton, New Jersey.

Colborn, T., Dumaonski, D. and Myers, J.P. (1996). “Our Stolen Future”. Little Brown & Co, London

Creaser, C. W., (1934). "The technique of handling the zebrafish (*Brachdanio rerio*) for the production of eggs, which are favourable for embryological research and are available at any specific time throughout the year", *Copeia* (4), 159-161.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M., (1998). "Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening", *Environmental Science & Technology* **32** (11), 1549-1558.

Eaton, R. C. and Farley, R. D., (1974). "Spawning cycle and egg production of Zebrafish, *Brachydanio rerio*, in the laboratory", *Copeia* , 195-209.

Eaton, R. C. and Farley, R. D., (1975). "Growth and the reduction of depensation of Zebrafish, Brachydanio rerio, reared in the laboratory", *Journal of Fish Biology* **10**, 121-173

Fenner-Crisp, P. A., Maciorowski, A. F., and Timm, G. E., (2000). "The endocrine disruptor screening program developed by the US Environmental Protection Agency", *Ecotoxicology* **9** (1-2), 85-91.

Gimeno S, Gerritsen A, Browmer T., Komen, H. (1996). “Feminisation of male carp”. *Nature* **384**, 221-222.

Harries, J. E., Runnalls, T., Hill, E., Harris, C. A., Maddix, S., Sumpter, J. P., and Tyler, C. R., (2000). "Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*)", *Environmental Science & Technology* **34** (14), 3003-3011.

HIsaoka, K. K. and Firlit, C. F., (1962). "Ovarian Cycle and Egg Production in the Zebrafish, *Brachydanio rerio*", Copeia (4), 788-792.

Huet, M. C., (2000). "OECD activity on endocrine disrupters test guidelines development", *Ecotoxicology* **9** (1-2), 77-84.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P., and Sumpter, J. P., (1996). "Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals", *Environmental Toxicology and Chemistry* **15** (2), 194-202.

Jones, J. C. and Reynolds, J. D., (1997). "Effects of pollution on reproductive behaviour of fishes", *Reviews in Fish Biology and Fisheries* **7**, 463-491.

Kime, D. E., (1995). "The effect of pollution on reproduction in fish", *Reviews in Fish Biology and Fisheries* **5**, 52-96.

Kime, D.E. (1998). "Endocrine Disruption in fish". Kluwer Academic Publishers, Boston, Dordrecht, London. 396 pages

Laale, H. W., (1975). "The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. A literature review", *Journal of Fish Biology* **10**, 121-173.

Laws, S. C., Carey, S. A., Kelce, W. R., Cooper, R. L., and Gray, L. E., (1996). "Vinclozolin does not alter progesterone receptor (PR) function in vivo despite inhibition of PR binding by its metabolites in vitro", *Toxicology* **112** (3), 173-182.

Legler, J., Broekhof, J. L. M., Brouwer, A., Lanser, P. H., Murk, A. J., Van der Saag, P. T., Vethaak, A. D., Wester, P., Zivkovic, D., and van der Burg, B., (2000). "A novel in vivo bioassay for (xeno-) estrogens using transgenic zebrafish", *Environmental Science & Technology* **34** (20), 4439-4444.

Melard, C., (1995). "Production of a high percentage of male offspring with 17-a-ethynylestradiol sex-reversed *Oreochromis aureus.* I. Estrogen sex-reversal and production of F2 pseudofemales", *Aquaculture* **130**, 25-34.

Metcalfe, T. L., Metcalfe, C. D., Kiparissis, Y., Niimi, A. J., Foran, C. M., and Benson, W. H., (2000). "Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to o,p '-DDT in water or through maternal transfer", *Environmental Toxicology and Chemistry* **19** (7), 1893-1900.

Metscher, B. D. and Ahlberg, P. E., (1999). "Zebrafish in context: Uses of a laboratory model in comparative studies", *Developmental Biology* **210** (1), 1-14.

Meyer, A.; Biermann, C.H. and Orti, G. (1993). "The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: An invitation to the comparative method." *Proc. R. Soc. Lond.* **252**, 231-236.

Miles-Richardson, S. R., Kramer, V. J. , Fitzgerald, S. D., Render, J. A., Yamini, B., Barbee, S. J., and Giesy, J. P., (1999). "Effects of waterborne exposure of 17 â-estradiol on secondary sex

characteristics and gonads of fathead minnows (*Pimephales promelas*)", *Aquatic Toxicology* **47** (2), 129-145.

OECD, Organisation for Economic Co-operation and Development, (1984). "OECD guidelines for testing of chemicals, section 2, Guideline 204: Fish, prolonged toxicity test: 14-day Study", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992a). "OECD guidelines for testing of chemicals, section 2, Guideline 203: Fish, acute toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992b). "OECD guidelines for testing of chemicals, section 2, Guideline 210: Fish, Early-life stage toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1998). "OECD guidelines for testing of chemicals, section 2, Guideline 212: Fish, Short-term test on embryo and sac-fry stages", Paris, France .

OECD, Organisation for Economic Co-operation and Development. (1999). Fish expert consultation meeting, London. Paris, France.

OECD, Organisation for Economic Co-operation and Development, (2000). "OECD guidelines for testing of chemicals, section 2, Guideline 215: Fish, Juvenile Growth Test", Paris, France .

Routledge, E. J. and Sumpter, J. P., (1996). "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen", *Environmental Toxicology and Chemistry* **15** (3), 241-248.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P., (1998). "Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach", *Environmental Science & Technology* **32** (11), 1559-1565.

Sohoni, P., Tyler, C. R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C. , Evans, M., Toy, R., Gargas, M., and Sumpter, J. P., (2001). "Reproductive effects of long-term exposure to bisphenol a in the fathead minnow (*Pimephales promelas*)", *Environmental Science & Technology* **35** (14), 2917-2925.

Staples, C. A., Dorn, P. B., Klecka, G. M., O'Block, S. T., and Harris, L. R., (1998). "A review of the environmental fate, effects, and exposures of bisphenol A", *Chemosphere* **36** (10), 2149-2173.

Strandberg, C. R., (1971). "Water Pollution", in: Smith, G.H.(ed): Conservation of natural resources, Wiley and Sons , 189-219.

Sumpter, J. P. and Jobling, S., (1995). "Vitellogenesis as a biomarker for estrogenic contamination of aquatic environment", *Environ Health Perspect Supplements* **7**, 173-178.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., and Servos, M., (1999). "Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil", *Science of the Total Environment* **225** (1), 81-90.

Tyler, C. R., Jobling, S., and Sumpter, J. P., (1998). "Endocrine disruption in wildlife: A critical review of the evidence", *Critical Reviews in Toxicology* **28** (4), 319-361.

Westerfield, M. (1995). “The zebrafish book, a guide for the laboratory use of zebrafish (*Danio rerio*).” 3rd edition. University of Oregon Press

Yokota, H., Tsuruda, Y., Maeda, M., Oshima, Y., Tadokoro, H., Nakazono, A., Honjo, T., and Kobayashi, K., (2000). "Effect of Bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*)", *Environmental Toxicology and Chemistry* **19** (7), 1925-1930.

Zacharewski, T., (1997). "In vitro bioassays for assessing estrogenic substances", *Environmental Science & Technology* **31** (3), 613-623.

Chapter 1

The gonadal development of the zebrafish (*Danio rerio*), Ham. Buc.

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Abstract

The zebrafish (*Danio rerio*) is used extensively in ecotoxicology research. Gonad histopathology is a suitable effect parameter to assess estrogen-active compounds. Therefore a detailed knowledge of the normal development is essential. This paper is an examination of gonad normogenesis in developing zebrafish. A total of 406 individuals were examined by light microscopy, from week 2 post-fertilisation up to adulthood. In 2-week-old zebrafish, the gonads contain primary germ cells (PGC) only. At 4 weeks old the first ovary-containing fish are found. In week 5 87 % of the dissected fish display gonads with the morphology of an early, non-functional ovary. This high percentage of fish containing ovaries is also found in weeks 6 and 7. The first testis appears in a 7-week-old zebrafish. All gonads start differentiating as ovaries irrespective of their genetic sex. In about half of the fish, the ovaries continue to grow, whereas in the other half, ovaries begin to transform into testes. The described developmental pattern of the gonads is interpreted as juvenile protogynic hermaphroditism. The alterations of ovarian morphology occurring between weeks 5 and 11 reflect the conversion of the protogynic ovaries of some individuals into testes. The structural change from ovaries into testes starts with a partial disintegration of the gonad wall and an atretic disintegration of the oocytes. In a later stage there was virtually no gonadal tissue visible at all inside the gonad. The first appearance of male germ cells marks the reorganisation of the gonad and the final stage of the transition. In adult zebrafish the sex ratio is 1male : 1 female. Counting the fish with “altered ovaries” as incipient males, the sex-ratio from week 8 onwards does not differ substantially from the adult sex ratio.

Key words

zebrafish, juvenile hermaphroditism, ovary, testis, endocrine disruption, gonad transformation

Introduction

The zebrafish, *Danio rerio*, has been used intensively in acute and chronic bioassays and is recommended as test organism in ecotoxicological guidelines, e.g., various OECD and ISO guidelines. In the laboratory, zebrafish can be stimulated to breed throughout the year, and development from the fertilised egg to the reproducing stage takes only about 3-4 months. Due to its short life cycle and its established use in ecotoxicity test protocols, the zebrafish is under consideration as a test species for the effects assessment of endocrine-active compounds, (EACs) (OECD 1999, OECD 2000, Fenner-Crisp et al. 2000, Huet 2000).

A critical question in the development of assay procedures with fish to test for an endocrine activity of substances is the selection of suitable effect endpoints, Histological examinations of gonads meet these criteria. During the 4th meeting of the OECD Task Force on Endocrine Disrupter Testing and Assessment (EDTA) in Paris on May 12th, 2001, it was generally agreed that gonad histopathology should be implemented as one core endpoint in the assessment of estrogen-active compounds. A number of reports have demonstrated that gonad histology responds sensitively to exposure to EACs: structural alterations range from, e.g., the appearance of ovo-testes to malformations of gametes and stromal tissue or an increased frequency of atretic oocytes (i.e. Gimeno et al. 1998, Kinnberg et al. 2000, Flammarion et al. 2000, Nolan et al, 2001).

If gonad histopathology is to be used as an endpoint in the evaluation of endocrine effects, it is necessary for the normal gonadal development of the test species to be well characterised. For the zebrafish, not much literature on gonad morphology is available. The report of Nagai et al.(2001) described the differentiation of the primordial germ cells (PGCs), Selman et al. (1993) illustrated the oocyte maturation, and van Ree (1977) reported on histological and enzyme histochemical studies of the zebrafish ovary. For testes development in the zebrafish no literature can be found at all.

To the best of our knowledge, the only published report of the ontogeny of the gonad morphology is the work of Takahashi (1977). This author described a juvenile hermaphroditism in the zebrafish. This observation is particularly important with respect to the use of zebrafish for EAC testing since gonadal features originating during early gonadal differentiation may be misinterpreted as an indication of adult sex change (Sadovy and Shapiro 1987). The present study extends the observations of Takahashi (1977) and provides a detailed description of the morphology of gonad differentiation in developing zebrafish. The morphological differentiation status of the gonads of individuals from early life stages up to adults was examined histologically. These results can serve as a basis for histological observation using the zebrafish as a model fish for assessing toxic effects caused by EACs.

Materials and Methods

Zebrafish were reared in the UFZ laboratory, Leipzig, Germany under standardised conditions at 26°C ± 1°C. The light/dark cycle was 12h/12h. Fish were fed daily with TetraMin® Hauptfutter (Tetra Werke, Melle, Germany) and nauplia larvae of the crustacean *Artemia* sp. *ad libitum*. Spawning of the zebrafish takes place when the light is switched on in the morning. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilised eggs.

Fertilised eggs were transferred from the spawning glasses into 4-L glass aquaria and reared under semi-static conditions. On day 6 post-fertilisation (pf), feeding was started daily with TetraMin® AZ 100 (Tetra Werke, Melle, Germany) and with freshwater rotifers (*Brachionus calyciflorus*). The tanks were cleaned once a day and water was changed once a week. From day 14 pf food was switched to the normal feeding regime as mentioned above. After week 4 pf, the fish were transferred into 25-L glass aquaria.

At least 20 fish were sampled every week, starting with week 2 pf and continuing to week 11 pf. In addition 153 adult, reproductively active fish were examined. The latter fish were taken randomly from 2 independent tanks, not fertilised on the same day.

For histological analysis, fish were anaesthetised in ice water, decapitated and fixed as a whole in Bouin's fluid for 24 h. Fixed tissue was dehydrated and infiltrated with Technovit 7100® (hydroxyethylmethacrylate) according the manufacturer's instructions (Kulzer GmbH, Germany) and sectioned (2-5 µm). Slides were stained in toluidin-methylene blue, dried overnight at 60°C and mounted with Entellan®. Altogether the gonads of 406 zebrafish were examined histologically.

Results

Maturing gonads

2-week-old zebrafish

The undifferentiated gonads are situated in a caudo-dorsal position in the coelomic cavity. The gonads contain approximately 10–20 primordial germ cells (PGCs) which are surrounded by a small rim of somatic cells. The roundish to slightly oval PGCs are distinguished from somatic cells by their relatively large size, approximately 12 μm diameter, and their histological features. The large nucleus measures 6-10 μm in diameter and contains a prominent nucleolus, a loose network of chromatin strands distributed throughout the karyoplasm and deeply staining granular material along the inner side of the nuclear membrane (Figure 1.1).

4-week-old zebrafish

The gonads are increased in size and are extended in an anterior-posterior direction. They contain germ cells with large nuclei and a small cytoplasmic rim; the germ cells are arranged in groups of 2–6 cells. Two different forms of germ cells can be distinguished (Figure 1.2). One germ cell type (type 1) displays a nucleus with a rather homogeneous, slightly basophilic karyoplasm; it contains one to several nucleoli. The second cell type (type 2) is larger than the first one, but again the cytoplasm is restricted to a small rim around the nucleus. The nucleus of type 2 contains densely arranged, thin chromatin threads throughout the light karyoplasm. Occasionally, chromatin strands and the nucleoli show a bouquet-like arrangement. Germ cell type 2 is usually more frequent than the first one, although there is pronounced inter-individual variation among the investigated fishes. The morphological features of cell type 1 are comparable to those of oogonia, and cell type 2 correspond in this respect to oocytes at the chromatin-nucleolar stage. Since male- and female-specific germ cells in developing fish are difficult to distinguish solely on the basis of cell morphological characteristics (e.g., Parmentier and Timmermans 1985), we interpret gonads containing only germ cell types 1 and 2 to be indifferent gonads. However, in three individuals out of 22 zebrafish at age 4 weeks, early perinucleolar oocytes were found to be present, identifiable by a few nucleoli in perinucleolar localization (Figure 1.2). The cytoplasm of these perinucleolar oocytes often contains small, localized areas of intense basophilia. These gonads were classified as ovaries .

The number of stroma cells – mainly fibroblast-like cells – in the gonads of 4-week-old zebrafish varies among individuals; in some fish, larger areas – particularly in the anterior

part – are occupied by stromal tissue, whereas in other individuals, stroma cells are restricted to a small layer at the gonad surface and most of the gonad tissue is populated by germ cells.

5-week-old zebrafish (10 –15 mm total length)

While the gonad morphology of 3 fish (10 %) is comparable to the indifferent gonad type as described for 4-week-old fish, the gonads of the majority (87 %) of the examined individuals now contain oocytes at the perinucleolar stage, so that these gonads can be classified as ovaries. The size of the oocytes is increasing, mainly because of an enlargement of the ooplasm (Figure 1.3). The latter stains strongly basophilic. In some ovaries, follicular cells start to develop around the oocytes. There is pronounced inter-individual variation in the percentage of gonad tissue occupied by perinucleolar oocytes: in some fish, up to 90 % of the gonadal tissue contains perinucleolar oocytes, whereas in others few perinucleolar oocytes are present, accounting for no more than 10 – 20 % of the gonadal size, while the remaining areas are occupied mainly by groups of type 1 and type 2 germ cells.

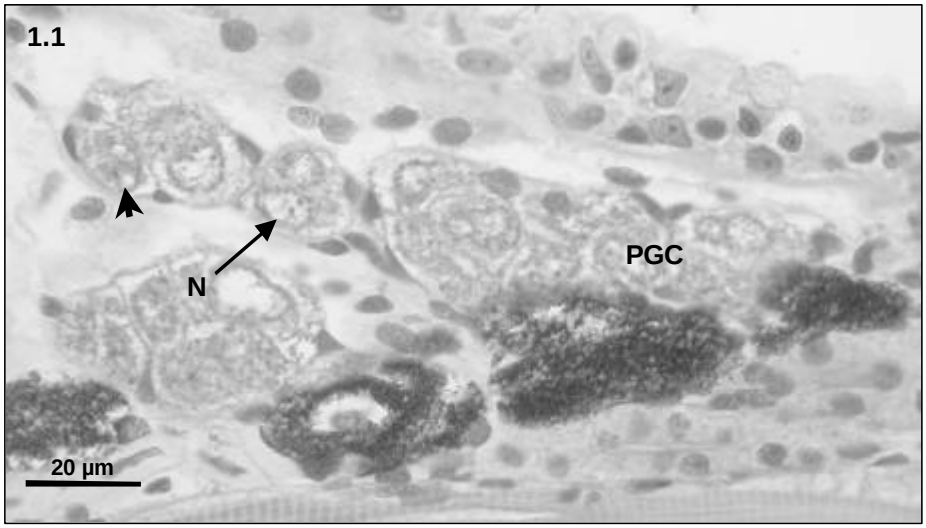
One individual out of the 31 examined zebrafish shows certain modifications of gonad morphology that are comparable to the “altered ovaries” described in detail for 6-week-old fish.

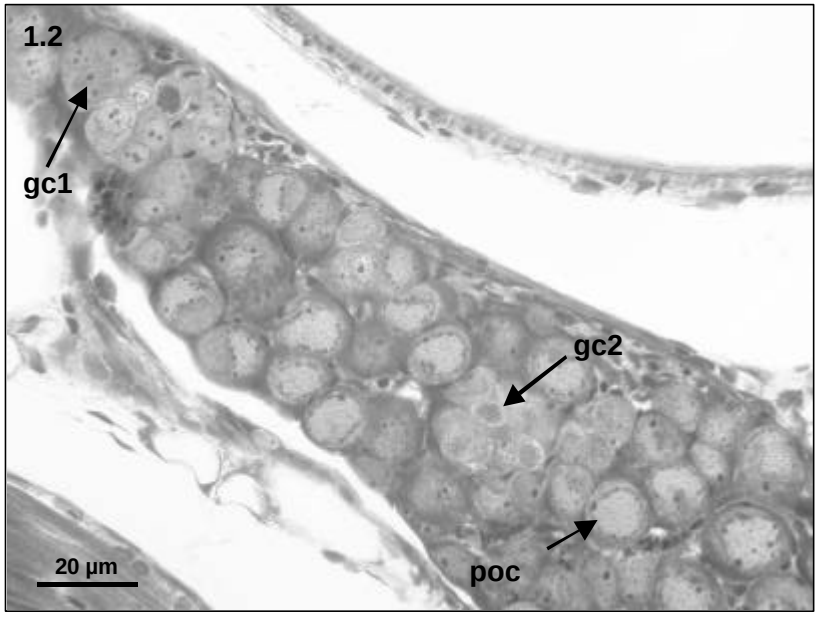
Figure 1.1: Undifferentiated gonad of a 15-day-old zebrafish with primordial germ cells (**PGC**). **N**: nucleus, with prominent nucleolus and deeply staining material along the inner side of the nuclear membrane. The **arrow** marks the network of chromatin strands. **Bar: 20 µm** (*Page 27, top*)

Figure 1.2: Part of an early ovary of a 5-week-old zebrafish. In germ cell type 1 (**gc1**) the presence of several nucleoli can be seen, as can the densely arranged chromatin threads of germ cell type 2 (**gc2**). The ovary is identifiable by the early perinucleolar oocytes (**poc**). **Bar: 20 µm** (*Page 27, bottom)*

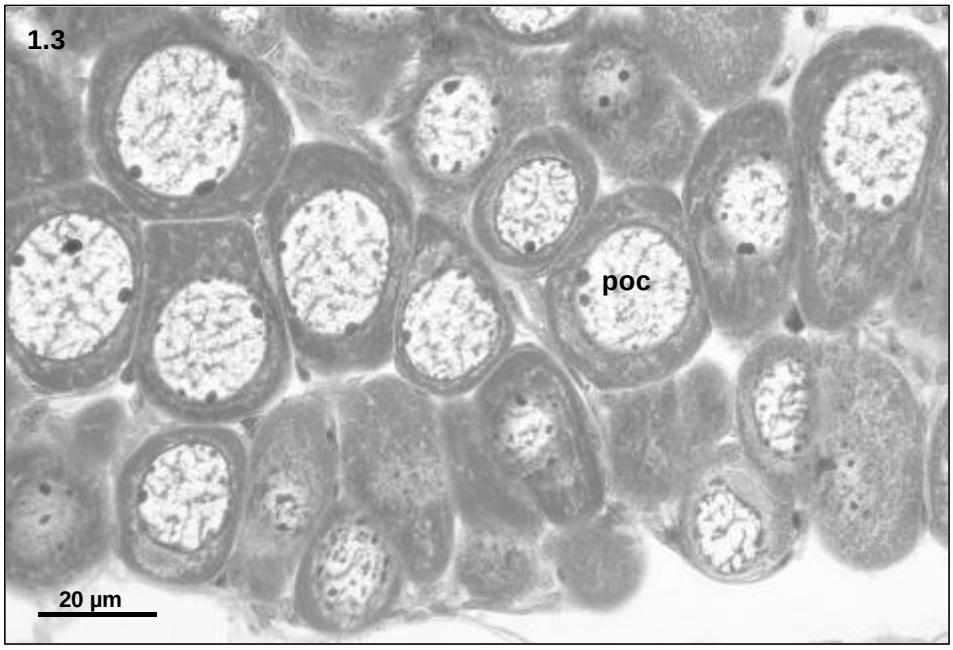
Figure 1.3: Part of a 5-week-old zebrafish ovary containing oocytes at the perinucleolar stage (**poc**). **Bar: 20 µm** *(Page 28, top)*

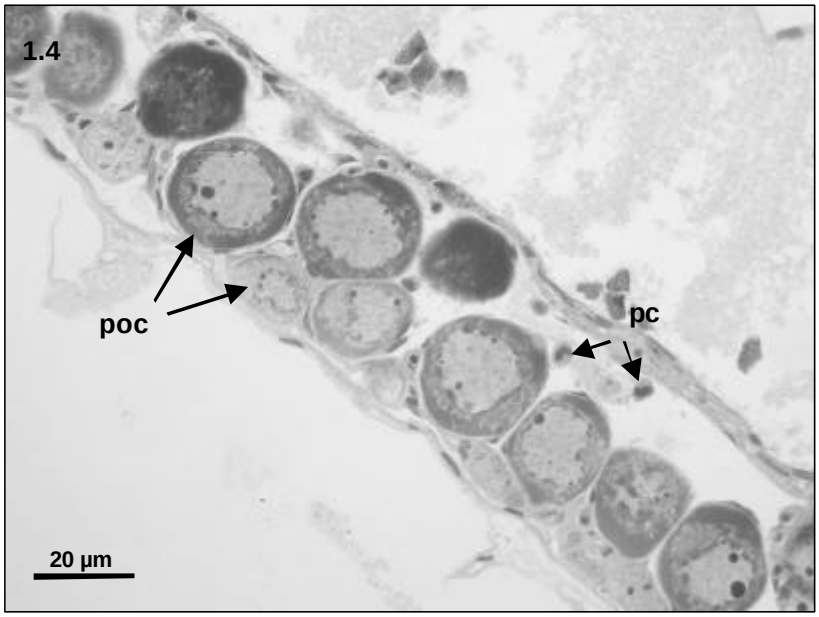
Figure 1.4: Gonad with altered ovarian morphology I:
Part of a gonad of a six-week-old fish. Phagocytes (**pc**) are found inside the ovary and between the perinucleolar oocytes (**poc**). **Bar: 20 µm** (*Page 28, bottom)*





Figures 1.1 and 1.2: Legends see page 26





Figures 1.3 and 1.4: Legends see page 26

6-week-old zebrafish (9 – 17 mm total length)

Gonads containing only type 1 and type 2 germ cells were no longer found. The majority of the examined fishes have rather large ovaries containing densely packed and well-differentiated perinucleolar oocytes, and variable numbers of type 1 and type 2 germ cells. In some fish, more than 90 % of the tissue is occupied by perinucleolar oocytes, and only at the cranial or caudal ends of the ovary do groups of undifferentiated germ cells occur. In other individuals, the relative portion of the ovary occupied by oocytes is reduced in favour of type 1 and type 2 germ cells.

In 19 % of the examined 6-week-old zebrafish, gonad morphology is clearly different from that described above. The sizes of the oocyte and of its nucleus are reduced and the shape of cell and nucleus is becoming increasingly irregular. The basophilia of the ooplasm may increase. Sometimes the nucleus may disintegrate and the oocyte turns into a highly basophilic residual body. The number of oocytes in the ovary is declining, and empty spaces may appear between the individual oocytes, sometimes infiltrated by macrophages (Figure 1.4). While the number of oocytes is severely decreased, the frequency of non-germ cells is increasing. Nests containing type 1 and type 2 cells are usually present, but are few in number. These alterations of gonad morphology can be restricted to a part of the ovary, or they can expand to the whole tissue. In the latter individuals, the ovary contains only very few oocytes; sometimes only one or two small oocytes are left (Figure 1.5). These altered ovaries are much smaller than the well-developed ovaries described above.

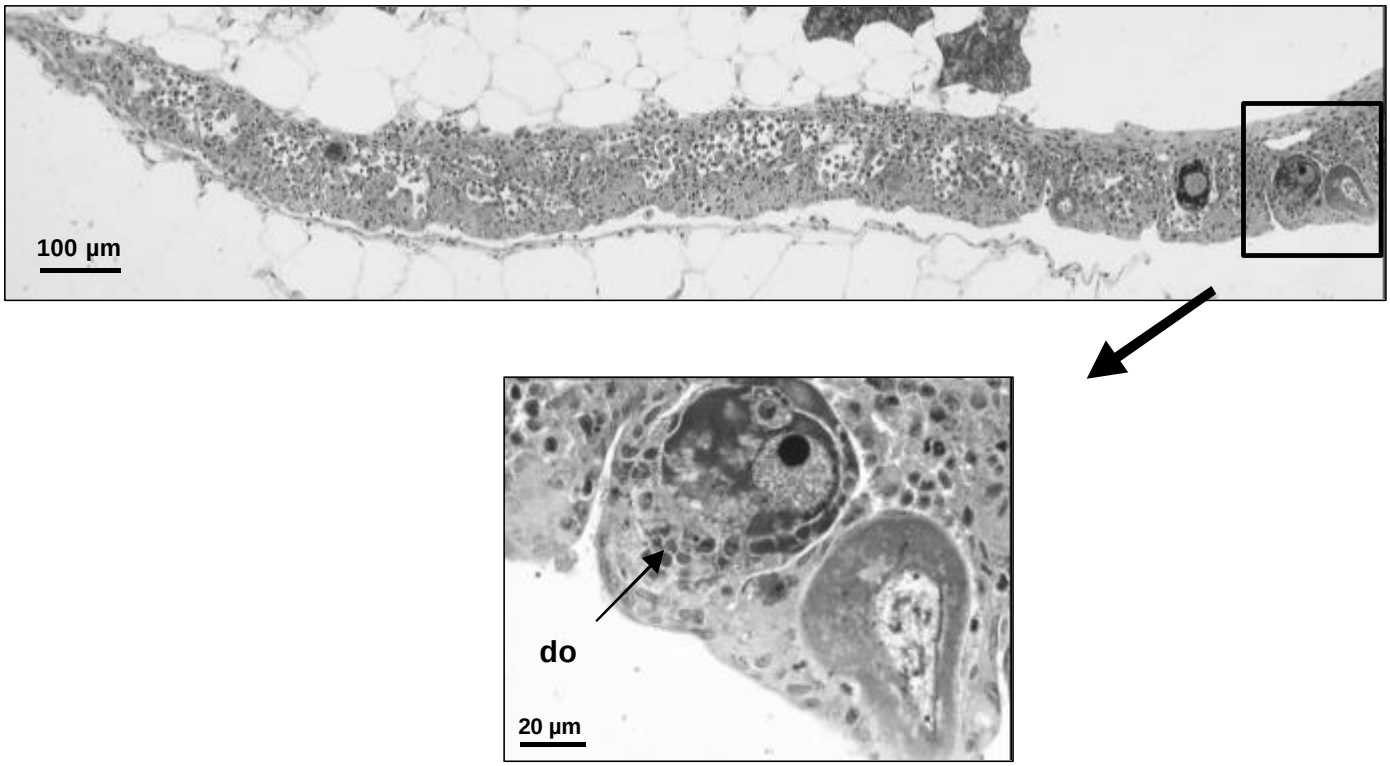


Figure 1.5: Gonad with altered ovarian morphology II:
Gonad of a eight-week-old fish
The gonad contains a few oocytes of altered morphology (**do**),

7-week-old zebrafish (13 – 22 mm total length)

The variation of gonad morphology described for 6-week-old zebrafish is still present in 7-week-old zebrafish. In morphological appearance the gonads range from large, well-developed ovaries to small ovaries with altered histological features. A new development is that in a few of these altered ovaries, the frequency of fibroblast-like and macrophagic non-germ cells is reduced, while the number of undifferentiated germ cells is clearly elevated. These correspond to the type 1 germ cells as described above: they have large nuclei with a prominent nucleolus, and a small cytoplasmic area. They are arranged in groups resembling spermatogonial cysts (Figure 1.6). Often, a lumen is present in the centre of the germ cell groups (Figure 1.7a). One of the examined 7-week-old fish shows a small group of spermatocytes. This gonad was classified as testis.

8-week-old zebrafish (13 – 23 mm total length)

In 8-week-old zebrafish pronounced variation of gonad morphology was found again. The percentage of fish containing well-differentiated ovaries is reduced compared to 7-week-old fish, whereas the number of fish showing gonads with altered ovaries and early testes was elevated. In the testes, both spermatocytes and spermatids are found (Figure 1.7b). 10 out of the 33 examined 8-week-old fishes possess “altered ovaries”.

9-week-old zebrafish (11 –21 mm total length)

In 9-week-old-zebrafish the number of individuals with testes is still further increased. The occurrence of altered ovaries is restricted to two out of 32 examined individuals.

10-week-old zebrafish (12 –23 mm total length)

Apart from one fish, the gonad of which shows the morphology of an altered ovary as described above, the remaining fish possess either a differentiated ovary or testis.

11-week-old zebrafish (12 –27 mm total length)

In 11-week-old zebrafish the same pattern as in 10-week fish is observed.

Figure 1.8 summarises the sex- ratio during the different periods of development.

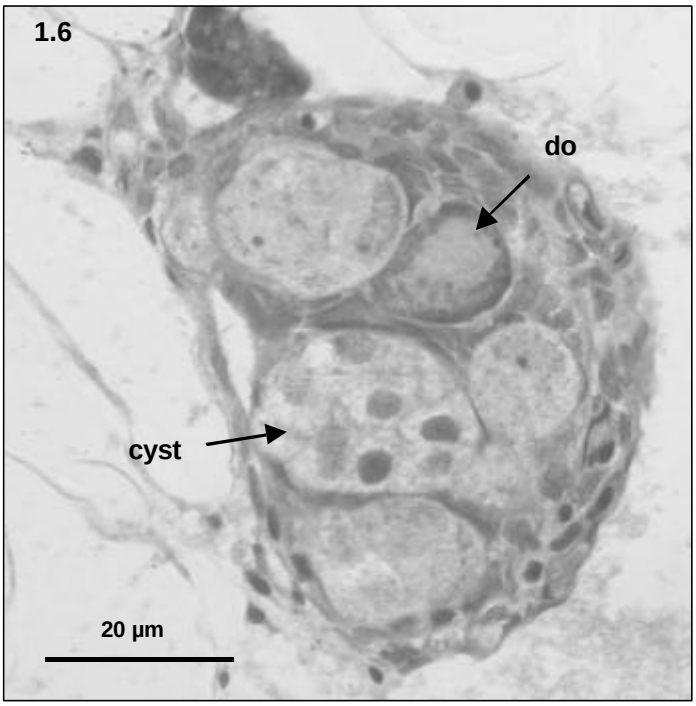
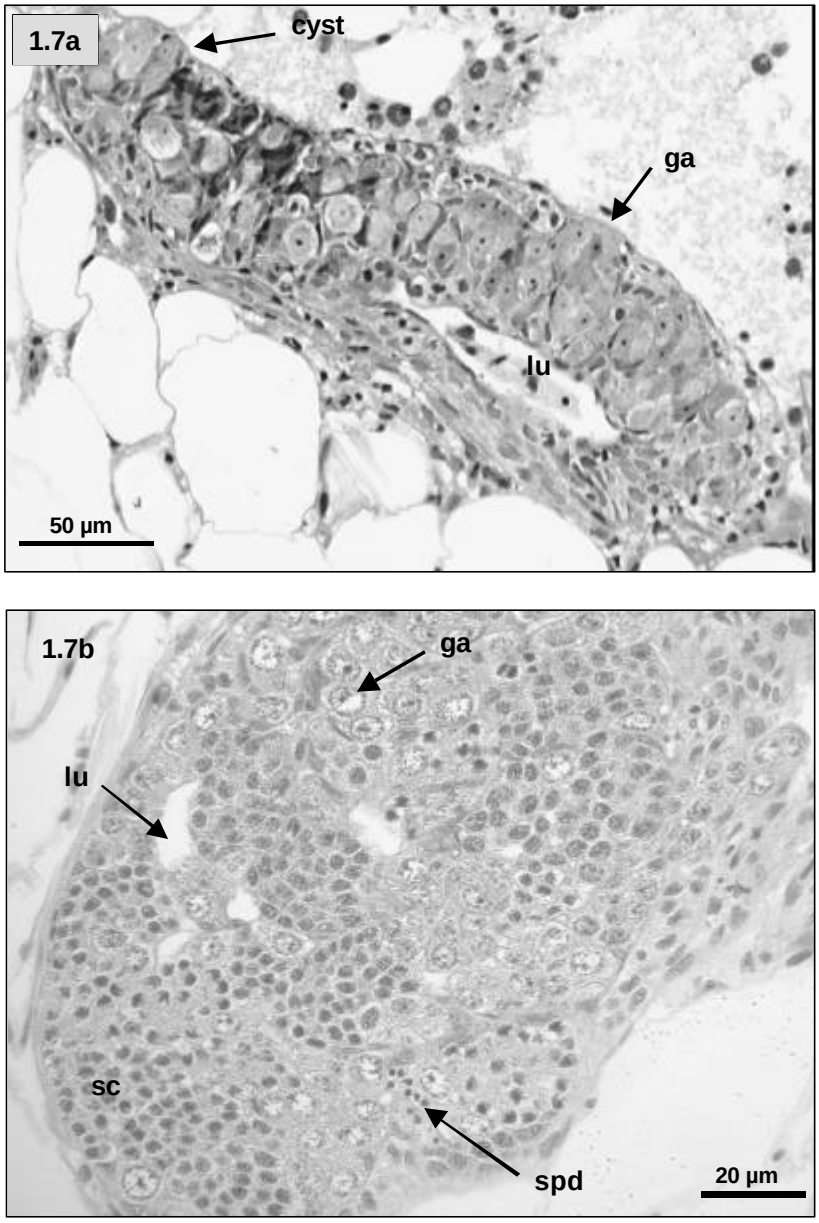


Figure 1.6: Gonad with altered ovarian morphology III:
Gonad of a seven-week-old fish.
While there are still oocytes present in the gonad, other parts of the gonad contain groups of germ cells resembling spermatogonian cysts (**cyst**)



Figures 1.7: Legend see page 34

Figure 1.7: Developing testes with male germ cells in different developmental stages
Fig. 1.7a: Very early testis of an 11-week-old fish with lumen formation (**lu**), spermatogonian cysts (**cyst**) and spermatogonia (**ga**)

Fig. 1.7b: Testis of an 8-week-old zebrafish with various male germ cells.
ga: spermatogonia **lu**: lumen; **sc** : spermatocytes , **sz**: spermatozoa (*Page 33*)

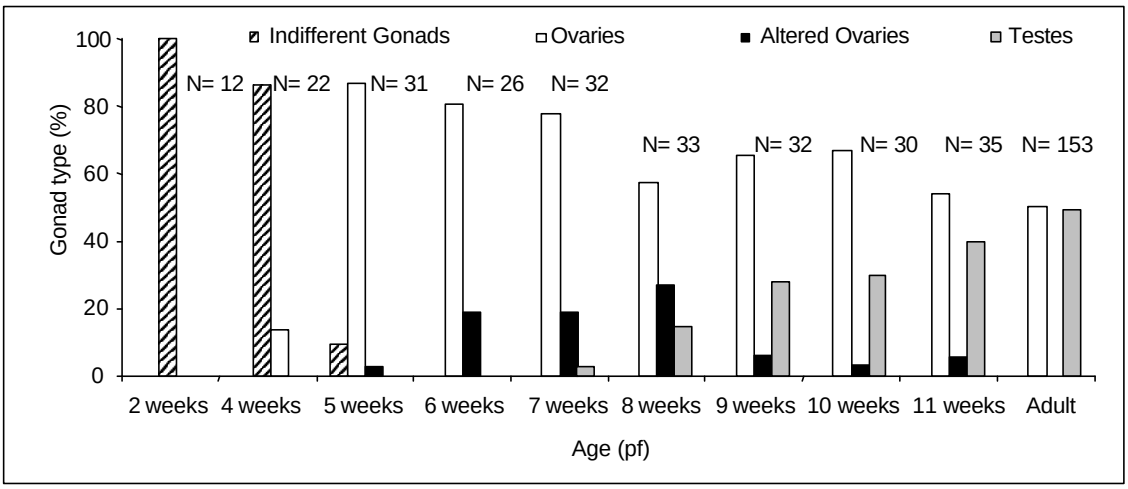


Figure 1.8: Distribution of morphological gonad types of the zebrafish with increasing age.
N: number of dissected individuals in the respective week.

Testes development was characterised by a decrease of undifferentiated tissue and an increase of male germ cells at different developmental stages, including spermatozoa (Figure 1.7b). In 4 out of 42 ripening testes a single isolated oocyte was found. The presence of these degenerated perinucleolar oocytes causes minimal disruption to the organisation of male tissue.

With the exception of one 10-week-old fish, ovaries contain oocytes in the perinuclear stage as the maximum developmental stage as long as they are non-reproductive. The average oocyte diameter grows slowly, increasing only from 33.3 µm to 46.5 µm between week 4 and week 11 post-fertilisation. Not until the fish reaches reproductive age are maturing or mature oocytes found.

Mature gonads

Spermatogenesis:

Cells in various stages of spermatogenesis were seen in separate cysts within a single tubule along the gonadal axes of the testis of mature zebrafish (Figure 1.9).

Oocyte maturation:

The development of zebrafish oocytes is divided into five stages, based on prominent morphological features. Perinucleolar oocytes, identifiable by a few peripherally located nucleoli as well as by small, localised areas of intense basophilia in the cytoplasm, mark the beginning of the primary growth stage. The beginning of oocyte maturation is identifiable by the appearance of cortical alveoli marks (yolk vesicles). Another prominent event occurring within this cortical alveolus stage is the formation of a vitelline envelope. During the vitellogenic stage, the oocyte increases in size, primarily due to the accumulation of yolk. In histological sections the main yolk bodies appear brighter than the surrounding matrix. During oocyte maturation, the germinal vesicle migrates toward the oocyte periphery. The nuclear envelope breaks down and the chromosomes proceed to the second meiotic prophase, at which point progress is arrested. In mature oocytes the nucleus is dissolved and the ooplasm consists of yolk bodies only. The oocyte is ready to be ovulated into the ovarian lumen. In a mature zebrafish ovary, oocytes at all stages are present, without dominant populations. After ovulation the follicle layer remains in the ovary. These postovulatory follicles are clear signs of recent spawning. Postovulatory follicles are irregularly shaped structures composed of columnar follicle cells. Those mature oocytes that have not been not ovulated are absorbed by follicle cells. The early phase of this process is characterised by the disintegration of some yolk globules and by a less regular shape. The zona radiata slowly dissolves, as indicated by a loss of striations and uneven diameter. In subsequent phases of atresia, granulosa cells enlarge and, upon rupture of the zona radiata, invade the degenerate oocyte. Figure 1.10 summarises the findings and characterisation. For a detailed description of zebrafish oocyte development see Selman et al. (1993).

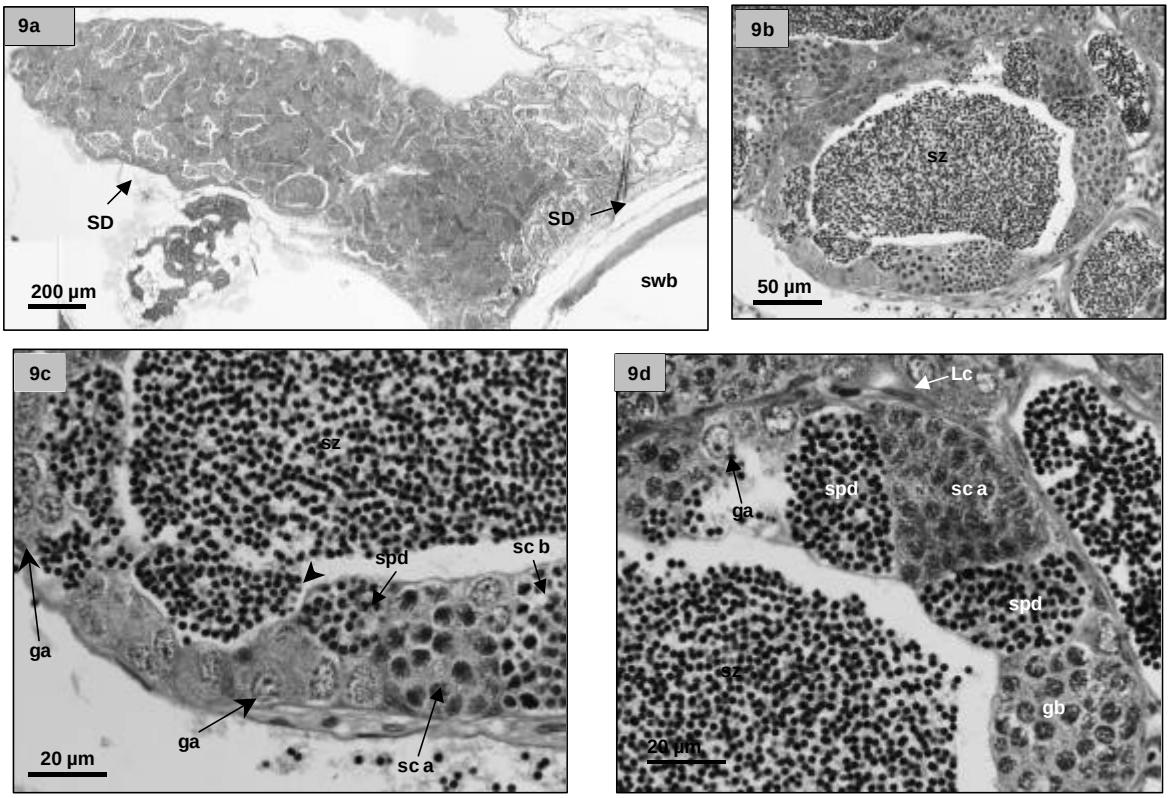


Figure 1.9: Transverse section (3µm) through the testes of mature male zebrafish to illustrate the normal testicular organisation. The single lobules are divided by connective tissue. Figure **9a** shows an overview. **9b** shows a single lobule with different developmental stages in the lobule wall and spermatozoa in the lobule lumen. **9c** and **9d**: Transverse section through a lobule to illustrate the different spermatogenetic cell types. The solid white arrow in Fig 9c marks the breakdown of lobule structure to release the spermatozoa into the lobule lumen.

SD: sperm duct, **swb**: swim bladder; **ga**: spermatogonia A; **gb**: spermatogonia B; **sc a**: spermatocytes A; **sc b**, spermatocytes B; **spd**: spermatids, **sz:** spermatozoa.
pay attention to the different scales

Figure 1.10.1 – 1.10.10: Zebrafish oocytes in different stages of development.

Fig. 1.10.1: Perinucleolar stage of primary oocyte growth. Several nucleoli appear at the periphery of the germinal vesicle (**GV**). During the primary growth stage, the oocyte grows from a diameter of approximately 10-20 µm to a diameter of ca. 100 µm. Although this is approximately a thousand-fold increase in oocyte volume, it is important to realise that ovaries containing oocytes in the primary growth stage only are still relatively small, having gonadosomatic indices of <2, and thus these ovaries are generally perceived as immature (Selman and Wallace 1989). Ovaries remain in this stage of oocyte development until shortly before maturing.

Fig. 1.10.2: Oocyte in advanced cortical alveolus stage. Cortical alveoli (**ca**) or yolk vesicles fill the oocyte cytoplasm. The germinal vesicle enlarges and becomes irregular in shape. **ve**: vitelline envelope or zona radiata

Fig. 1.10.3: Enlargement of Fig. 10.2. The vitelline envelope is in turn surrounded by follicle cells. Follicle cells are organised into an inner monolayer of granulosa cells and an outer layer of theca cells (**TC**). The pore channels through the zona radiata are visible. Through those pore canals oocyte microvilli contact granulosa cells (Takashima and Hibiya 1995). (**ca**) Cortical alveoli

Fig. 1.10.4: Vitellogenic oocyte (**voc**). During this major growth stage the oocyte increases in size primarily due to the accumulation of yolk. As yolk bodies accumulate centripetally within the oocyte, cortical alveoli are progressively displaced towards the periphery On both sides, oocytes in a less developed stage are seen. **Coc**: Cortical alveolus stage, **Poc**: Primary growth stage

Fig. 1.10.5: Oocyte maturation. During this stage, meiosis is reinitiated. The nucleus migrates towards the oocyte periphery (future animal pole?). The nuclear envelope breaks down. ca: cortical alveoli, **N**: nucleus, **ve**: vitelline envelope or zona radiata

Fig. 1.10.6: Enlargement of Fig.10.5. **ne**: nuclear envelope

Fig. 1.10.7: Mature oocyte (**moc**). At the end of maturation the yolk bodies lose their crystalline main bodies and develop a homogeneous interior. Prior to ovulation the follicle cells retract from the oocyte and their microvillar processes withdraw from the pore canals of the vitelline envelope (Selman et al. 1993).

Fig. 1.10.8: Postovulatory follicle (**POF**). The follicle layer is easy to identify. After ovulation the follicle layer remains in the ovary. These postovulatory follicles are clear signs of recent spawning. Postovulatory follicles are irregularly shaped structures composed of columnar follicle cells and an underlying connective tissue theca, and degenerate rapidly. 24h after spawning the postovulatory follicle has greatly shrunken or collapsed on itself. The prominent underlying connective tissue theca is still present but not as abundant

Fig. 1.10.9: á-stage atretic oocyte (**A1**): the vitelline envelope slowly dissolves, as indicated by the uneven diameter In subsequent phases of the alpha atresia, granulosa cells enlarge and, upon rupture of the vitelline envelope, invade the degenerate oocyte The alpha stage ends when the resorption of the oocyte is complete

Fig. 1.10.10: Advanced phase of á-stage atretic oocyte (**A2**)

pay attention to the different scales. *(Pages 38 and 39)*

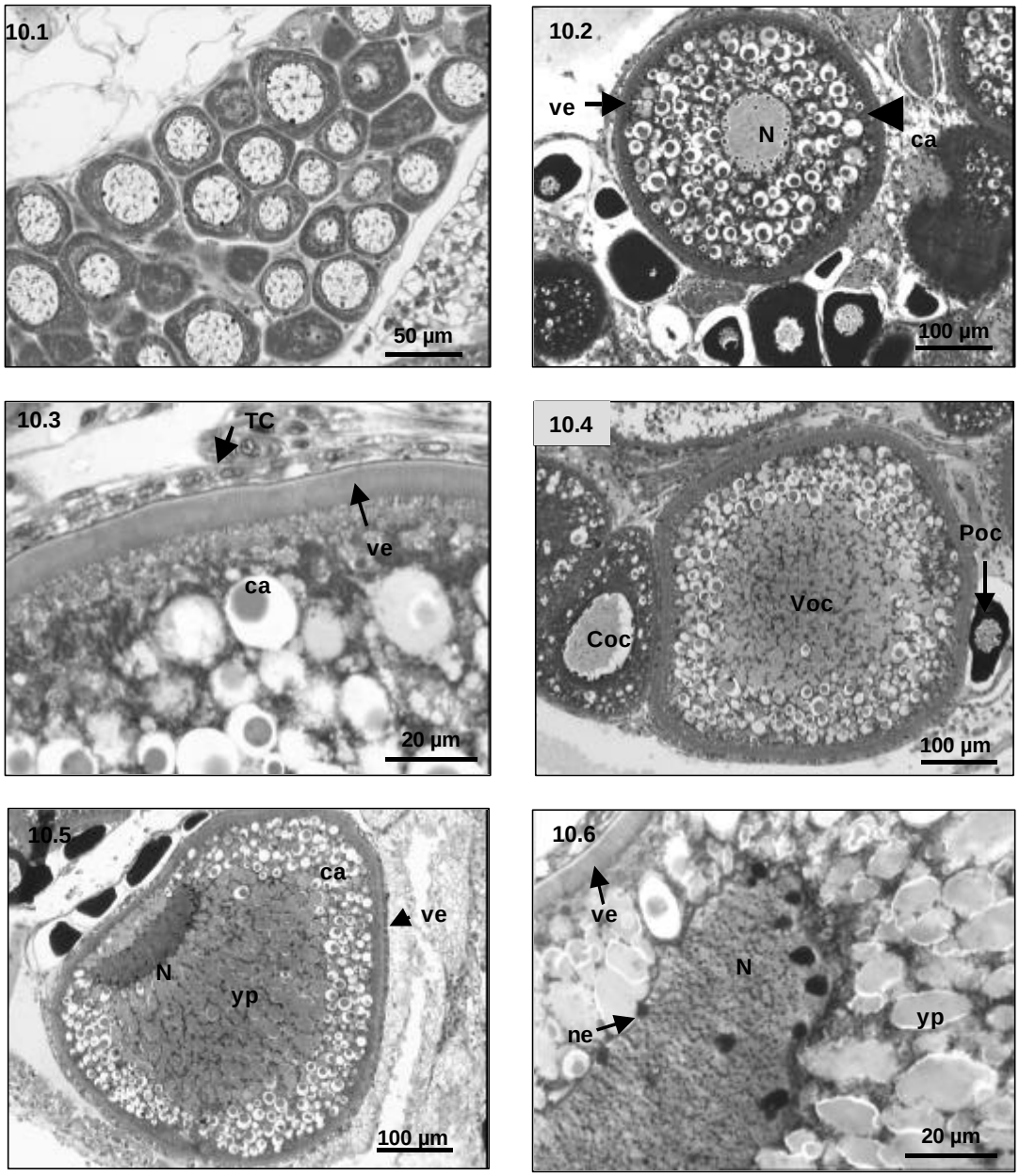


Figure 1.10, part 1: Legend see page 37

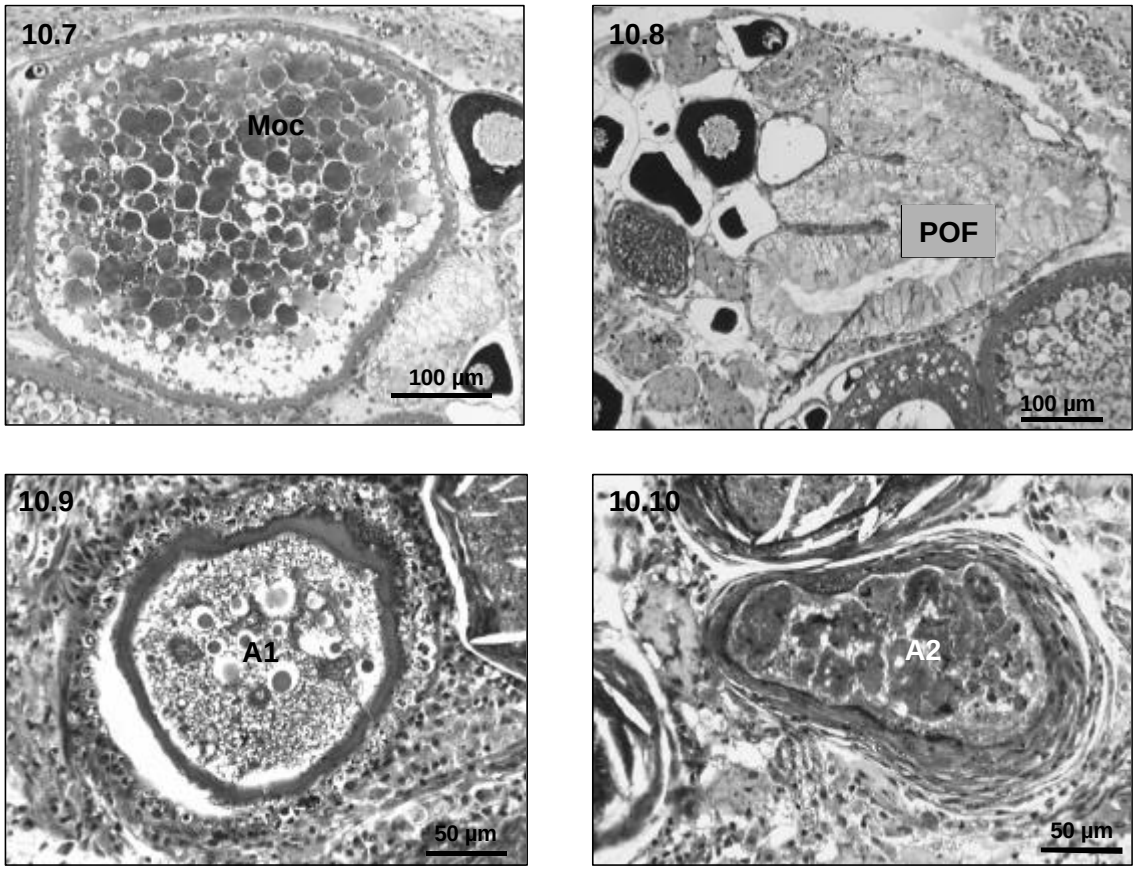


Figure 1.10, part 2: Legend see page 37

Discussion

To assess disruptive effects of chemicals on morphologically altered development, a detailed knowledge of the normal characteristics is necessary. This study provides a histological description of the gonadal development during the ontogeny of the zebrafish.

Gonadal development varies considerably in fish. The majority of fish species are gonochoristic; that is, undifferentiated primordial germ cells (PGCs) develop into ovaries or testes during ontogenesis. PGCs develop next to the dorsal wall of the coelomic cavity at the site of the future gonadal ridges. During the larval period, the gonad anlage gradually arises from the PGCs and surrounding somatic cells (Yamamoto 1969, Timmermans 1987), and grows into morphologically differentiated gonads during puberty. A sex change does not occur. In addition to gonochorism, several forms of hermaphroditism are found, including sequential hermaphroditism (either protandry or protogyny) as well as simultaneous hermaphroditism. Protrandrous fishes develop first as males and then later change to females, whereas protogynous fishes develop first as females and then later become males. Simultaneous hermaphrodites are capable of releasing viable eggs or sperm during the same spawning (Sadovy and Shapiro 1987, Helfman et al. 1997, Nakamura et al. 1998). Protogyny is by far the most common form of hermaphroditism, probably reflecting the fact that most teleosts, including gonochoristic species, differentiate first as non-functional females (Helfman et al. 1997). In most gonochoristic fish species male gonads develop later than female gonads (i.e. Takashima and Hibiya 1995). In that case undifferentiated gonads should be found as long as no males are visible.

In zebrafish gonad development the most characteristic pattern is the occurrence of altered ovaries between week 5 and week 11 and the non-existence of undifferentiated gonads from week 5 onwards, whereas the first males are not found before week 7. Another prominent feature is the simultaneous decrease in numbers of these altered ovaries and the rising percentage of males with increasing age. This pattern of gonadal development indicates a protogynous hermaphroditism in the zebrafish, where the altered ovaries are the transitional form between ovaries and testes. This transition took place before mature oocytes developed. That suggests that the protogyny observed in juvenile zebrafish is a nonfunctional protogyny.

A premature change of gonadal sex is not unusual in teleosts. This pattern is suspected or known from eels, hagfishes, lampreys, minnows, salmonids, cichlids, butterflyfishes, wrasses, parrotfishes, gobies and belontiid paradise fishes (Colombo & Grandi 1996, Helfman et al. 1997).

The exact stage at which the phenotypic gender is determined in fishes is controversial. Although genetic determination probably applies to most fishes, in many fishes, sex determination may be not fixed at fertilisation or even during ontogeny (Helfman et al. 1997). Sexual differentiation of developing gonads in fish is considered to be under the control of steroid hormones (Baroiller et al. 1999, Jalabert et al. 2000, Nagahama 2000, Piferer 2001). In the late sixties Yamamoto (1969) suggested that sex steroids are the natural inducers of sex in fish. Since then, numerous studies including recent molecular work (see Baroiller et al. 1999, Nagahama 2000) have corroborated and extended the original hypothesis of Yamamoto (1969), although contradictory observations do exist (e.g., Kawahara and Yamashita 2000). After sexual differentiation the zebrafish shows the typical pattern of sexual development for a synchronously spawning fish (i.e., Selman & Wallace, 1989, Takashima & Hibiya 1995, Tyler & Sumpter 1996), where spermatogenesis and oogenesis are continuous processes.

The period of gonadal sexual differentiation of zebrafish reared in our laboratory fell between day 40 and day 70 pf. Whereas the principle of juvenile hermaphroditism in zebrafish is clearly demonstrated, the timing of the period of sexual differentiation may vary between different strains and between laboratories. The growth of the fish, and therefore the age at the onset of sexual differentiation and the time when reproduction begins, depends on several factors. Besides abiotic parameters such as temperature, light, food availability, oxygen, pH and water quality, space is an essential parameter. If an individual fish does not have the minimum amount of space, it will not grow.

These results can serve as a basis for histological observations of the zebrafish as a model fish for assessing toxic effects caused by endocrine-altering chemicals (EACs). It is essential to keep in mind the phenomena of juvenile hermaphroditism when using zebrafish as a model species in aquatic toxicology; otherwise the normal ontogeny could be misinterpreted as a reaction to EACs.

Acknowledgement

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References

Baroiller, J.F., Guguin, Y. and Fostier, A. (1999). "Endocrine and environmental aspects of sex differentiation in fish." *Cell Molec Life Sci* **55**:910-931.

Billard, R. (1986). "Spermatogenesis and spermatology of some teleost fish species." *Reprod. Nutr. Dévelop.* **26**, 877-920.

Colombo, G. & Grandi, G. (1996). "Histological study of the development and sex differentiation of the gonad in the European eel". *Journal of Fish Biology* **48**, 493-512.

Fenner-Crisp, P. A, Maciorokowski A. F & Timm, G. E. (2000). "The endocrine disruptor screeening program developed by U.S. Environmental Agency." *Ecotoxicology* **9**. 85-91.

Flammarion P., Brion F., Babut, M., Garric J., Migeon B., Noury P, Thybaud E., Tyler C.R. Pallazzi X. 2000. "Induction of fish vitellogenin and alterations in testicular structure: preliminary results of estrogenic effects in chub (*Leuciscus cephalus*)". *Ecotoxicology* **9**: 127-135

Gimeno S, Komen H, Gerritsen AGM, Bowmer T. (1998) "Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during sexual differentiation." *Aquatic Toxicology* **43**: 77-92

Helfman, G. S., Colltte, B. B. & Facey, D. E. (1997). *The diversity of fishes.* Blackwell Science.

Huet, M.-C. (2000). "OECD activity on Endocrine Disrupters test guidelines development". *Ecotoxicology* **9**. 77-84.

Jalabert,B., Baroiller, J.F., Breton, B., Fostier, A., Le Gac, F., Guiguen, Y. and Monod, G. (2000). "Main neuro-endocrine, endocrine and paracrine regulations of fish reproduction, and vulnerability to xenobiotics". *Ecotoxicology* **9**:25-40.

Kawahara, T., Yamashita, I. (2000) "Estrogen-independent ovary formation in the medaka fish, *Oryzias latipes*". Zool Sci 17:65-68.

Kinnberg K., Korsgard B. & Bjerregaard. P. (2000). Concentration-dependent effects of nonylphenol on testis structure in adult platyfish *Xiphorus maculatus*. *Marine Environmental Research* **50**: 169-173.

Nagahama, Y. (2000). "Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish." In: Norberg B et al. (eds). Reproductive physiology of fish. University of Bergen Press, Bergen. pp. 211-222.

Nagai, T., Yamaha, E., & Arai, K. (2001). Histological differentiation of primordial germ cells in zebrafish. *Zoological Science* **18**. 215-223.

Nakamura, M. and Kobayashi. T., Chang XT, Nagahama Y (1998). "Gonadal sex differentiation in teleost fish." *J Exp Zool* **281**, 362-372.

Nolan, M., Jobling, S., Brighty, G., Sumpter, J.P. & Tyler, C.R. (2001). “A histological description of intersexuality in the roach”. *Journal of Fish Biology* **58**, 160-176.

Organisation for Economic Co-operation and Development, (1999). *Fish Expert Consulting Meeting*, London, Paris Cedex 16, France.

Organisation for Economic Co-operation and Development, (2000). OECD guidelines for testing of chemicals, section 2, Guideline 215: Fish, Juvenile Growth Test. Paris, Cedex 16, France.

Sadovy, Y. & Sharpio, D. Y. (1987). “Criteria for the diagnosis of hermaphroditism in fishes”. *Copeia*, 136-156.

Selman, K. & Wallace, R. A. (1989). “Cellular aspects of oocyte growth in Teleosts”. *Zoological Science*, **6**, 211-231.

Selman, K., Wallace, R. A., Sarka, A. & Qi, X. (1993). “Stages of oocyte development in the zebrafish *Brachydanio rerio*.” *Journal of Morphology* **218**, 203-224.

Takahashi, H. (1977). “Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*.” *Bull. Fac. Fish. Hokkaido Univ*. **28** (2), 57-65.

Takashima, F. & Hibiya, T. (1995). “An Atlas of Fish Histology: Normal and Pathological Features”, 2nd edn. New York: Gustav Fischer Verlag.

Timmermanns, L. P. M., (1987). "Early development and differentiation in fish", *Sarsia* **72**, 231-339

Tyler, C. R. & Sumpter, J. P. (1996) “Oocyte growth and development in teleosts.” *Reviews in Fish Biology and Fisheries*. **6**, 287-318.

van Ree, G.E. (1977). “In vitro induction of nuclear breakdown in oocytes of the zebrafish, *Brachydanio rerio* Ham. Buc., Effects of the composition of the medium and of protein and steroid hormones.” *Proc. Kon. Ned. Akad. Wetensch. Series C*, **80**, 353-371.

Wallace, R. A. & Selman, K. (1981) “Cellular and dynamic aspects of oocyte growth in teleosts.“ *American Zoologist* **21**, 325-343.

Yamamoto, T. (1969). “Sex differentiation.” In: Hoar WS, Randall D.J. (eds) Fish Physiology, Vol. III. Academic Press, New York, pp. 117-175.

Chapter 2

Effect of 17α-Ethynylestradiol and Bisphenol A on the Life-Cycle of the Zebrafish (*Danio rerio*)

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Abstract

The aims were to determine how chronic exposure to estrogens affects the gonadal development and compare the histological findings with reproductive alterations, and secondly to address possible qualitative differences of the fish response after exposure to a highly potent xenoestrogen and to a weakly estrogenic chemical. Fish full-life-cycle tests were conducted with the zebrafish exposed to 3 ng 17α-ethynylestradiol (EE2) for 14, 21, 42 days and permanent exposure, and permanent exposure to bisphenol A (BPA) at nominal concentrations of 94, 188, 375, 750 and 1500 µg/L starting with fertilised eggs. Neither in the early life stage toxicity test nor in somatic growth were any difference from the controls visible. An inhibitory effect on fertilisation occurred in fish exposed to 375, 750 and 1500 µg/L BPA. Histopathological effects in mature ovaries were found In females exposed to 750 and 1500 µg/L BPA, but not in testes. Permanent exposure to 3 ng EE2 completely inhibited reproduction. When the exposure ended before the period of sexual differentiation, it did not cause irreversible effects. Fertilisation, the most sensitive parameter in zebrafish reproduction, was reduced only in fish exposed for 43 days. These reductions were no longer visible when the reproductive parameters were measured a second time after 2 more weeks of recovery in tap water. Histological observation of permanently exposed fish revealed a total missing of male gonads as long as the fish were exposed, indicated a prevention of the transition from female to male gonads. Only after stopping the exposure the transition from female to male gonads was possible, but after a recovery period of 3 weeks in tap water the fertilisation and number of eggs laid per female were still reduced. A morphological analysis of the gonads demonstrated that following all exposure periods a significant proportion of the females did not participate in reproduction, even after an additional 50 days recovery in tap water.

KEYWORDS: zebrafish, *Danio rerio*, 17α-ethynylestradiol, Bisphenol A, gonadal development, gonad histology, reproduction, sexual differentiation

Introduction

A number of chemicals in the environment have beenshown or are suspected to have an endocrine-active potential (Toppari et al., 1996, Tyler et al., 1998). The incidence of hermaphroditic wild fish near sewage treatment works (STW) in the UK indicated the presence of estrogenic substances (Harries et al. 1996, Harries et al., 1997) The nature of the inducers has not yet been clearly elucidated.

One hypothesis implicated 17-α-ethynylestradiol (EE2) (Arcand-Hoy and Benson 1998). EE2 is one of the most commonly used active ingredients for oral contraception and has been detected in sewage treatment at the low ng/L range in various countries, e.g., in Germany (Stumpf et al. 1996) and in the UK (Desbrow et al., 1998, Routledge et al., 1998). Segner et al. (2002) showed that an EE2-concentration of 10 ng/L in water caused irreversible reproductive failure in zebrafish (*Danio rerio*) and Schäfers et al. (2002) demonstrated a lowest effect concentration (LOEC) for reproductive effects of EE2 on the zebrafish at 1.1 ng/L. Congruent observations were reported by Länge et al. (2001) for the fathead minnow (*Pimephales promelas*). These authors considered the overall no-observed-adverse-effect concentration to be 1.0 ng/L.

Another possible candidate for the estrogenic potency of the effluents is Bisphenol A, a high production volume chemical used to make polycarbonate plastics and other chemicals (Feldman and Krishnan 1995, Staples et al., 1998). It is known as a weak estrogen, as indicated by in vivo studies in mammals (Bond et al. 1980) and in fish (Sohoni et al., 2001). These authors mentioned effects on breeding at and above 640 µg/L in life-cycle tests with the fathead minnow and (Yokota et al., 2000) found that the LOEC for endocrine effects in the early life stage of medaka (*Oryzias latipes*) was between 355 and 1800 µg/L. BPA is widely distributed in water but as reviewed by Staples et al. (1998) surface water concentrations measured in effluents in Europe and the United States were at least one order of magnitude lower (up to 25 µg/L), than the measured effect concentrations.

In this study fish full-life-cycle (FFLC) studies were conducted with EE2 and BPA, in order to investigate the effects of a very potent synthetic estrogen, EE2, in comparison to a weakly estrogenic chemical, BPA, using the zebrafish as a model species. The zebrafish, *Danio rerio*, (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae) has a relatively short life cycle of about 4 months, and, in the laboratory, can be stimulated to breed throughout the year. For this reason, zebrafish is suitable for assessing toxic effects of chemicals on development and reproduction (Nagel 1994, Nagel and Isberner 1998, Andersen et al., 2000). Additionally there are test protocols in place, including OECD guidelines (OECD 1984, OECD 1992a, OECD 1992b, OECD 1998, OECD 2000) that recommend zebrafish as representative of aquatic vertebrates, for chemical toxicity assessments.

The aim of this study was to determine if chronic exposure of zebrafish to estrogens modifies or disrupts gonadal development and to evaluate if this is associated with reproductive alterations. To this end, we selected a potent synthetic estrogen, EE2, and a weak xenoestrogen, BPA. For EE2, only one test concentration – 3 ng/L - was selected. Previous experiments on the concentration dependency of EE2 effects showed that 3 ng/L

EE2 is in the lower range of the concentration-effect curve of the estrogenic activity of EE2. Zebrafish was exposed to EE2 during different developmental periods in order to explore the importance of exposure stage and exposure duration for the induction of estrogenic effects in zebrafish. The concentration dependency of endocrine effects of environmental estrogens was studied using the xenoestrogen, BPA. Fish were exposed to the BPA concentrations 94, 188, 375, 750 and 1500 µg/L during a full-life cycle. Gonadal development and oocyte growth were measured after histological analysis and beside the reproductive performance parameters the somatic growth was measured.

Materials and methods

The experiments were conducted at the Fraunhofer Institute of Microbiology and Applied Ecology Schmallenberg Germany.

Study organism

The origin of the zebrafish strain was the West Aquarium GmbH in 37431 Bad Lauterberg, Germany. Fertilised eggs for the tests were obtained from parental fish reared in the laboratory,. Parental fish were kept in water of the same quality as used in the tests. The water temperature was maintained at 26 °C ± 1 °C. Light/dark cycle was 12 h/12 h. Animals were fed daily with TetraMin^R Hauptfutter (Tetra Werke, Melle, Germany) and nauplia larvae of the crustacean *Artemia sp., ad libitum*. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilised eggs. Purified drinking water was prepared according to OECD-guidelines. The water purification included filtration with activated charcoal and aeration.

Test substances and concentrations

EE2: 17α-e thinylestradiol (MM296.4, CAS 57-63-6) was obtained from Sigma (Deisenhofen, Germany) with a purity of 98%. The test concentration of nominal 3 ng EE2/L was selected on the basis of previous full-life-cycle experiments (EC50 = 1.1 ng EE2/L) and of a 28d prolonged toxicity test with juvenile and adult zebrafish (EC_{50}: 100 ng EE2/L).

The fish were exposed in two replicates for 14, 21, 43 and 125 days, respectively and two untreated vessels served as controls. In all cases exposure began with fertilised eggs under flow-through conditions. For each exposure duration, an automatic syringe was used for dosing the mixing chambers with test substance, dilution water being constantly supplied

hydrostatically. A stock solution of 10 mg EE2/10 ml acetone was prepared and diluted with sterile aqua dest. to a concentration of 1 mg/L. The nominal test concentration was 3ng EE2/l. The average test concentration was between 80 and 93 % of the target value, with a mean of 2.6 ± 0,18 ng/L EE2.

BPA: Because of the rapid degradation of BPA in the stock solution, the test was performed semi-statically with three exchanges of the test solution per week. The procedure guaranteed an exposure to peak (= nominal) concentrations. The mean measured concentrations at nominally 94, 188, 375, 750 and 1500 µg/L were in good agreement with the target values (not less then 80 % and not more than 120 % of the nominal values) directly after water exchange. Due to the fact that these concentrations were reached on nearly 50% of the days of the study, effect concentrations were related to the initial rather to the mean concentration values.

Chemical analysis

EE2: The water samples (approx. 1 l) were acidified with hydrochloric acid (10 µL 30% acid per 100 ml of sample). After addition of the Internal Standard (IS; EE2-D_4; CDN Isotopes, Pointe-Claire, Canada) the extraction was carried out by "solid phase extraction" (SPE). The whole sample was sucked through a 3M Octadecyl EMPORE™-extractions disks (Varian, Harbor City, USA) during 30 minutes. The elution of the disks was performed with 10 ml of acetone. The elution solvent was reduced to 1 ml using a stream of nitrogen. The EMPORE™ extracts were cleaned by adsorption chromatography using 1 g activated silica gel and n-hexane / acetone solvent mixtures. The cleaned solvents were reduced to approx. 300 µl by a gentle stream of nitrogen, transported into a GC micro vial and evaporated further to dryness. Then 50 µl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS-No. 24589-78-4, Pierce, Illinois, USA), was added to the residue. The mixture was heated up to 70 °C for 1 hour to form the non-polar trimethylsilyl (TMS) ethers of the analytes. The gas chromatographic separation was performed in a non-polar capillary column and the mass selective measuring was carried out with ion trap GC/MS/MS (GC Varian Saturn 3400, MS Varian Saturn 4D). The MS/MS function was used for the mass selective trapping of the analyte ions (parent ions), but not for the production of daughter ions. The method for the EE2 analysis of water samples was performed at the IUCT, Schmallenberg, and is described in detail by Böhmer and Kurzawa (2000).

BPA: Depending on the nominal concentration samples from 10 to 100 ml were taken and filled up to 100 ml with aqua dest. After addition of the internal standard (BPA-d16 in MeOH) the samples were acidified with hydrocloric acid (1N). The extraction was carried out with 50 and 30 dichloromethane, dried out with natriumsulfate and reduced to 10 ml. 50µg of the extract were transferred to Microvilas and derivated with 50 µl N-methyl-N-trimethylsilyltrifluoracetamid (MSTFA) for 30 minutes. BPA was measured by GC/MC in the range from 0 – 1000 ng/L; analysis was performed by the method of the internal standard (Bruchert and Müller, pers. com)

Test conditions

pH-value, oxygen concentration, and temperature were measured directly before adding the fertilised eggs and afterwards once per working day.

EE2 experiments: The mean temperature ± standard deviation for all test vessels (water bath including all test vessels) was 25.6 ± 0.4°C until the end of the experiment, single values ranging from 25.0 to 26.3 °C. The pH-values ranged from 7.7 to 8.1. The mean content of dissolved oxygen was in the range of 7.3 ± 0.7 to 7.5 ± 0.7 mg/L for all test vessels during the study period, single values ranging from 77 % to 109 %.

BPA experiments: The mean temperature ± standard deviation for all test vessels (water bath including all test vessels) was 25.3 ± 0.3°C until the end of the experiment, single values ranging from 24.8 to 25.8 °C. The mean pH-values The pH-values ranged from 7.7 to 8.1± standard derivation for the ten test vessels varied between 8.0 ± 0.2 and 8.1 ± 0.3, single values ranging from 7.4 to 8.9. The mean content of dissolved oxygen was in the range of 7.2 ± 0.9 to 7.5 ± 0.7 mg/L for all test vessels during the study period, single values ranging from 54 % to 123 %.

Rearing of zebrafish

The studies were mainly performed according to (Nagel 1994), starting with exposure of 100 fertilised eggs per test vessel. The light/dark cycle for all of the test periods was adjusted to 12 h/12 h. The fish were exposed as fertilised eggs. Glass aquaria measuring 29 x 22 x 21 cm (length x depth x height; total volume=13.4 l) with cages of 20 x 9 x 9 cm (length x depth x height) for the eggs were used as test vessels during the first early life stage test (until day 35 to 42); these aquaria with a total volume of 13.4 l were placed into glass aquaria with a total volume of 29 l (40 x 27 x 27 cm; length x depth x height). Volume of test solution was 25 l per vessel. Hatching and survival rates was determined by photography followed by

digital image processing. After 2-3 weeks the larvae were transferred from the cages into the glass aquaria (total volume = 13.4 l), mentioned above. On day 42 pf, the juvenile fish were photographed, the survival rate was determined and the length of the animals was measured. The animals were transferred from the smaller glass aquaria (13.4 l) into the glass aquaria with a total volume of 29 l. The number of fish was reduced to 50 animals per vessel. On day 75pf, the number of fish was reduced again to 30 fish per vessel. Fish were again photographed at day 75. The survival rate and the fish length were determined (figures 2.1a and 2.1b).

Effect endpoints: length measurements

For each test concentration fish length was determined by photographing the single test vessels, following by digital analysis. Mean values ± standard deviation were calculated and the data from the different treatments were compared by the following statistical methods (SPSS program package): When data were shown to be normally distributed (Levène-Test), ANOVA was performed, followed by Scheffle's test for the investigation of significant differences between the treatments and the untreated control. If variances were inhomogeneous, Dunnett's T3 test was used.

Egg production and fertilisation

For the onset of spawning egg production was evaluated semi-quantitatively by collecting the eggs in spawning trays. The trays were covered with a lattice (stainless steel). Artificial "spawning-trees", were fixed onto the lattice (modified method according to (Nagel 1986). The spawning trees stimulate the spawning and concentrate it over the spawning trays. Mating behaviour and spawning were observed for the first time between day 80 and day 84 pf for fishes exposed to BPA and to EE2 respectively. During the next days the egg production was checked daily to make sure that eggs were produced continuously in every test vessel. Only when active and continuous spawning was evident in each aquarium, the quantitative determination of egg production begun. After 20 days of daily counts, the reproduction assessment was finished, the fish were sampled for histology, and were sexed on the basis of histological analyses of the gonads. The number of eggs per aquarium was related to the number of females per tank in order to calculate the numbers of eggs per female and day. The percentage of fertilised eggs per vessel and day is a measure of the fertilisation success. For each test concentration, the mean value and standard deviation of the total number of eggs per female and day, and of the fertilisation per day (n = 20-25

counts) were calculated. To create a normal distribution fertilisation rate values (0-100%) were z-transformed (arc sin (square root x), egg numbers were log-transformed. With the transformed data, ANOVA was performed. In case of significant differences between the mean values, Scheffle's Test was used to find out which mean values were significantly lower than the mean value found for the control.

Reversibility of reproductive effects of EE2

After determination of egg number and the fertilisation, the fishes of one replicate of each exposure treatment, including one control vessel were transferred to uncontaminated tap water in the same flow-through system for a further two weeks to find out whether possible reproductive effects are reversible.

Histological analysis

321 fish, aged from day 75 pf to day 176 pf, exposed to EE2 and 158 fish exposed to BPA, aged 75 pf and 110 pf, were analysed histologically. Fish were anaesthetised in ice water and were fixed for 24 h in Bouin' s fluid. While the young stages were fixed as a whole, larger fish, were decapitated first. For a better infiltration of the fixative, an incision was made on the dorsal side. Fixed tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax (Leica Histowax). Serial sections were cut at 7 µm, collected onto glass slides, and stained with PAS (Periodic-Acid Schiff's). For the histological analysis an Olympus BX 60 microscope was used. Figures 2.1a and 2.1b summarize the various parameters measured at the different times.

In mature testes the testicular sex cell development was investigated. For this purpose, the number of the different cell types of up to 5 gonads per treatment and age were counted in four sections (1mm²), taken from different parts of the gonad. The cell types were divided into: I: spermatogonia (a and b); II: spermatocytes, III: spermatids, IV: spermatozoa and V: non germ cells. The proportions of the different cell types were determined by pooling the numbers in the different sections of one testes. The oocytes of the zebrafish were classified according to (Selman et al., 1993). The classification scheme of Selman et al. is based both on morphological criteria and on physiological and biochemical events. These authors subdivided the oocyte development into five stages: STAGE I: follicle phase of primary growth (perinucleolar oocytes) STAGE II: cortical alveolus stage, STAGE III: vitellogenesis, STAGE IV: oocyte maturation, STAGE V: hydrated oocyte (mature egg). See Chapter 1 for more details. For determining the diameter of the perinucleolar oocytes up to 50 oocytes per female were

measured. Slides were photographed by an Olympus DP 10 digital camera and the oocyte diameter were measured using SIS-analysis® software package.

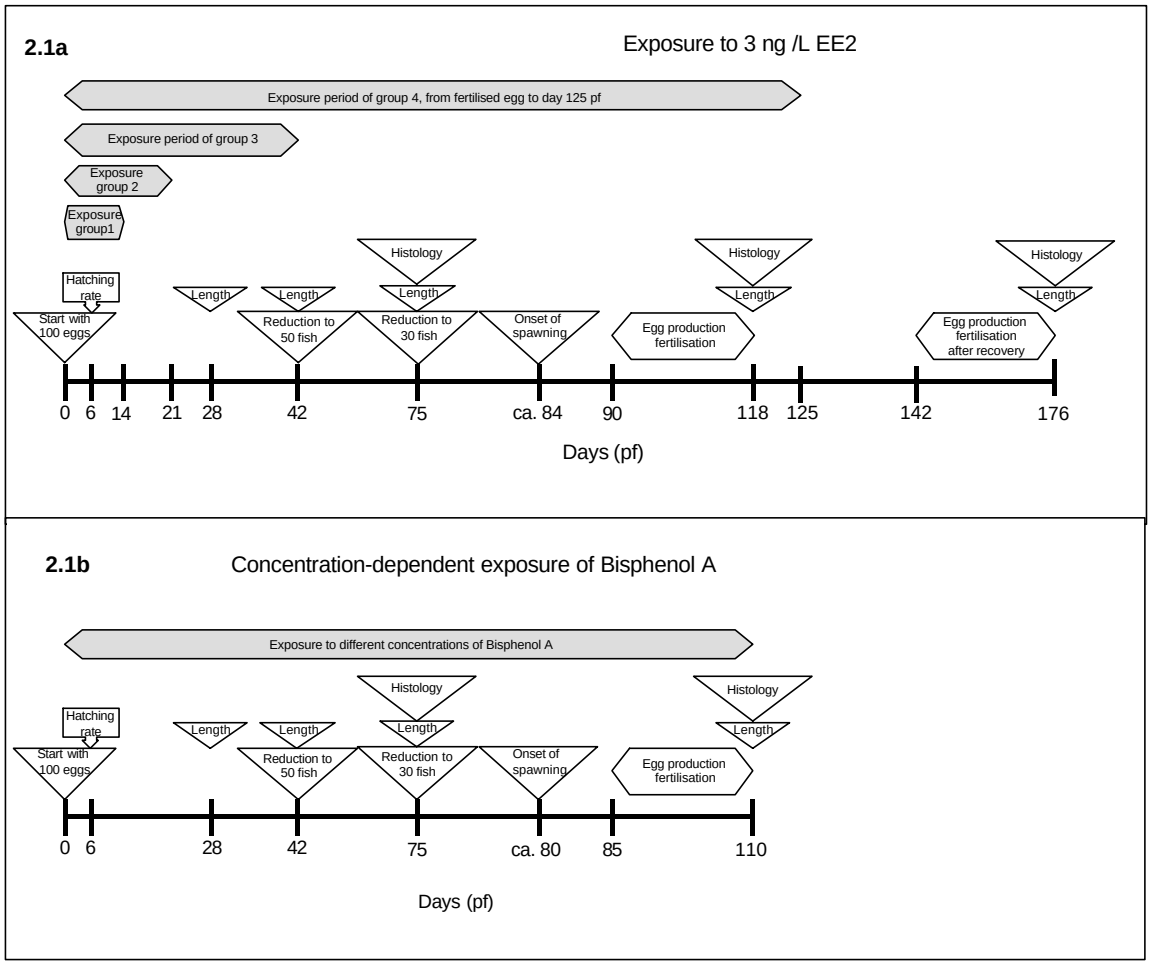

Figure 2.1a: Life-cycle test with 3 ng EE2. Summary of the time course, with different endpoints determined at different times.

Figure 2.1b: Life-cycle test with different concentrations of BPA. Summary of the time course, with different endpoints determined at different times.

Results

Effect of exposure on body length

As shown in Figures 2.2a and 2.2b neither in the fish exposed to 3 ng/L EE2 for different periods nor in the fish exposed to different concentrations of bisphenol A could any alterations in the body length be observed.

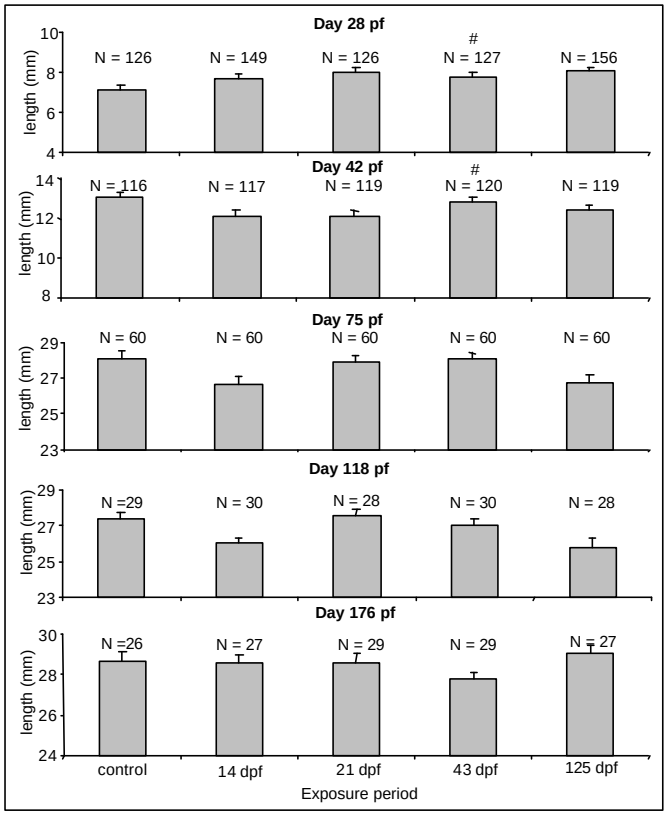

Figure 2.2a: Total length (mm) of zebrafish at different ages, exposed for different periods to 3 ng EE2, starting on the day of fertilisation. Exposure was stopped on day 128 pf to evaluate a possible recovery of reproductive parameters.
N = number of fishes per exposure period;
#: permanent exposure up to this time.

Data generated at the Fraunhofer Institute

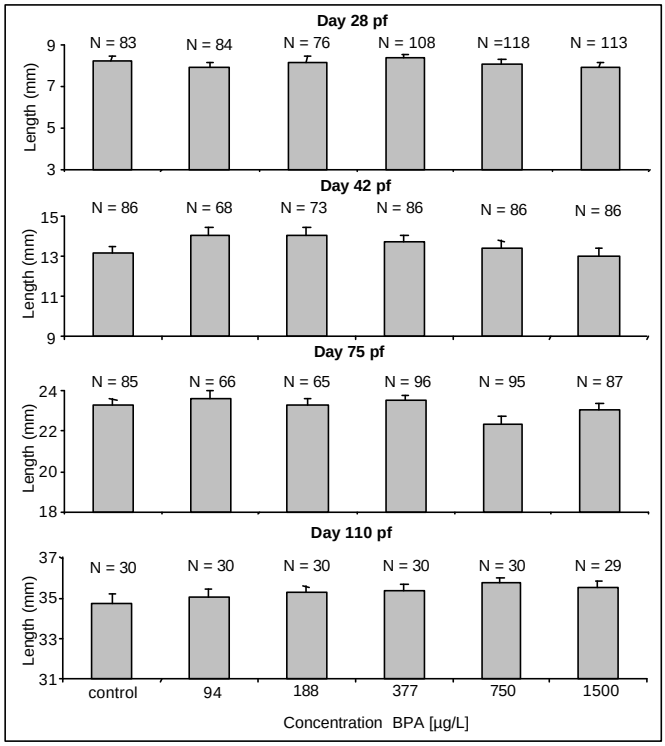

Figure 2.2b: Total length (mm) at different ages of zebrafish exposed to different concentrations of bisphenol A
N: number of fishes

Data generated at the Fraunhofer Institute

Reproduction

Start of spawning, egg number per female and fertilisation

Exposure to 3 ng /L EE2 for different periods: Time until first spawning was not influenced by exposure until14, 21 or 43 days pf (Figure 2.3). All groups started to spawn between day 83 and day 85 pf. The total number of eggs per female and the fertilisation when fish were exposed to 3 ng EE2 during the first 14 or 21 days pf showed no statistically significant differences from the control group. In the group exposed for 43 days pf a difference in the number of eggs laid per female also could not be observed, however, fertilisation was slightly but significantly reduced (83.5 % fertilization compared to 90 % in the control; (Figure 2.4). Fish exposed continuously from fertilization up to the age when control individuals started to spawn showed neither mating behaviour nor female egg laying. Even when the treatment was extended until day 125 pf, spawning was not initiated. When the fishes were transferred into non-contaminated water after 125 days of exposure to 3 ng EE2/L, spawning was initiated at 142 dpf. However, the number of eggs per female as well as the fertilization success remained significantly below those of control fish of the same age (29 eggs per female compared to 54 in the control) as well as a reduced fertilisation (21.7 % compared to 91.7 % in the control group) was observed (Figures 2.5 and 2.6).

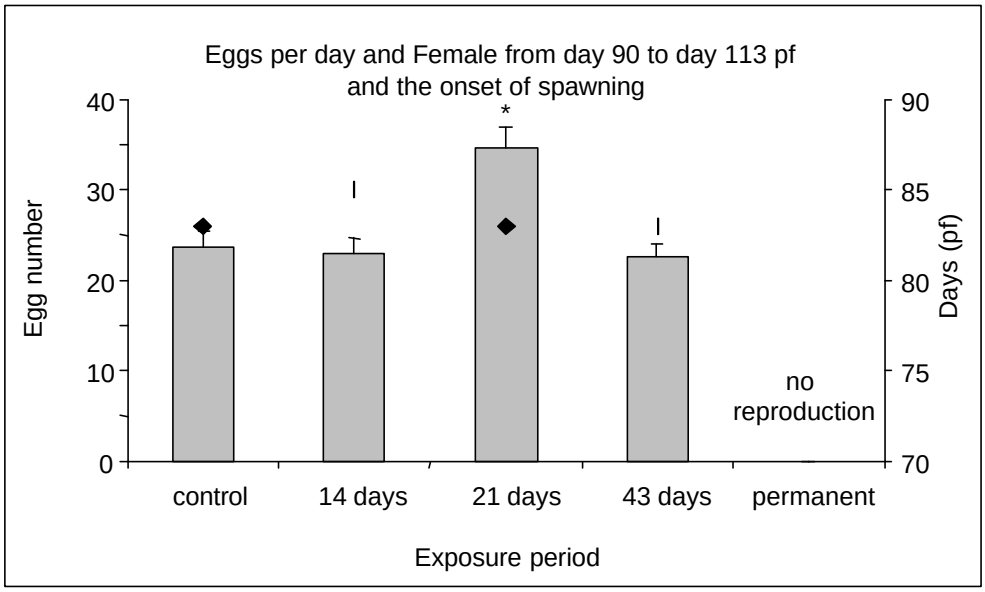

Figure 2.3: Number of eggs per female and day and the onset of spawning of zebrafish exposed to 3 ng EE2 over different periods, started with fertilised eggs.
Columns: mean(± SE) number of eggs per female, pooled data from all fishes per exposure period
Rhombus: mean onset of spawning in days post-fertilisation, average of two test vessels per exposure period
*significant $p < 0.05$; Scheffle's Test; one way ANOVA
Data generated at the Fraunhofer Institute.

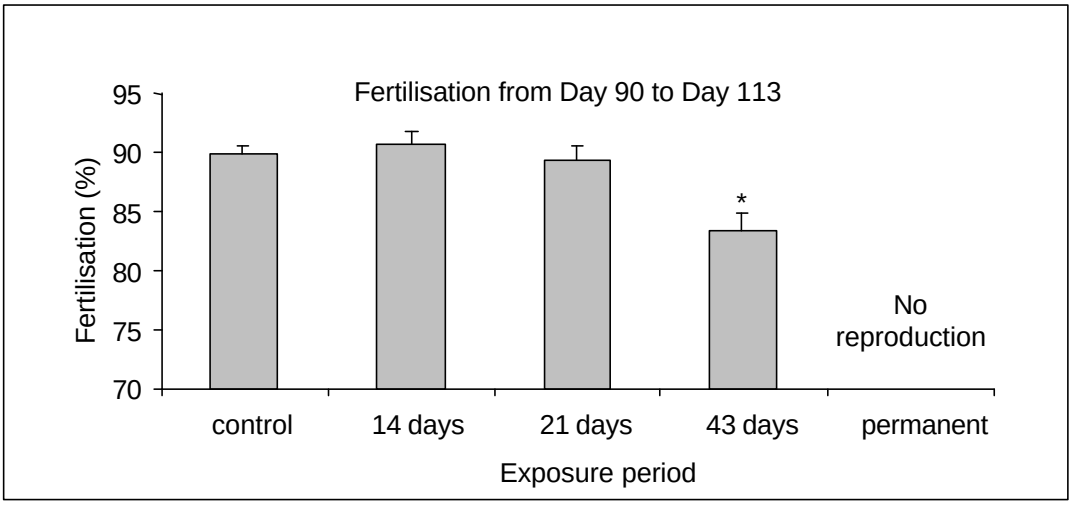

Figure 2.4: Fertilisation of zebrafish exposed to 3ng EE2/L over different periods, starting with fertilised eggs. (*significant difference from the control $p < 0.05$; Scheffle's Test; one way ANOVA). Data generated at the Fraunhofer Institute.

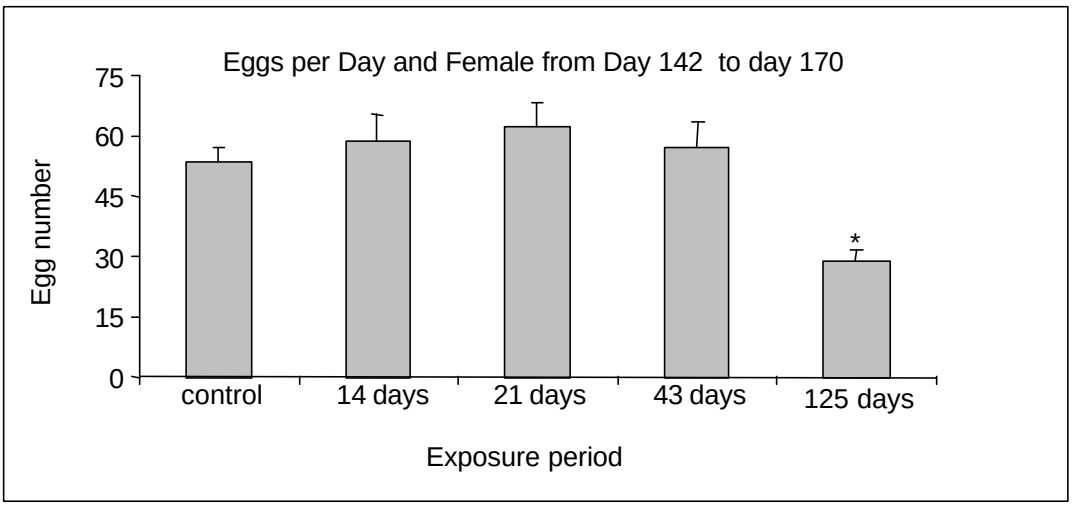

Figure 2.5: Mean number (± SE) of eggs per female and day of zebrafish exposed to 3 ng EE2 over different periods, starting with fertilised eggs. Second counting after stopping the exposure at 125 dpf to evaluate a possible recovery of the reproductive parameters, *significant $p < 0.05$; Scheffle's Test; one way ANOVA
Data generated at the Fraunhofer Institute.

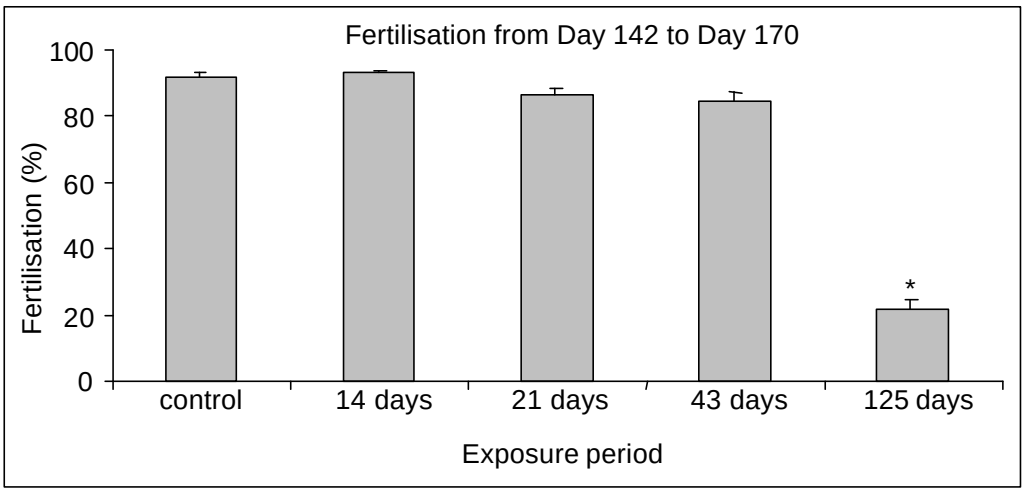

Figure 2.6: Fertilization of zebrafish exposed to 3ng EE2/L over different periods, starting with fertilised eggs. Second counting after stopping the exposure at 125 dpf to evaluate a possible recovery of the reproductive parameters *significant difference from the control $p < 0.05$;Scheffle's Test; one way ANOVA
Data generated at the Fraunhofer Institute

Continuous exposure to different concentrations of BPA:

Neither in the time until the first spawning nor in the number of eggs laid per female and day could any statistically significant difference between the control group and any exposure group be found (Figure 2.7).

Mating behaviour was observed on day 77 pf, and on day 84 the fish in the last test vessel (at 750µg/L BPA) started to spawn. The number of laid eggs varied from 24 eggs laid per female and day at the highest concentration to 41 eggs laid by per day and female zebrafish exposed to 188 µg/L, whereas the control fish laid 34 eggs per day and female. Fertilisation in the groups exposed to 94 and 184 µg/L showed no statistical difference from the control group, although in fish, exposed to higher concentrations there was showed a significant, concentration-related reduction. In fish exposed to 375 µg/L the fertilisation was reduced to 62.7 % in those fish exposed to 750 µg/L to 56 %, and in those fish exposed to 1500 µg/L to 38.6 %, compared to the control with 84.3 % fertilised eggs (Figure 2.8).

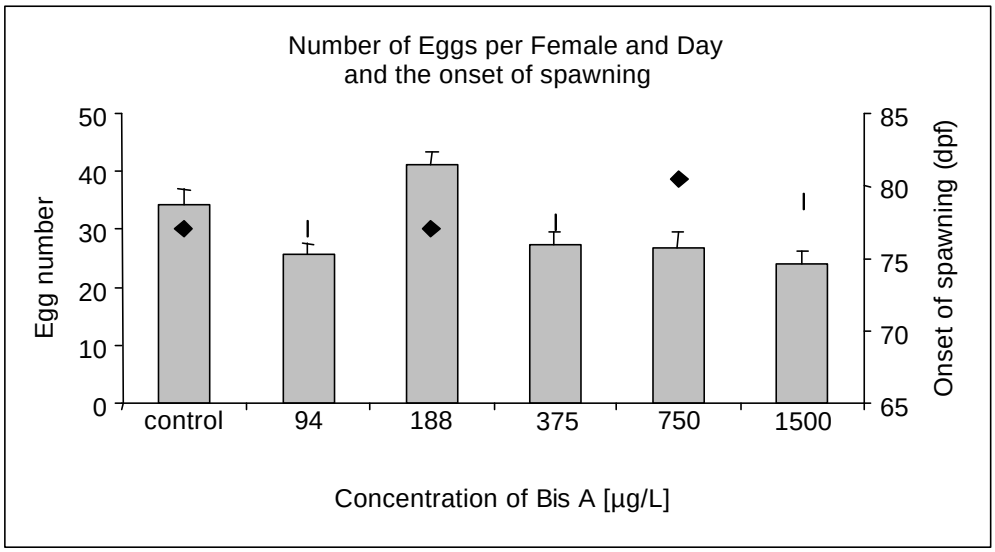

Figure 2.7: Number of eggs per female and day and the onset of spawning of zebrafish permanently exposed to different concentrations of bisphenol A
Columns: mean(± SE) number of eggs per female, pooled data from all fishes at each concentration
Rhombus: mean onset of spawning in days post-fertilisation, average of two test vessels per concentration
Data generated at the Fraunhofer Institute.

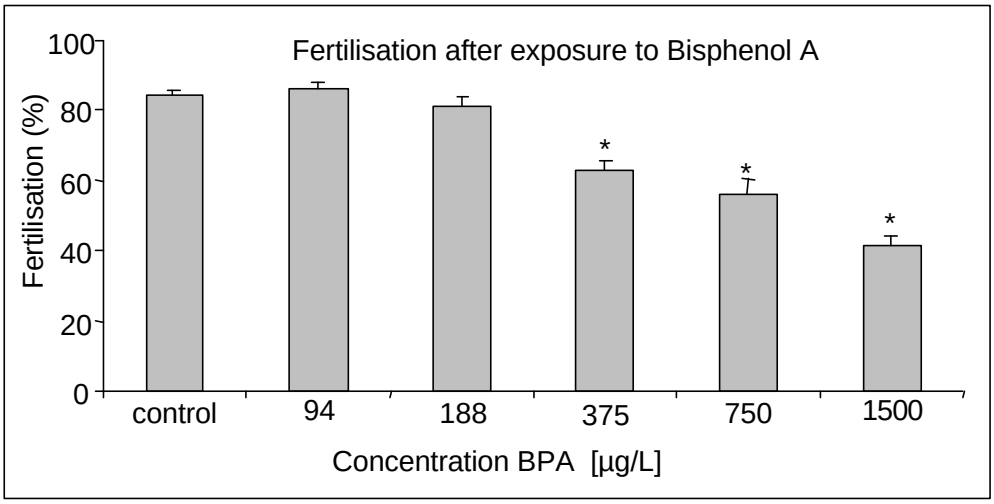

Figure 2.8: Fertilisation of zebrafish permanently exposed to different concentrations of bisphenol A.
*significant difference from the control group, $p < 0.05$; Scheffle's Test; one way ANOVA
Data generated at the Fraunhofer Institute.

Gonadal morphology

The ontogeny of gonad morphology in non-exposed zebrafish was described in detail in Chapter 1. Here we describe the alterations of gonad morphology at different ages after exposure to 3 ng/L ethynylestradiol or to different concentrations of bisphenol A. In exposed fishes cellular structures of the gonad were identical to those observed in control groups: that is, pathological alterations of gonadal structures did not occur, although, the relative frequencies of the individual developmental stages differed between treatments and controls.

Exposure to 3 ng/L EE2 over different developmental periods

321 fish exposed to 3 ng/L EE2 (97 fish aged 75 dpf, 115 fish aged 118 dpf and 109 fish at the age of 178 dpf) were histologically analysed

Ovarian morphology

75-day-old zebrafish

75-day-old zebrafish were exposed for 21 days, 43 days or continuously.

1 of the control fish and 2 fish exposed for 21 days displayed mature ovaries containing oocytes of all maturation stages, from perinucleolar oocytes up to mature oocytes. In all other dissected ovaries of 75-day-old fish previtellogenic oocytes only were found. Figure 2.9 illustrates the variation of the gonads found at this age. Two major forms of ovarian morphology with immature oocytes could be observed. Ovaries of the first form were composed almost exclusively of densely packed perinucleolar oocytes; only in the periphery of the gonadal tissue did a few nests of germ cells occur (Figures 2.9a). The second form of ovarian morphology was characterised by alterations of oocyte morphology and tissue structure. These alterations of cell and gonad morphology did not take place uniformly throughout the gonad but could be restricted to a specific part of the gonad. In the altered areas, the oocytes decreased in size, and the shape of both the whole cell and the nucleus was becoming irregular. The close contact between the oocytes was lost, so that empty spaces appeared between the oocytes. These stroma-rich gonads were usually much smaller in size than the oocyte-rich gonads (Figures 2.9c). For a detailed description see Chapter 1.

Size of perinucleolar oocytes

Fish for which the exposure was stopped on day 21 pf or day 43 pf did not differ in oocyte size from control fishes could be observed. However the diameter of the perinucleolar

oocytes in continuously exposed fish was significantly reduced compared to control fish (mean 29 mm compared to 42.7 mm in the control) (Figure 2.11a). Oocyte diameter of 71-day-old fishes exposed during the first 14 days pf to 3 ng/L EE2 was not measured.

118-days–old zebrafish

118-day-old zebrafish that had been exposed for 21 days, 43 days or continuously were evaluated

When the fish started to spawn around day 85 pf reproductive parameters were measured for 20 days. After this period, the 118-day-old fishes were fixed and gonad morphology was examined histologically. In the exposed groups, the percentage of ovaries containing immature oocytes only in the exposed groups differed significantly from the control group and was closely related to the exposure period (Table 2.1). The same pattern was found with respect to the size of the perinucleolar oocytes. The oocyte diameter was reduced, in all exposed groups. The extent of the reduction was dependent of the exposure period and was significant in the group exposed for 43 days and in permanently exposed fish (Figure 3.11).

Fish of the control group:

Only one out of 14 fish was found, to contain only immature, previtellogenic oocytes. The average oocyte diameter was 62.9 ± 2.0 mm

Fish exposed for 21 days:

4 out of 11 fish contained only immature, previtellogenic oocytes. The average oocyte diameter was 53.7 ± 2.1 mm

Fish exposed for 43 days:

7 out of 15 fish contained only immature, previtellogenic oocytes. The average oocyte diameter was 52.4 ± 1.9 mm

Fish continuously exposed:

18 out of 26 fish contained only immature, previtellogenic oocytes. The average oocyte diameter was 46.8 ± 1.9 mm

Mature ovaries in adult, exposed fish partly showed morphological changes such as areas with rather heterogeneous size distribution of oocytes as well as an increased frequency of non-germ cells. For illustration of the various ovarian types and morphological alterations see Figure 2.10. In half of the mature ovaries those morphological changes were found (Table 2.1).

176-days –old zebrafish

176-day-old zebrafish had been exposed for 21 days, 43 days or 125 days. After these exposure periods, they were transferred into non-contaminated water and kept until d 176 pf. The strong relation in oocyte diameter to the exposure period that was found on day 118 pf was no longer discernible in 176-day-old fish. At the end of the experiment no concentration-related difference of the oocyte diameter could be found in the different groups (Figure 2.11c).

In zebrafish of the control group one fish out of 12 hold immature oocytes only.

Following exposure for 21 days 6 out of 13 fish contained immature gonads

Following exposure for 43 days 8 out of 16 fish contained gonads with immature oocytes only.

Following exposure for 125 days 4 out of 19 females contained gonads with immature oocytes

In all exposed groups mature females was observed with altered areas, whereas in the control group no ovary was found with impaired areas. The percentage of ovaries with altered areas increased with the exposure period (Table 2.1).

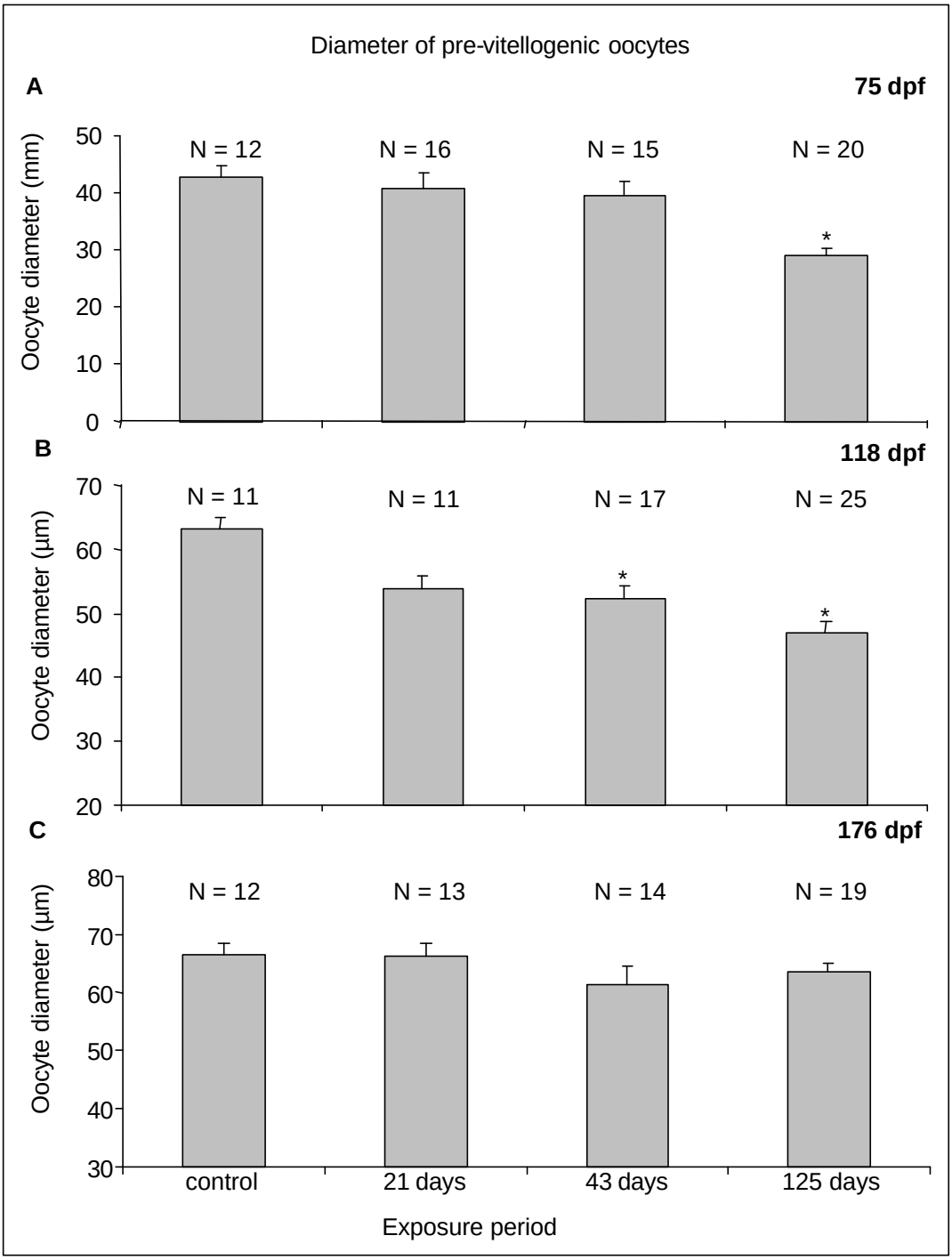

Figure 2.11: Mean sizes ± SE of pre-vitellogenic oocytes of zebrafish, exposed for different periods to 3 ng EE2, starting with fertilised eggs . Up to 50 oocytes were measured per individual female (pooled data from females of all groups in each treatment)

* significant difference to the control group; $p < 0.05$, Scheffles's Test, one-way-ANOVA

A: Day 75 pf **B**: Day 118 pf **C**: Day 176 pf **N** = number of fishes

Table 2.1: The morphological appearance of ovaries in zebrafish, exposed to 3ng/L EE2 over different periods, starting with the fertilised eggs.
***** significant different from the control group at the respective age, Chi² P < 0.05,
[1] Exposure was stopped on day 125 pf.

Age (dpf)	Exposure period (dpf)	Immature ovaries, containing premature oocytes only	Number of fish with maturing or mature ovaries	Mature ovaries only		Percentage of ovarian-like gonads (Number of total examined fish)
				Ovaries with morphologically normal appearance	Ovaries containing altered areas	
75 dpf	**control** (N = 14)	13	1	1	--	58% (24)
	21 days (N = 17)	15	2	2	--	58% (29)
	43 days (N = 15)	15	0	--	--	62% (24)
	permanent (N = 20)	20	0	--	--	100% (20)
118 dpf	**control** (N = 14)	1	13	13	0	46% (30)
	21 days (N = 11)	4	7*****	7	0	39% (28)
	43 days (N = 15)	7	8*****	7	1	50% (30)
	permanent (N = 26)	18	8*****	4	4*****	96% (27)
176 dpf	**control** (N = 12)	1	11	11	0	48% (25)
	21 days (N = 13)	6	7*****	5	2	46% (28)
	43 days (N = 16)	8	8*****	5	3*****	55% (29)
	permanent[1] (N = 19)	4	15	5	10*****	70% (27)

Figure 2.9: (page 64) Gonads of zebrafish prior to reproduction (7µm-section) The pictures demonstrate the wide range of zebrafish gonad development under exposed as well as under unexposed conditions. Oocytes were staged according to Selman et al. (1993). For details see text and Chapter 1.

A: Part of a premature ovary of an 75-dpf old fish permanently exposed to 3ng/L ethynylestradiol (EE2). The ovary contains previtellogenic oocytes (p**oc**) in the resting stage. As long as they are not reproductive, ovaries contain oocytes in the follicle phase (stage 1b) as the maximum developmental stage. The ovary is attached to the liver (**L**).

B: Part of a mature ovary of an 75-dpf control fish. All oocyte developmental stage are visible, beginning from previtellogenic oocytes (**Poc**) up to mature oocytes (**moc)**. **coc**: cortical alveolus stage oocytes, **voc:** vitellogenic oocytes

C: Part of an ovary with altered morphology, exposed for 21 days to 3 ng/L EE2. The oocytes (**do**) have begun to degenerate. Non-germ-tissue (**ngt**) is visible in the inner part of the gonad. For a detailed description see Chapter 1.

D: Premature testis of an 57 dpf old zebrafish, permanently exposed to 750 µg/L Bisphenol A (BPA). Spermatogonia (**sg**); spermatocytes (**sc**) and spermatids (**spd**) are already visible in addition to the lumen (**lu**).

E: Part of a mature testis, containing male germ cells at all developmental stages. (77 dpf, exposed for 43 days to 3 ng/L EE2). spermatozoa (**sz**); spermatids (**spd**) spermatocytes (**sc**) and spermatogonia (**sg**).

F: Testis of a 75-dpf old **z**ebrafish, permanently exposed to 1500 µg BPA. Beside the male germ cells several degenerated oocytes (**do**) are visible. In contrast to a altered ovary germ cells only were found inside the gonad; spermatids (**spd**) spermatocytes (**sc).**

Figure 2.10: (page 65) Gonads of zebrafish at the end of the experiments, illustrating the different appearances of zebrafish gonads, exposed to estrogens with different exposure regimes. The frequency of the different forms were shown in Figure 2.9 and 2.10, and in Tables 2.2 to 2.5. (7µm-section)

A: Part of an ovary of a 176-dpf old zebrafish, exposed for 125 days to 3 ng/L EE2. All oocyte developmental stages are visible, beginning from previtellogenic oocytes (**poc**) up to mature oocyte (**moc)**. **coc**: cortical alveolus stage oocytes, **voc:** vitellogenic oocytes

B: Another part of the same ovary as described in A. In this area all oocytes at the various developmental stages are degenerated (**do**).

C: Both gonads of a 110-dpf old fish permanently exposed to 750 µg/L BPA. In both gonads mature male as well as mature female germ cells were visible. The vitellogenic oocytes (**voc**) as the most developed stage were degenerated. **poc:** previtellogenic oocytes, (**coc**) cortical alveolus stage oocytes. It was not be clear from histological examination whether this fish had spawned.

D: Part of an ovary of a 176-dpf old fish, exposed for 43 days to 3 ng/L EE2. This fish did not take part in reproduction. The ovary contained previtellogenic oocytes (**poc**) only.

E: Mature testis of an control fish, age: 176 days. Germ cells at all developmental stages were seen. spermatozoa (**sz**); spermatids (**spd**) spermatocytes (**sc**) and spermatogonia (**sg**).

F: Mature testis of a176-dpf old fish exposed for 125 days to 3 ng/L EE2. In one part of the testis several degenerated oocytes (**do**) were found, but the other part was full of spermatozoa (**sz**).

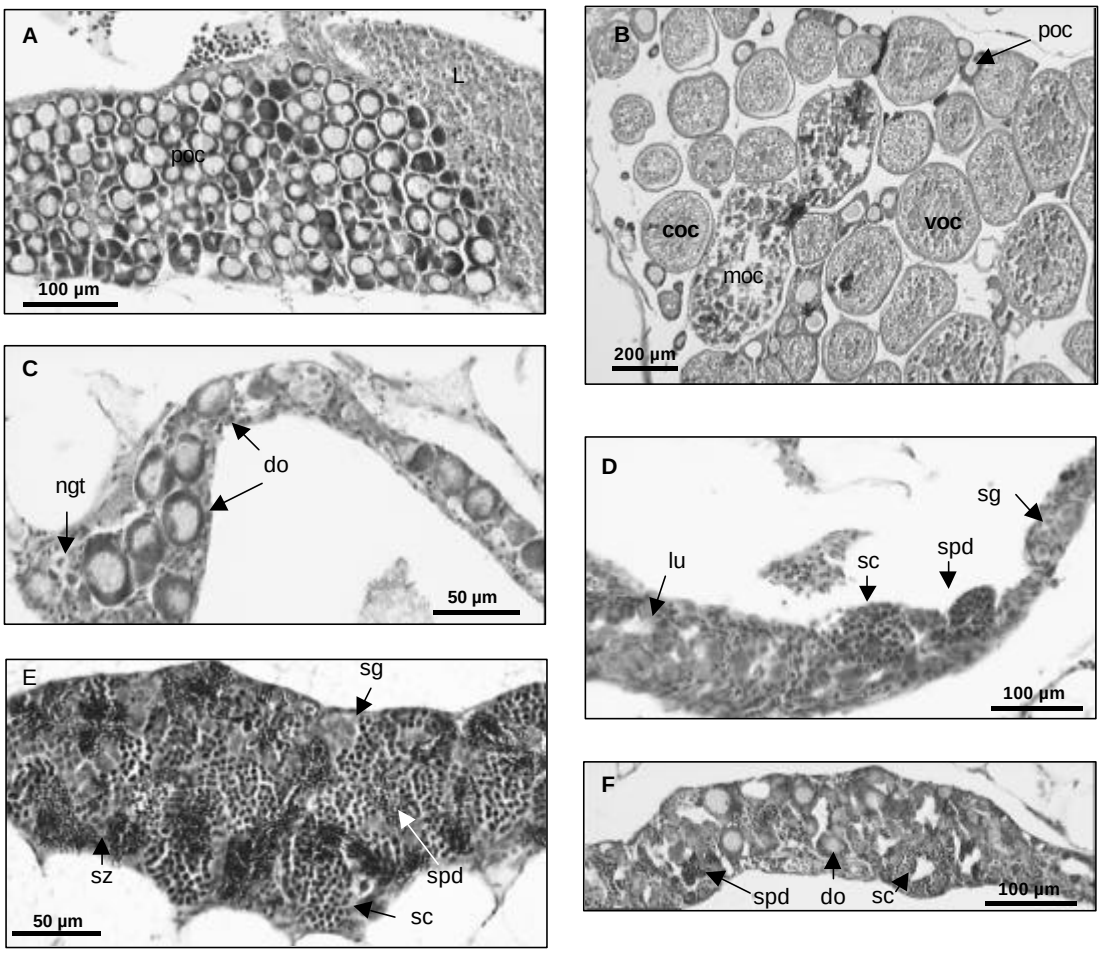

Figure 2.9: Legend see page 63

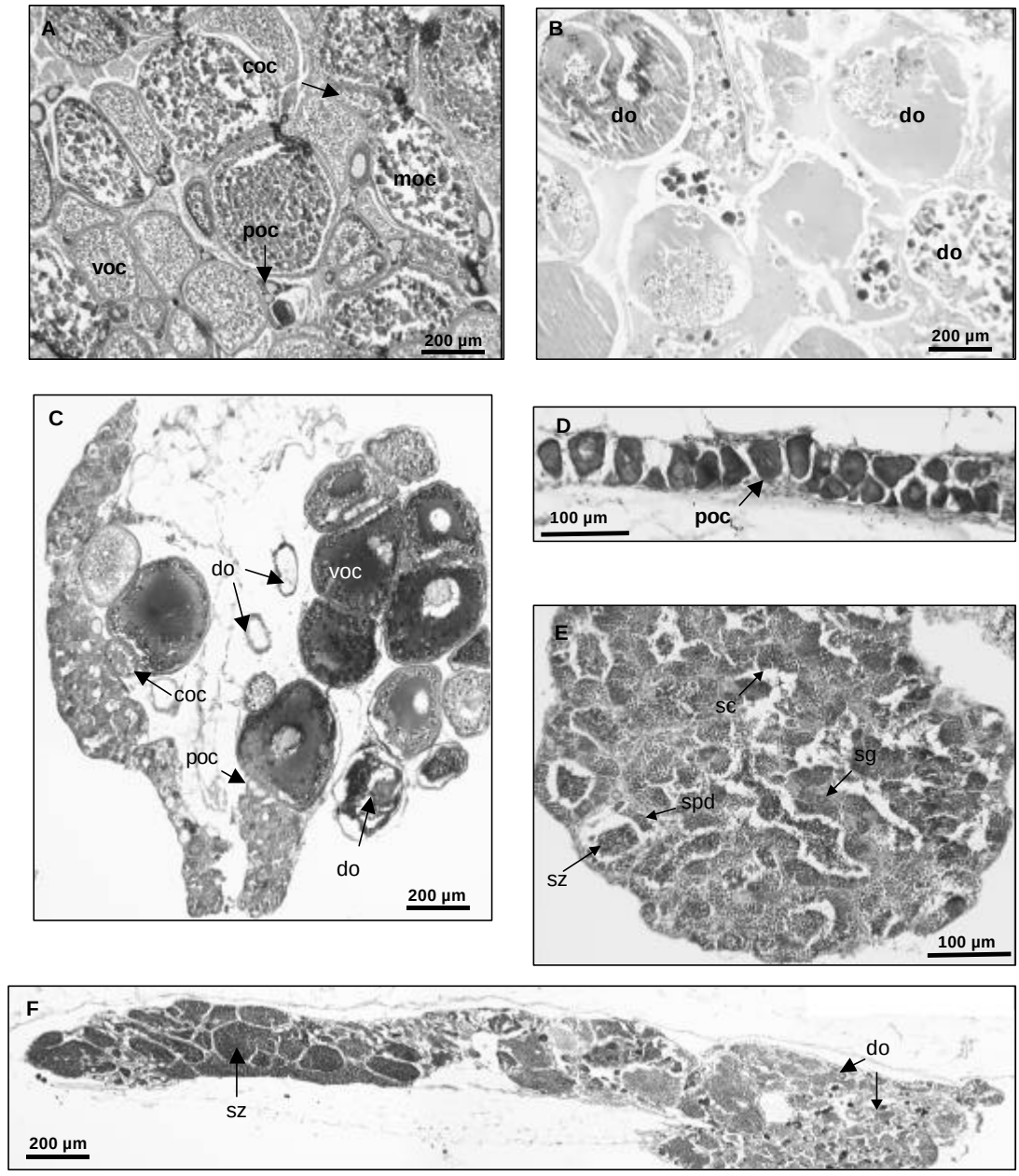

Figure 2.10: Legend see page 63

Testes Morphology

Fish exposed to 3 ng/L EE2 during different developmental periods

75-day-old fish

75-day-old zebrafish were exposed for 21 days, 43 days or continuously

On day 75 pf the increase of the percentage of immature testes was correlated with exposure period (Table 2.2).

In control fish 4 out of 10 fish contained immature testes.

Following exposure for 21 days 6 out of 10 fish contained immature testes.

Following exposure for 43 days 7 out of 9 fish contained immature testes.

In permanently exposed fish no fish with testes were found at all.

In testes of 75-day old zebrafish no effect of EE2 on the proportion of the different sex cell types could be found (Figure 2.12a)

118-day-old fish

118-day-old zebrafish that had been exposed for 21 days, 43 days or continuously were evaluated

On day 118 pf all males in all groups had sperm-containing testes and no EE2-related effect on the proportion of the different sex cell types was detectable (Figure 2.12b).

176-day-old fish

176-day-old zebrafish had been exposed for 21 days, 43 days or 125 days. After these exposure periods, they were transferred into non-contaminated water and kept until day 176 pf.

At the end of the experiment on day 176 pf all males in all groups had sperm-containing testes and again no EE2-related effect on the proportion of the different sex cell types could be found (Figure 2.12c).

Degenerated perinucleolar oocytes were found in only 5 out of 112 dissected testes from all groups and ages including the control group.

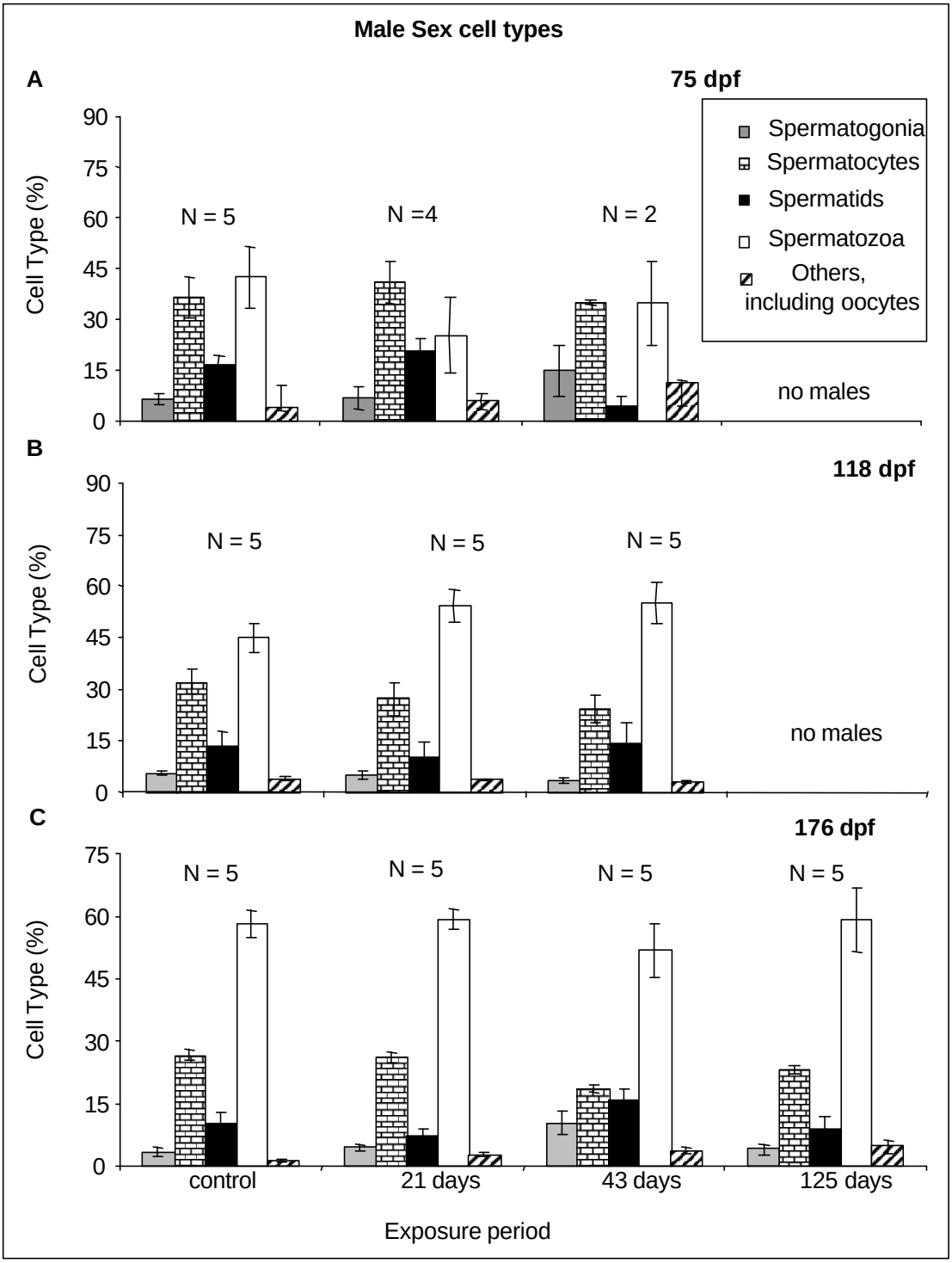

Figure 2.12: Testicular cell types in zebrafish, exposed to 3 ng EE2 for different exposure periods, starting with fertilised eggs. Values are means ± SE (pooled data from males of all groups in each treatment)

N: number of fishes. **A**: Day 75 pf **B**: Day 118 pf **C**: Day 176 pf

Table 2.2: The maturity stages of the testes of zebrafish exposed to 3ng/L EE2 over different periods, starting with the fertilised eggs. Testes determined as immature as long as they contained no sperm. An individual fish can contain both sperm and oocytes in one and the same testis. [1] Exposure was stopped at day 125 pf.

Age	exposure period (dpf)	Immature testes	Sperm-containing testes	Testes containing oocytes	Percentage of testes-like gonads (Number of total examined fish)
75 dpf	**control** (N = 10)	4	6	1	42% (24)
	21 days (N = 10)	6	4	2	42% (29)
	43 days (N = 9)	7	2	0	38% (24)
	permanent (no testes)	--	--	--	0% (20)
118 dpf	**control** (N = 15)	0	15	0	54% (30)
	21 days (N = 17)	0	17	0	61% (28)
	43 days (N = 12)	0	12	1	50% (30)
	permanent (no testes)	--	--	--	4% (27)
176 dpf	**control** (N = 9)	0	7	0	52% (25)
	21 days (N = 11)	0	11	0	54% (28)
	43 days (N = 12)	0	12	0	45% (29)
	permanent (N = 7)	0	7	2	30% (27)

Gonad type

75-day-old fish

On day 75 pf the percentage of the fish containing ovary-like gonads and testis-like gonads was statistically not different in fish that had been exposed for the first 21 days, for the first 42 days and in control fish (Figure 2.13a). In the group exposed for 21 days two fish with altered ovarian morphology were found. Both fish had a total length of 21 mm, which is below the mean of 27.9 mm TL in that group as seen in Figure 2.1. In fish exposed to 3 ng EE2/L continuously from fertilization to 75 dpf, all 20 dissected fish contained ovaries.

118-day-old fish

Whereas in the groups exposed for 21 days and 42 day no significant difference to the control group could be observed in the permanently exposed fish again ovary-like gonads only could be found. One fish was found to contain both maturing male and non-atretic female germ cells (Figure 2.13b).

176-day-old fish

When stopping the exposure had been stopped on day 125 pf, at the end of the experiment (on day 176 pf) no difference in the gonad types from the control group could be observed in any of the exposed groups (Figure 2.13c).

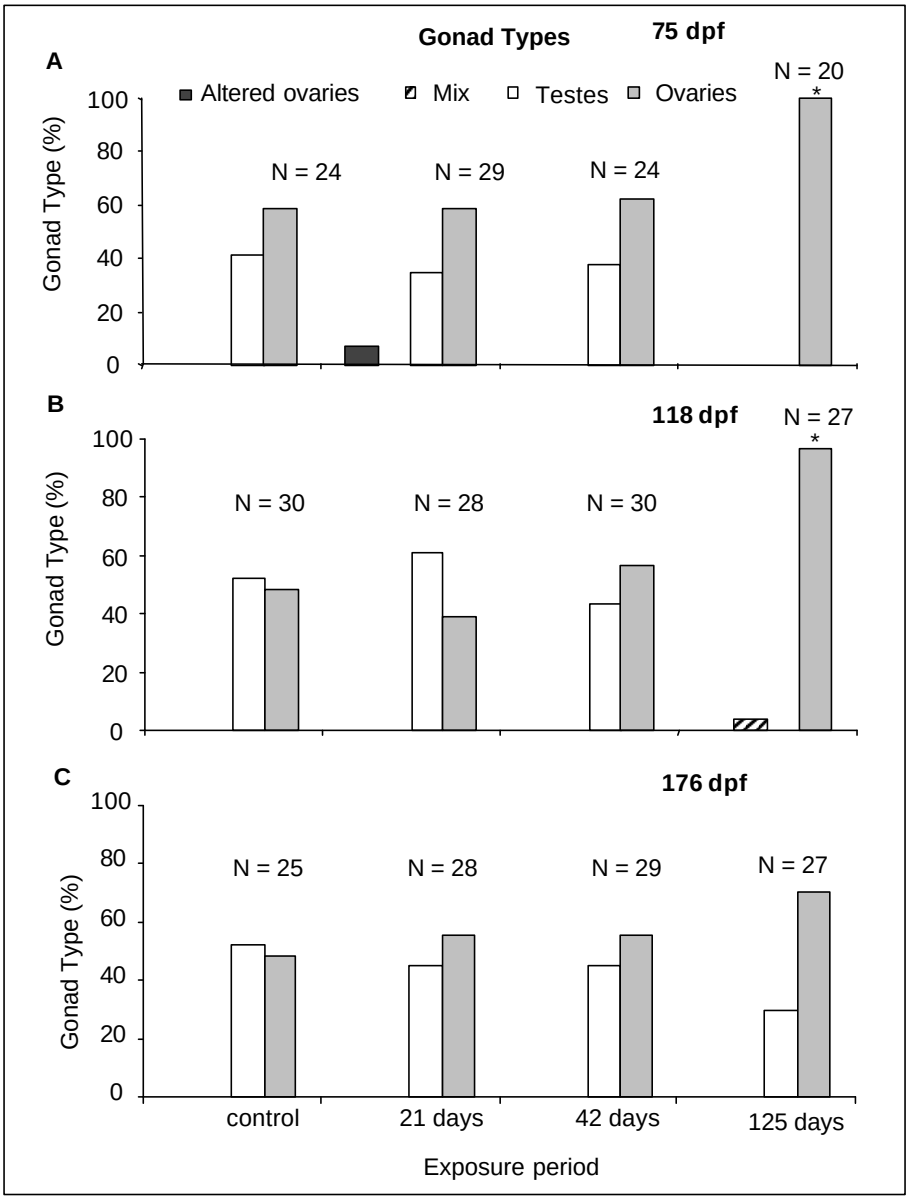

Figure 2.13: Relation of the different gonad types in zebrafish at different age after exposure to 3ng/L EE2 of different exposure periods, starting with fertilised eggs. Exposure was stopped at day 125 pf.
A: 75 dpf old zebrafish **B**: 118 dpf old zebrafish; **C**: 176 dpf old zebrafish
N: Number of fishes * significant difference to the control, $p < 0.05$; Chi^2-Test

Exposure to different concentrations of BPA

158 fish exposed to BPA (48 fish aged 75 dpf and 120 fish aged 110 dpf) were histologically analysed. Due to technical reasons samples at the concentrations of 94 µg/L and 188 µg/L were not available

Ovarian morphology

Fish were continuously exposed to the BPA concentrations of 94, 188, 375, 750 and 1500 µg/L.

75-day-old zebrafish

As summarised in Table 2.3 the majority of the ovaries of the 75 pf old fish hold premature oocytes only. The only exception were found in females exposed to 375 µg/L BPA. In this group 7 out of 11 female zebrafish had ovaries, containing maturing or mature oocytes.

No difference in the oocyte diameter could be found between the single affected group and the control on day 75 pf (Figure 2.14a).

110-day-old zebrafish

At the end of the experiment all fish without any exception took part in the reproduction.

No difference in the oocyte diameter could be found (Figure 2.14b).

Some mature ovaries in adult, exposed fish showed the same morphological changes as described above in the EE2 exposed fish such as areas with rather heterogeneous size distribution of oocytes as well as an increased frequency of non-germ cells. For illustration of the various ovarian types and morphological alterations see Figure 2.10. At the two highest concentration the percentage of fishes with those morphological changes differed significantly from the control group (table 2.3).

In control fish 2 out of 19 fish displayed such morphological changes.

Following exposure to 375 µg/L BPA 3 out of 23 fish displayed such morphological changes.

Following exposure to 750 µg/L BPA 12 out of 23 fish displayed such. morphological changes.

Following exposure to 1500 µg/L BPA 10 out of 23 fish displayed such morphological changes.

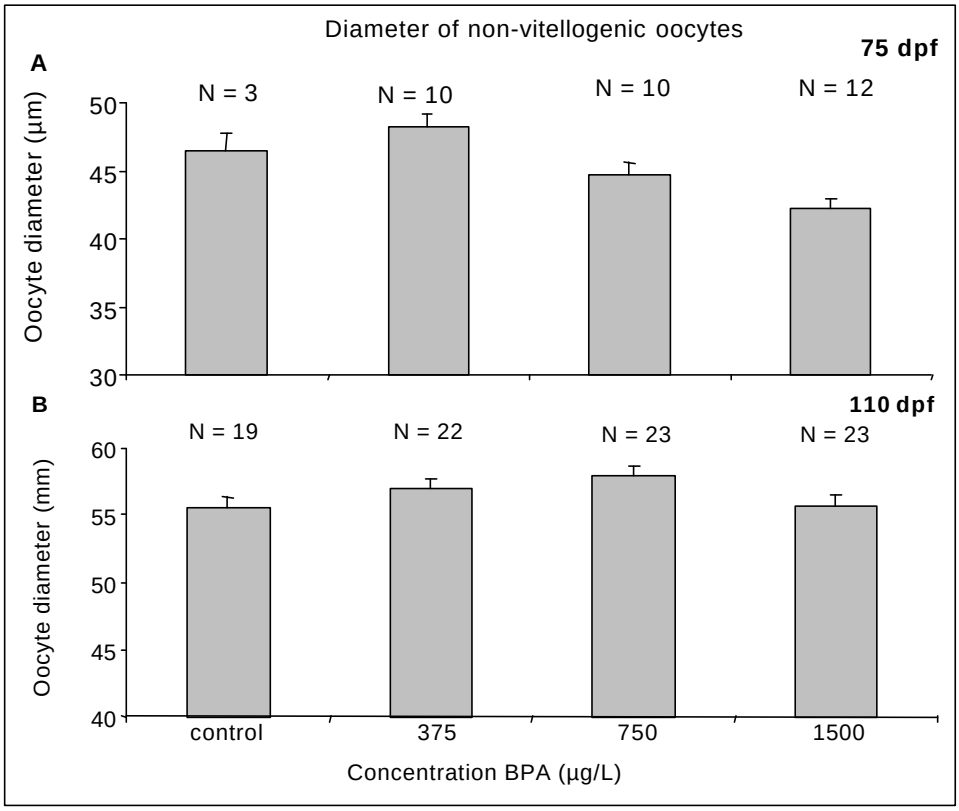

Figure 2.14: Mean sizes ± SE of pre-vitellogenic oocytes of zebrafish, exposed to different concentrations of Bisphenol A . Up to 50 oocytes were measured per individual female (pooled data from females of all groups in each treatment) **N** = number of fishes
A: Day 75 pf **B**: Day 110 pf

Table 2.3: The morphological appearance of zebrafish ovaries, permanently exposed to graded concentrations of BPA, starting with the fertilised eggs. ***** significantly different from the control group at the respective age, Chi² $P < 0.05$.

Age (dpf)	Concentration BPA/L	Immature ovaries, containing immature oocytes only	Number of fish with maturing or mature ovaries	Mature ovaries only		Percentage of ovarian-like gonads (Number of total examined fish)
				healthy ovaries	ovaries containing impaired parts	
75 dpf	**control** (N = 3)	2	1	1	0	38% (8)
	375 µg (N = 11)	4	7	7	0	79% (14)
	750 µg (N = 10)	10	0	--	--	77% (13)
	1500 µg (N = 12)	11	1	1	0	92% (14)
110 dpf	**control** (N = 19)	0	19	17	2	66% (29)
	375 µg (N = 22)	0	22	19	3	70% (31)
	750 µg (N = 23)	0	23	11	12	79% (28)
	1500 µg (N = 23)	0	23	13	10	79% (29)

Testes morphology

Fish were continuously exposed to the BPA concentrations 94, 188, 375, 750 and 1500 µg/L

A proportion of the 75-day old zebrafish hold sperm-containing testes. The small amount of fish excluded further analysis.

At the end of the experiment at day 110 pf all males had sperm containing testes. Degenerated perinucleolar oocytes were found in only 3 out of 43 dissected testes only in all groups and ages including the control group (Table 2.4)

Male sex cell types

No effect of BPA on the proportion of the different sex cell types in the testes of zebrafish could be found (Figure 2.15).

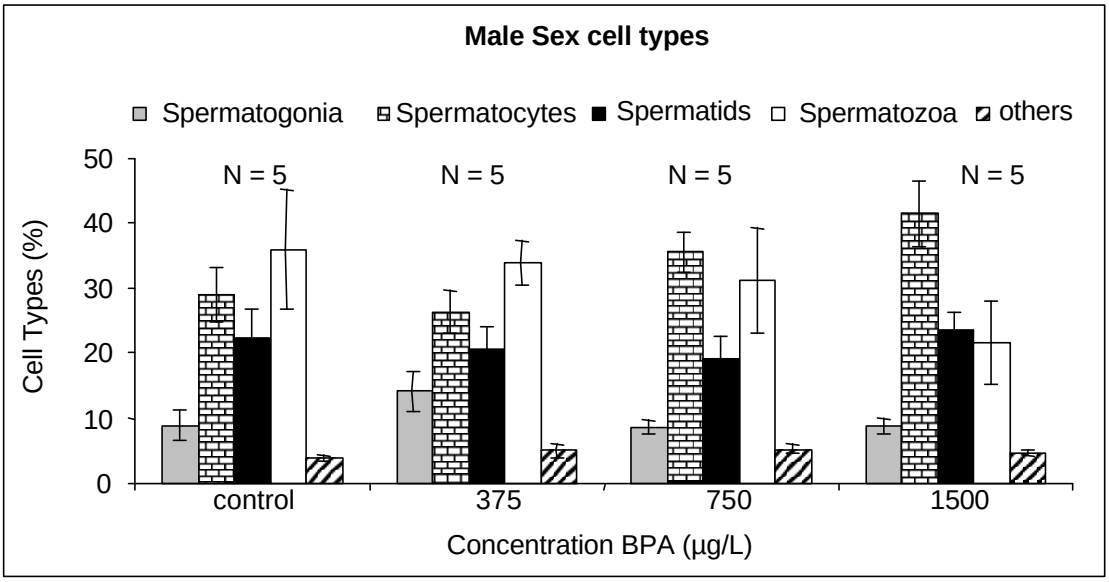

Figure 2.15: Testicular cell types in zebrafish, exposed to different concentrations of Bisphenol A for 110 days, starting with fertilised eggs. Values are means ± SE (pooled data from males of all groups in each treatment)
N: number of fishes.

Table 2.4: The maturity stages of the testes of zebrafish permanently exposed to graded concentrations of BPA starting with the fertilised eggs. Testes were determined as immature as long as they contained no sperm. An individual male can contain both sperm as well as atretic oocytes in one and the same testis

Age	Concentration BPA/L	Immature testes	Sperm-containing testes	Testes containing oocytes	Percentage of testes-like gonads (Number of total examined fish)
75 dpf	**control** (N = 5)	2	3	0	62% (8)
	375 µg (N = 3)	2	1	0	21% (14)
	750 µg (N = 3)	2	1	0	23% (13)
	1500 µg (N = 1)	1	0	1	8% (14)
110 dpf	**control** (N = 10)	0	10	0	34% (29)
	375 µg (N = 9)	0	9	1	30% (31)
	750 µg (N = 5)	0	5	0	21% (28)
	1500 µg (N = 6)	0	6	1	21% (29)

Gonad type

No difference in the relation of the different gonad types could be observed for any concentration (Figure 2.16a)

No difference in the relation of the different gonad types was observed for any concentration (Figure 2.16b). One fish was found containing both maturing male and female germ cells in the group exposed to 750 µg/L at day 110 pf.

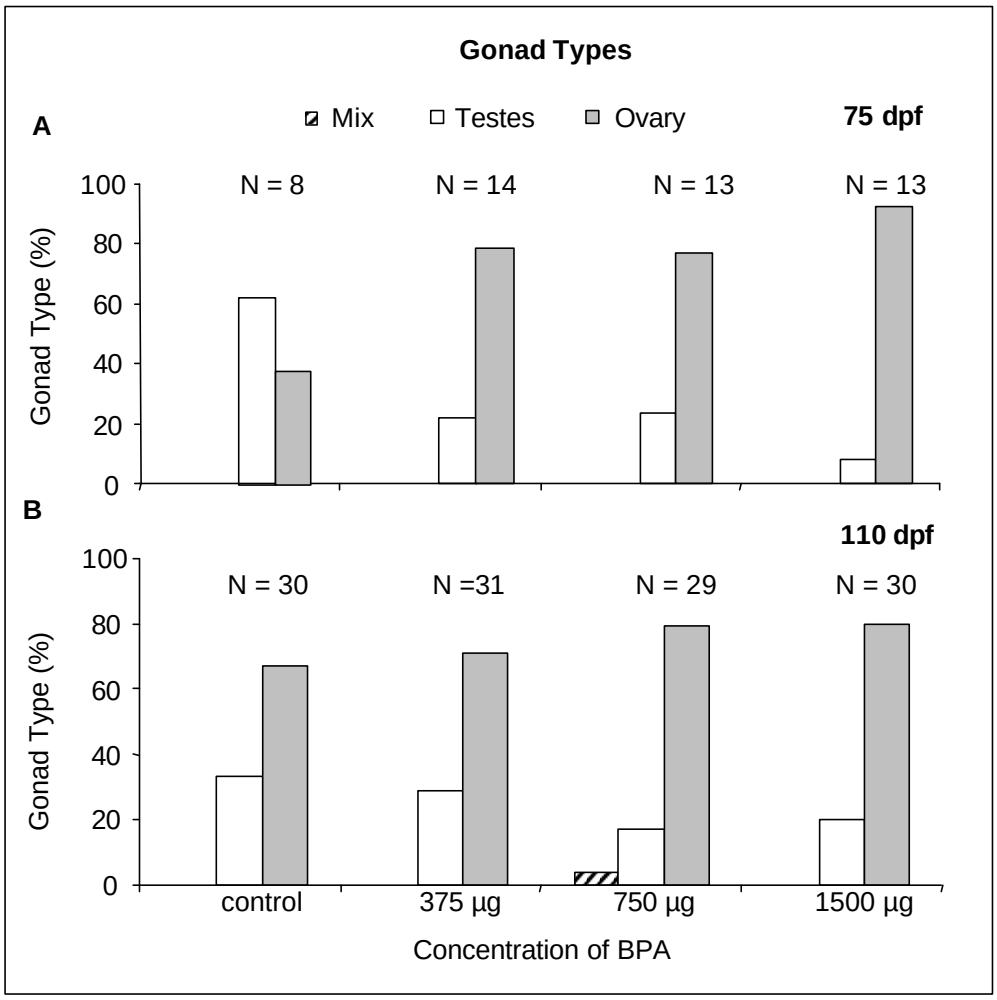

Figure 2.16: Relation of the different gonad types in zebrafish after permanent exposure to different concentration of Bisphenol A
A: 75 dpf old zebrafish **B**: 110 dpf old zebrafish
N: Number of fishes

Discussion

The aim of this study was to determine how gonadal development was effected by chronic exposure to estrogens and to compare the histological findings with reproductive alterations. Permanent exposure to environmentally realistic concentration of EE2 had a strong impact on gonad development. As long as the zebrafish were exposed ovaries only were found. Not before the exposure had been stopped, zebrafish containing testes were seen. And this observation was not correlated to the exposure period. This could be a statistical error due to the small sampling size, but more probably indicating a remaining of the protogynic stage of development of the genetic males. It seemed that the transformation from female to male gonads could continue only after termination of the exposure. This was supported by own experiments (Chapter 3).

After exposure had been stopped, the number of females that contained immature ovaries at the end of the experiment, and did not participate in reproduction, was related to the exposure period. These alterations in gonad morphology affects the reproductive success, as is evident in several population-relevant endpoints such as time to the onset of spawning, fertilisation, and the number of eggs laid by females.

BPA caused effects in reproductive parameters only at concentrations of 375 µg/L and higher, which more than a magnitude greater than the 25 µg/L found in aquatic environment (Staples et al. 1998)

Gonadal development

No zebrafish containing testes were found as long as they were exposed to 3 ng/L EE2 and consequently reproduction in zebrafish exposed to 3 ng/L EE2 can occur only after exposure had been stopped. This study confirmed the results of former experiments in which zebrafish were exposed to 10 ng/L EE2 (Segner et al. 2002) and is in agreement with the studies of Legler et al. (2001). As long as the fish were exposed, only individuals containing ovaries were found, but after a recovery period males also appeared. Weber et al. (2001) also observed in 60-dpf old zebrafish permanently exposed to 10 ng/L EE2 and 100 µg/L nonylphenol respectively also. Lower concentrations or weaker estrogens like bisphenol A allow male to develop as shown in the present study and confirmed by Weber et al. (2001), and Legler et al. (2001). In case of ethynylestradiol it may be speculated that the assimilation of external EE2 substituted for the falling endogenous estrogen level in genetic males at the beginning of the sexual differentiation, which prolonged the period of juvenile hermaphroditism in exposed fish and therefore postponed the onset of mating. This thesis

was supported by Fenske (2002) who found a vitellogenin induction in exposed males but not after stopping the exposure.

For the fathead minnow Länge et al. (2001) mentioned that the percentage of males following exposure to 1.0 ng/L EE2 decreased; there was an absence of males in fish exposed to 4.0 ng/L. The authors also found an increasing number of fish containing ovotestes. It should be pointed out, that the fathead minnow has a gonochoristic gonadal development (van Aerle, pers. comm.), in which ovaries and testes develop directly from the undifferentiated gonad. For the medaka, which also is a species with a gonochoristic gonadal development, Papoulias et al. (2000) were able to induce a sex reversal following an injection of 0.5 or 2.5 ng/egg, but they found no intersex. This was confirmed by Scholz and Gutzeit (2000) when they exposed juvenile medaka to 100 ng/L EE2: all genetic males developed immature ovaries. The explanation presented by Shibata and Hamaguchi, (1988) involved the sexual bipotentiality of the spermatogonia. These authors exposed adult male medaka to estradiol, and depending on the exposure period, found ovotestes or ovaries. These effects were reversible as testes reappeared after fish had had the opportunity to recover in tap water. Sohoni et al. (2001) found none of these effects in the fathead minnow after exposure to BPA. None of the testes examined by the authors contained oocytes.

This contrasts with the juvenile hermaphroditism in the zebrafish, where all gonads first differentiate into ovaries. In our experiments single perinucleolar oocytes were found in a small percentage (less than 5 %) in sperm-containing, reproductively active, testes distributed over all ages and concentrations. This could be interpreted in terms of the genesis of the testis. As male zebrafish are going through the period of juvenile hermaphroditism it is quite possible that occasional oocyte is not absorbed completely for some reason.

The absence of testes could have different reasons in a gonochoristic species than in protogynic hermaphrodite like the zebrafish. In the latter it is quite possible that the absence of males was caused by a retardation of the transforming from female to male gonads. In gonochoristic fish the not existing males are probably due to a phenotypic sex reversal.

Histological alterations in female zebrafish

Female zebrafish exposed to EE2 did not show exposure-related alteration in ovarian maturation on day 75 pf. On day 118 after reproduction parameters were determined the percentage of females containing immature oocytes only increased with the exposure period. In the permanently exposed fish less than 30 % of the females contained maturing oocytes. This can be explained by the absence of males. Mating behaviour is the cue for female egg

production and therefore for oocyte maturation, as demonstrated in other small tropical fishes such as *Encrasicholina heteroloba* (Wright 1992), *E. punctifer* (Maack and George 1999), *Engraulis ringens* (Alheit 1984) and *E. encrasicolus ponticus (Arianov et al., 1996).* This effect seemed not to be reversible. At the end of the experiment after a recovery period of a minimum of 50 days still a high percentage of the females were still not participating in reproduction. It may be speculated that those fish are not retarded females, but genetic males, that did not transform into functional males; however without the possibility to identify sex-specific genes in the zebrafish, this hypothesis can not be clarified. The retardation of oocyte development in the permanently exposed fish is also seen in the size of the pre-vitellogenic oocytes. The size of the oocyte is a sign of maturation, as described in detail by Selman et al. (1993). A percentage of the mature ovaries contained disturbed areas. The number of females exhibiting such deficiencies was correlated with the exposure period. These disturbed areas are the only effect visible in mature females exposed to BPA., and only if the concentration was to 750 or 1500 µg/L BPA. As seen also in fertilization, for the production of effects in the ovaries a threshold concentration seemed to be necessary. Besides the direct estrogen effect on the ovaries another explanation could be the incomplete mating behaviour of the male zebrafish, indicated by the diminished fertilization.

Histological alterations in male zebrafish

In male zebrafish exposed to EE2 on day 75 pf a retardation of testicular development was visible, which correlate with the exposure period. On day 118 pf, after spawning and on day 176 at the end of the experiment, all male zebrafish were reproductively active. In males exposed to BPA no such retardation could not be found. Nor was a correlation between the exposure concentration and the proportion of the different male germ cells discernible, after exposure to either 3 ng/L EE2 or BPA. This is in contrast to Sohoni et al., (2001), who found that in the fathead minnow an effect on the proportion of the different sex cell types in the testes of males was affected, with a positive concentration-related effect of BPA on the proportion of spermatogonia and an inhibitory effect on the proportion of the testis occupied by spermatozoa. This finding was corroborated by Weber et al. (2001), who found significantly more acellular areas in zebrafish testes with a larger proportion of spermatogonia after estrogenic exposure. Miles-Richardson et al. (1999) reported an increase of Sertoli cells in testes of male fathead minnows following an exposure to 17-â estradiol. One of the major functions of Sertoli cells is to phagocytize germ cells that degenerate in the normal course of spermatogenesis (Le Gac and Loir 1998)Hence Miles-Richardson et al. (1999) speculated that the histological lesions were due to an arresting of

in germ cell maturation with subsequent degeneration of spermatozoa. This effect was reversible, as Sertoli-cells lesions disappeared after exposure was discontinued.

As described above histological alterations are not found in mature males, so the reason for the reduced fertilization observed in this study does not reside in gonad morphology. Reduced fertilisation may have different causes: effect on released sperm only, or on the eggs by changing the egg membrane to the effect that penetration of intact spermatozoa is hindered or prevented (Bresch et al. 1990). This could not be resolved in these studies.

Male mating behaviour is able to recover after a period without exposure, but fertilisation is obviously not. This result confirmed the experiment of Schäfers et al (2002) for permanent exposure to 10 ng/L EE2. Normal mating behaviour was observed following an appropriate period of recovery but the fertilisation was still significantly reduced. The time the exposed fish needed for recovery was even longer and the reproductive performance lower compared to the fish exposed to 3 ng/L EE2 (Schäfers et al. 2002, Segner et al., 2002). Scholz and Gutzeit (2000) found a reduced reproduction after exposure of juvenile medaka to 10 and 100 ng/L EE2, but not after exposure to lower concentrations. In fathead minnows, exposed for up to 301 days to EE2 Länge et al. (2001) reported no reduction in reproductive parameters at concentration below 4 ng/L and a total failure of reproduction at 4 ng/L and above.

Exposure to BPA demonstrated a threshold concentration in the reduction of the fertilisation. Only at concentrations of 375 µg/L and higher was fertilization significantly reduced. Sohoni et al. (2001) found a reduction in the reproductive output after exposing fathead minnows to only 1280 µg/L BPA and Shioda and Wakabayashi (2000) reported a significant decrease in the egg number after exposure medaka at 10µmol/L, the highest concentration tested.

It should be pointed out that the zebrafish is a different spawning type than the medaka and the fathead minnow, which makes it difficult to compare the reproductive output of the three species. Medaka and fathead minnow are pair-breeding fishes. For measuring the reproductive performance one male medaka and two females were kept together (Shioda and Wakabayashi, 2000) and for the fathead minnow single pairs were transferred to breeding tanks (Sohoni et al., 2001). On the other hand, the zebrafish is a group spawner, so that up to 30 individuals were kept together for measuring the reproduction. A reproductive failure of a single individual could easily be masked by other fish of the same sex. This is not possible in pair-breeding fishes and could lead to an high variability as mentioned by Sohoni et al. (2001).

Due to the nature of reproduction, estrogen-related reproductive effects can be found not earlier than in mature fish, whereas effects on gonadal development are seen in immature fish, before the onset of spawning already on day 75 pf (this study) and vitellogenin induction (VTG) even earlier at day 42 pf (Fenske 2002). On the other hand, fertilization appears to be is a more sensitive parameter than gonad histopathology and longer lasting than vitellogenin induction.

Only the combination of VTG, gonad histopathology and reproductive success provides a more complete understanding of the estrogenic potency of a potential test substance in a life-cycle test. As VTG can detect estrogenic effects earlier than gonad histology and reproduction, it can act as a reliable indicator of current estrogenic exposure. If the estrogenic potency of a substance were thus detected, the life-cycle test could easily be prolonged and extended to measure the reproductive success of the exposed population and to use histopathology for analysis of altered gonad morphology.

Conclusions

Permanent exposure to the environmental realistic concentration of 3 ng EE2 completely inhibited reproduction. This effect – absence of male in continuously estrogen-exposed fish maybe explained by the inability of developing genetic males to transform under the influence of EE2 their protogynic ovaries into testes. This does obviously not reflect a feminization of genetic males but a retarded differentiation under exposure to estrogen as controls do during the period of gonadal sexual differentiation the influence of EE2. Only after the stopping of the exposure, the transition from females to male gonads was possible, however fertilization and number of eggs were still reduced compared to controls. Stopping the exposure before the period of sexual differentiation did not cause effects on gonadal irreversible effects.

BPA caused effects in fertilization only and at concentrations more than a magnitude higher than those found in aquatic environment.

Acknowledgement

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References

Alheit, J., (1984). "Spawning frequency and sex ratio in the Peruvian anchovy, *Engraulis ringens*", *Calcofl Rep*. **25**, 43-52.

Andersen, L., Bengtsson, B.-E., Björk, M., Gessbo, A., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K. L., Lundgren, A., Petersen, G. I., Steinholz, A., and Örn, S., (2000). "Zebrafish for Testing Endocrine Disrupting Chemicals", Chemical Nordic council, Copenhagen. TemaNord 555.

Arcand-Hoy, L. D. and Benson, W. H., (1998). "Fish reproduction: an ecologically relevant indicator of endocrine disruption", *Environmental Toxicology and Chemistry* **17** (1), 49-57.

Arianov, D. P., Lisovenko, L. A., Bulgakova, Yu, and Oven, L. S., (1996). "Reproductive Ecology of the Black Sea Anchovy (*Engraulis encrasicolus ponticus*): 1. Cicadian Rhythms of Oogenesis and Spawning", *Journal of Ichthyology* **36** (4), 514-526.

Böhmer, W., Kurzawa, B.: Bestimmung von 17α-Ethinylestradiol mittels GC/MS/MS nach SPE-Extraktion an EMPORE disks, *GIT Labor-Fachzeitschrift*, 10/2000.

Bond, G.P.; McGinnis, P.M.; Cheever, K.L.; Harris, S.J.; Platnik H.B. and Neimier, R.W. (1980): Proceedings from the 19th Annual Meeting of the Society of Toxicology, Washington, D.C.

Bresch, H., Beck, Ehlermann, D., Schlazus, H., and Urbaneck, M., (1990). "A long-term Toxicity Test comprising reproduction and growth of Zebrafish with 4-Chloroaniline", *Arch. Environ. Contam. Toxicol.* **19**, 419-429.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M., (1998). "Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening", *Environmental Science & Technology 32* (11), 1549-1558.

Feldman, D. and Krishnan, A., (1995). "Estrogens in unexpected places: possible implications for researchers and consumers", *Environ Health Perspect Supplements* **7**, 129-133.

Fenske, M. (2002). "Developmental exposure of zebrafish (*Danio rerio* Hamilton-Buchanan, 1822) to endocrine active substances: Impact on VTG, aromatase and sexual differentiation" PhD-thesis at the University of Halle-Wittenberg. Germany

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E. J., Rycroft, R., Sumpter, J. P., and Tylor, T., (1996). "A survey of estrogenic activity in United Kingdom inland waters", *Environmental Toxicology and Chemistry* ***15*** (11), 1993-2002.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, M., Sumpter, J. P., Taylor, T., and Zaman, N., (1997). "Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout", *Environmental Toxicology and Chemistry* **16** (3), 534-542.

Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., and Sumpter, J. P., (2001). "Effects of the synthetic estrogen 17α-ethynylestradiol on the life-cycle of the fathead minnow (*Pimephales promeales*)", *Environmental Toxicology and Chemistry* **20** (6), 1216-1227.

Le Gac, F. and Loir, M. (1998). "Male Reproductive System, Fish" in: Knobil, E. and Neill, J.D. (Eds). *Encyclopedia of Reproduction*, **3**, 20-30.

Legler, J., van der Zee, M., Lanser, P., Jonkers, C. C. H., van der Ven, L., Vethaak, A., van der Burg, B., and Murk, A. J., (2001). "Biological validation of the transgenic zebrafish assay: Effects of (xeno-)estrogens on sexual differentiation and male reproduction", in: Proceedings of the 22th annual meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, USA .

Maack, G. and George, M. R., (1999). "Contributions to the reproductive biology of *Encrasicholina punctifer* Fowler, 1938 (Engraulidae) from West Sumatra, Indonesia", *Fisheries Research*, **44** (2), 113-120.

Miles-Richardson, S. R., Kramer, V. J. , Fitzgerald, S. D., Render, J. A., Yamini, B., Barbee, S. J., and Giesy, J. P., (1999). "Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*)", *Aquatic Toxicology,* **47** (2), 129-145.

Nagel, R., (1986). "Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio,* Ham.-Buc.)", *Journal of Applied Ichthyology* , 173-181.

Nagel, R., (1994). "Complete life cycle tests with zebrafish- a critical assessment of the results", in: Müller, R. and Lloyd, R. Sublethal and chronic effects of pollutants on Freshwater Fish. FAO Fishing New Book, Blackwell Science, 371 pages , 188-195.

Nagel R., Isberner K. (1998). "Testing of chemicals with fish – a critical evaluation of tests with special regard to zebrafish." In: Braunbeck, T, Hinton D. E, Streit B (eds). Fish Ecotoxicology. Birkhäuser, Basel. pp. 337-352.

OECD, Organisation for Economic Co-operation and Development, (1984). "OECD guidelines for testing of chemicals, section 2, Guideline 204: Fish, prolonged toxicity test: 14-day Study", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992a). "OECD guidelines for testing of chemicals, section 2, Guideline 203: Fish, acute toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992b). "OECD guidelines for testing of chemicals, section 2, Guideline 210: Fish, Early-life stage toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1998). "OECD guidelines for testing of chemicals, section 2, Guideline 212: Fish, Short-term test on embryo and sac-fry stages", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (2000). "OECD guidelines for testing of chemicals, section 2, Guideline 215: Fish, Juvenile Growth Test", Paris, France .

Papoulias, D. M., Noltie, D. B., and Tillitt, D. E., (2000). "An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethynylestradiol", *Aquatic Toxicology* **48** (1), 37-50.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G. C., Waldock, M., and Sumpter, J. P., (1998). "Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach", *Environmental Science & Technology* **32** (11), 1559-1565.

Schäfers, C.; Böhmer, W.; Schmitz, A. and Wenzel, A. (2002). "The relevance of xenoestrogenic effects for fish populations: Full life cycle tests with zebrafish (*Danio rerio*)." *Environmental Toxicology and Chemistry*, in press

Scholz, S. and Gutzeit, H. O., (2000). "17-alpha-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (Oryzias latipes)", *Aquatic Toxicology* **50** (4), 363-373.

Segner, H., Fenske, M., Maack, G., Schäfers, C. and Wenzel, A. 2002: "Life-time exposure to Ethynylestradiol induces irreversible infertility in the zebrafish Danio rerio" in prep.

Selman, K., Wallace, R. A., Sarka, A., and Qi, Xiaoping, (1993). "Stages of oocyte development in the zebrafish Brachydanio rerio", Journal of Morphology 218, 203-224.

Shibata, N. and Hamaguchi, S., (1988). "Evidence for the Sexual bipotentiality of spermatogonia in the fish, Oryzias latipes", *Journal of Experimental Zoology* **245**, 71-77.

Shioda, T. and Wakabayashi, M., (2000). "Effect of certain chemicals on the reproduction of medaka (Oryzias latipes)", *Chemosphere* **40** (3), 239-243.

Sohoni, P., Tyler, C. R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C. , Evans, M., Toy, R., Gargas, M., and Sumpter, J. P., (2001). "Reproductive effects of long-term exposure to bisphenol a in the fathead minnow (*Pimephales promelas*)", *Environmental Science & Technology* **35** (14), 2917-2925.

Staples, C. A., Dorn, P. B., Klecka, G. M., O'Block, S. T., and Harris, L. R., (1998). "A review of the environmental fate, effects, and exposures of bisphenol A", *Chemosphere* **36** (10), 2149-2173.

Stumpf, M., Ternes, T.A., Haberer, K. & Baumann, W. Nachweis von natürlichen und synthetischen Östrogenen in Kläranlagen und Fließgewässern (Determination of natural and synthetic estrogens in sewage plants and river water, German with abstract in English). *Vom Wasser **87***, 251-261 (1996).

Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J., Jegou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Muller, J., RajpertDeMeyts, E., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebaek, N. E., (1996). "Male

reproductive health and environmental xenoestrogens", *Environmental Health Perspectives* **104**, 741-803.

Tyler, C. R., Jobling, S., and Sumpter, J. P., (1998). "Endocrine disruption in wildlife: A critical review of the evidence", *Critical Reviews in Toxicology* **28** (4), 319-361.

Weber, L. P., Abdel-Hamide, N., Hill, R. L., and Janz, D. M., (2001). "Developmental estrogenic exposure alters gametogenesis and produces organ toxicity in zebrafish", in: Proceedings of the 22th annual meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, USA

Wright, P. J., (1992). "Ovarian development, spawning frequency and batch fecundity in *Encrasicholina heteroloba* (Rupell, 1858)", *Journal of Fish Biology* **40**, 833-844.

Yokota, H., Tsuruda, Y., Maeda, M., Oshima, Y., Tadokoro, H., Nakazono, A., Honjo, T., and Kobayashi, K., (2000). "Effect of Bisphenol A on the early life stage in Japanese medaka (*Oryzias latipes*)", *Environmental Toxicology and Chemistry* **19** (7), 1925-1930.

Chapter 3

Life-stage-dependent sensitivity of zebrafish (*Danio rerio*) to estrogen exposure

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Abstract

The aim of this study was to identify possible sensitive periods for estrogenic exposure during zebrafish (*Danio rerio*) development. To this end, zebrafish were exposed to 10 ng/L ethynylestradiol (EE2) during the protogynic stage (from day 15 to 42 post-fertilisation pf), during the stage of gonadal sexual differentiation (from day 43 to day 71 pf) and during the premature gonad stage (day 72 to 99 pf). Somatic growth, the onset of spawning, number of eggs per female, fertilisation and sex ratio of the adult, after termination of the experiment, were determined. Exposure to EE2 led to a reduction in total length, whatever life stage was exposed, but the effect was reversible Furthermore, the onset of spawning was delayed and both the number of laid eggs and the number of fertilized eggs produced by adult 157-day-old fish were reduced significantly when the developing fish was exposed to EE2 during days 43 and 71 post-fertilization. Fish of 213 days pf still showed a significant reduction of fertilization success, whereas the number of laid eggs per female was no longer different from control fish. When zebrafish was exposed to EE2 during the protogynic or during the premature developmental stage, this did not result in significant alterations of reproductive parameters, i.e. egg number per female and fertilization success, in the adult stage. In a second experiment, zebrafish were exposed during the phase of gonadal sexual differentiation (days 43 to 71 pf) to 1.67, 3 and 10 ng/L ethylnyestradiol to examine the concentration-dependence of the reproductive effects. In addition to the reproductive parameters the gonads were examined histologically directly after the exposure, at the onset of spawning and after termination of the experiment. Histologically analysis at the and of the exposure period revealed a total prevention of the transformation of the male gonads from a concentration of 3 ng/L EE2 and more. All dissected gonads at 3 and 10 ng/L EE2 were ovaries in the protogynic stage of development. After a month of recovery, at day 140 pf, testes were found in all exposed groups. A concentration-related skewing of the gonadal sex ratio at the end of the experiments, after giving the fish the possibility to recover, could not be found. The fertilization success were significant reduced after exposure to 3 ng and 10 ng/L EE2. The onset of spawning was delayed in fish exposed to 10 ng/L only.

Keywords:

zebrafish; *Danio rerio*, 17α-ethynylestradiol, gonadal development, gonad histology, reproduction, sexual differentiation

Introduction

There is considerable concern over the potential hazardous effects of endocrine-active chemicals (EACs), i.e. natural and man-made substances that interfere with the endocrine system of vertebrates (i.e. Colborn and Clement 1992, Kavlov et al. 1996, Toppari et al. 1996). Evidence from field studies, particularly from the aquatic environment, suggests a relationship between exposure to environmental hormones or hormone mimics and the manifestation of developmental and reproductive alterations in exposed organisms (e.g. Purdom et al. 1994, Giullette et al. 1996, Jobling et al. 1996, Oberdörster and Cheek 2001, Rodgers-Gray et al. 2001). EACs have the potential to disrupt or modulate hormone-regulated processes in the exposed organisms and/or to alter the synthesis, secretion, transport, binding, action or metabolism of endogenous hormones. By these effects, EACs have the potential to affect organism homeostasis, development, reproduction and behaviour Most notable among the EACs are compounds that act as agonists or antagonists of steroid sex hormones, particularly estrogens.

Steroid sex hormones are phylogenetically conserved and share a common primary mechanism of action, i.e. binding to cytoplasmic and nuclear receptors. In vertebrates, sex steroids have an “organizational” role with respect to developmental processes such as sex determination and differentiation, and an “activational” role in the regulation of the sexual cycle of mature animals (Guilette et al. 1995, Bigsby et al. 1999). Assessment of the potential effects of environmental estrogens and estrogen mimics on animal development and reproduction is particularly complicated due to (1) life-stage-specific differences in sensitivity to steroid action, (2) the possible translation of subtle physiological alterations during development into lasting, irreversible reproductive dysfunction of the adult organism, (3) differences between species and taxa in steroid action, and (4) the pleiotropic actions of sex steroid hormones within the organism.

Schäfers et al. (2002) demonstrated that long-term life cycle exposure of zebrafish to EE2 concentrations as low as 1.7 ng leads to significant alterations of reproductive parameters. In the literature, it has been discussed whether there exist periods in fish development which may be specifically sensitive to estrogen action, so that short-term exposure during these periods may induce lasting alterations in sexual differentiation and reproductive performance. Therefore, in the present study, we explore whether exposure of zebrafish to estrogens during a restricted period of development will lead to effects similar or identical to those of chronic life cycle exposure.

As experimental model, we selected the zebrafish, *Danio rerio*, Hamilton-Buchanan 1822, (Teleostei, Cyprinidae) because the zebrafish has a relatively short life cycle of about 4

months, and, in the laboratory, can be stimulated to breed throughout the year. For this reason, zebrafish are suitable for assessing toxic effects of chemicals on development and reproduction (Nagel 1993, Nagel and Isberner 1998, Andersen et al. 2000). Additionally test protocols are available, including OECD guidelines (OECD 1992a, 1992b, 1998) that recommend zebrafish as a model species for chemical toxicity assessments.

The test compound chosen in this study was ethynylestradiol (EE2). This substance is present in contraceptive pills and occurs in surface waters at concentrations that have been shown to induce developmental and reproductive disturbances in fish (Stumpf et al. 1996; Harries et al. 1997, Desbrow et al. 1998; Ternes et al. 1999 Tyler et al., 1998 and Länge et al., 2001). The zebrafish were treated with EE2 during three different life stages, i.e. during the protogynic phase (in our conditions: day 15 to day 42 post fertilisation [pf]), during the period of final sexual differentiation (day 43 to day 71 pf) and during the premature stage (day 72 to onset of spawning at day 99 pf). Possible adverse effects of EE2 on zebrafish development and reproduction were assessed by measuring somatic growth, development of gonadal morphology and reproductive parameters.

Materials and methods

Study organism

The origin of the zebrafish strain was the West Aquarium GmbH in 37431 Bad Lauterberg, Germany. Fertilised eggs for the tests were obtained from parental fish reared in the laboratory of the UFZ, Leipzig, Germany. Parental fish were kept in water of the same quality as used in the tests . The water temperature was maintained at 26 °C ± 1 °C. Light/dark cycle was 12 h/12 h. Animals were fed daily with TetraMin^{R} Hauptfutter (Tetra Werke, Melle, Germany) and nauplia larvae of the crustacean *Artemia sp., ad libitum*. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilised eggs. Purified drinking water was prepared according to the OECD-guideline (1993). The water purification included filtration with activated charcoal and aeration.

Test substances and concentrations

The tests were performed under semistatic conditions, with a total exchange of the test solution every third day. 17α-ethynylestradiol (MM296.4, CAS 57-63-6) was obtained from Sigma (Deisenhofen, Germany) with a purity of 98%. A stock solution of 10 mg EE2/10 ml acetone was diluted with sterile aqua dest. to a concentration of 1 mg/L. The nominal test

concentrations were 1.67, 3 and 10 ng EE2/L. The required amount of the stock EE2 solution was added during the refilling of the aquaria to ensure an even distribution of the test chemical. A solvent control was prepared in the same way, but without EE2. These solvent control aquaria contained the same amount of acetone as the 10 ng EE2 test concentration (less than 0.001%).

Chemical analysis

The chemical analysis was carried out by the Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany. Samples of 1 L volume were taken from the experimental aquaria and prepared for chemical analysis. The samples were acidified with hydrochloric acid (10 µL 30% HCl per 100 mL of sample). After addition of the internal standard (internal standard: EE2-D_4; CDN Isotopes, Pointe-Claire, Canada) the extraction was carried out by solid phase extraction (SPE). The whole sample was sucked for 30 minutes through 3M Octadecyl EMPORE™ extractions disks (Varian, Harbor City, USA) The elution of the disks was performed with 10 mL of acetone. The elution solvent was reduced to 1 mL using a stream of nitrogen. The EMPORE™ extracts were cleaned by adsorption chromatography using 1 g activated silica gel and n-hexane/acetone solvent mixtures. The cleaned solvents were reduced to approx. 300 µL by a gentle stream of nitrogen, transported into a GC micro vial and evaporated further to dryness. Then 50 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS-No. 24589-78-4, Pierce, Illinois, USA), was added to the residue. The mixture was heated up to 70 °C for 1 hour to form the non-polar trimethylsilyl (TMS) ethers of the analytes. Gas chromatographic separation was performed in a non-polar capillary column and the mass selective measuring was carried out with ion trap GC/MS/MS (GC Varian Saturn 3400, MS Varian Saturn 4D). The MS/MS function was used for the mass selective trapping of the analyte ions (parent ions), but not for the production of daughter ions. The method for the EE2 analysis of water samples is described in detail by Böhmer and Kurzawa (2000).

Rearing of zebrafish

The experiments started with larvae at an age of 15 days post-fertilisation (pf). 80 individuals were placed into one glass aquarium of 20 x 20 x 15 cm (length x depth x height; total volume = 6 L) containing a test solution of 5 L volume. In order to maintain stable water temperature, the aquaria were placed into larger glass aquaria with a total volume of 29 L (40 x 27 x 27 cm; length x depth x height). Oxygen concentration, pH value and temperature were measured regularly. The mean temperature ± standard deviation for all test vessels (all

concentrations and control) was 26.0 °C ± 0.6 °C. Single values ranged from 24.5 to 27.5 °C. The mean pH-value for the test vessels was 8.2 ± 0.2, with single values ranging from 7.7 to 8.4. The mean oxygen saturation values varied from 70.9 % to 94.6% with a mean value of 87.2 % ± 4.6 % (equivalent to 6.96 mg/L).

At day 43 pf the number of zebrafish per vessel was reduced to 60 individuals and the fish were transferred into large aquaria containing 20 L of exposure solution. At day 71 pf, the number of fish per aquarium was reduced to 40. In order to measure the reproduction parameters, the fishes were grouped at day 99 pf, shortly before the onset of spawning, in breeding groups of 4 females and 8 males per vessel.

Exposure experiments: Stage-specific exposure of developing zebrafish to 10 ng EE2/L

Three exposure regimes were applied: a control, with fish reared in non-contaminated water, an EE2 treatment in which fish were exposed during different life stages to 10 ng EE2/L, and a solvent control. The test concentration of 10 ng EE2/L (nominal) was selected on the basis of previous full life cycle experiments (EC_{50}: 1.1 ng EE2/L). and of a 28-d prolonged toxicity test with juvenile and adult zebrafish (EC_{50}: 100 ng EE2/L).

Zebrafish were exposed to 10 ng EE2/L from:

day 15 to day 42 pf (protogynic period)

day 43 to day 71 pf (period of sexual differentiation of gonads)

day 72 to day 99 (pre-mature stage).

For each period, 4 exposure replicates, 4 replicates of the solvent control and 4 replicates of controls without solvent addition were used. Altogether the experiment started with 2,240 zebrafish larvae in 28 test vessels.

Exposure experiments: Treatment with different EE2 concentrations during days 43 – 71 pf

For the period of sexual differentiation (days 43 - 71 pf), the EE2-concentration dependence was examined by exposing developing zebrafish to nominal test concentrations of 1.67, 3 and 10 ng EE2/L. The experiment was conducted with 3 replicates per concentration and 3 additional control vessels. The experiment started with 960 zebrafish larvae in 12 aquaria.

Effect endpoints: body length

For the stage-specific experiment fish length was measured at the end of each exposure period — day 43 pf, day 67 pf, day 99 pf — and additionally on day 141 pf and day 185 pf. For the second experiment the length was measured before the exposure period on day 43 pf, in the middle of the exposure period on day 57 pf, at the end of the exposure, on day 71 pf and shortly before spawning, on day 99 pf. Body length was determined by photographing the individual test vessels, with subsequent digital analysis of the photographed fish. Mean body lengths ± standard deviation were calculated and the data from the different treatments were compared by the following statistical methods (SPSS program package) When data were normally distributed according to the Levène test, ANOVA was performed, followed by Scheffle's test to evaluate significant differences between treatments and controls. If variances were inhomogeneous, Dunnett's T3 test was used.

Effect endpoints: reproductive parameters:

On day 99 pf, spawning groups comprising 4 females and 8 males were arranged in every test vessel. Spawning trays were placed at the bottom of the aquaria from day 110 pf. The trays were covered with a lattice (stainless steel). Artificial "spawning-trees", were fixed onto the lattice (modified method according to (Nagel 1986). The spawning trees stimulate the spawning and concentrate it over the spawning trays. Mating behaviour and spawning were observed for the first time on day 124 in two aquaria with fish that had been exposed from day 15 to day 42 pf. During the next 14 days the egg production was checked every day to make sure that eggs were produced continuously in every test vessel. Only when active and continuous spawning was evident in each aquarium quantitative determination of egg production begun. After 25 days of daily counts, the reproduction assessment was finished, the fish were sampled for histology, and were sexed on the basis of histological analyses of the gonads. The number of eggs per aquarium was related to the number of females per tank in order to calculate the numbers of eggs per female and day. The percentage of fertilised eggs per vessel and day is a measure of the fertilisation success. For each test concentration, the mean value and standard deviation of the total number of eggs per female and day, and of the fertilisation per day (n = 20-25 counts) were calculated. To create a normal distribution, fertilisation values (0-100%) were z-transformed (arc sin (square root x)), and egg numbers were log-transformed. With the transformed data, ANOVA was performed. In case of significant differences between the mean values, Scheffle's Test was used to find out which mean rate values were significantly lower than the mean value found for the control.

Effect endpoint: gonadal sex ratio

For determining the sex ratio of the adult fish of the first experiment, after termination of the experiments the zebrafish from all test vessels were sexed by macroscopical examination of the gonads after dissection of the body. Altogether 609 fish from the stage-specific treatments were sexed.

At the end of the second experiment 331 fish were sexed in the same way, as described above. The gonads of the fish of the spawning tanks were additionally examined histologically.

The gonadal sex of 71- and 140-dpf-old fish was determined by histological examination. 229 zebrafish from the second experiment, exposed from day 43 to day 71 pf were examined histologically, as described below. At the end of the exposure period, on day 71 pf, at the beginning of daily counts of the reproductive parameters, and on day 140 pf up to 20 fish were taken per concentration for histological analysis.

Statistics

Determination of the sex ratio of mature zebrafish with fully developed gonads by macroscopically identification of the gonads agreed very well with histological observation. Chi^2 -test was used to find possible statistically significant differences between the various treatments.

Histological analysis

For histological examination, fish anaesthesized in ice water were fixed in Bouin' s fluid for 24 h. While the tiny young stages were fixed as a whole, larger fish were decapitated first. To facilitate infiltration of the fixative, an incision was made in the dorsal surface of the fish.

Fixed tissues were dehydrated through a graded series of ethanol and embedded in paraffin wax (Leica Histowax). Serial sections were cut at 7 µm and collected onto glass slides, stained with PAS (Periodic-Acid Schiff's) and analysed by light microscopy. 71-day-old fishes were dehydrated and infiltrated with Technovit 7100® (hydroxyethylmethacrylate) according the manufacturer's instructions (Kulzer GmbH, Germany) and sectioned (2-5 µm). Slides were stained in toluidin-methylene blue, dried overnight at 60°C and mounted with Entellan®. For the histological analysis an Olympus BX 60 microscope was used.

The oocytes of the zebrafish were classified according to (Selman et al., 1993). The classification scheme of Selman et al. is based on morphological criteria and on physiological and biochemical events. These authors subdivided the oocyte development into five stages: STAGE I: follicle phase of primary growth (perinucleolar oocytes), STAGE II: cortical alveolus

stage, STAGE III: vitellogenesis, STAGE IV: oocyte maturation, STAGE V: hydrated oocyte (mature egg).

For determining the diameter of the perinucleolar oocytes, up to 50 oocytes per female were measured. Slides were photographed by an Olympus DP 10 digital camera and the oocyte diameters were measured using the SIS-analysis® software package.

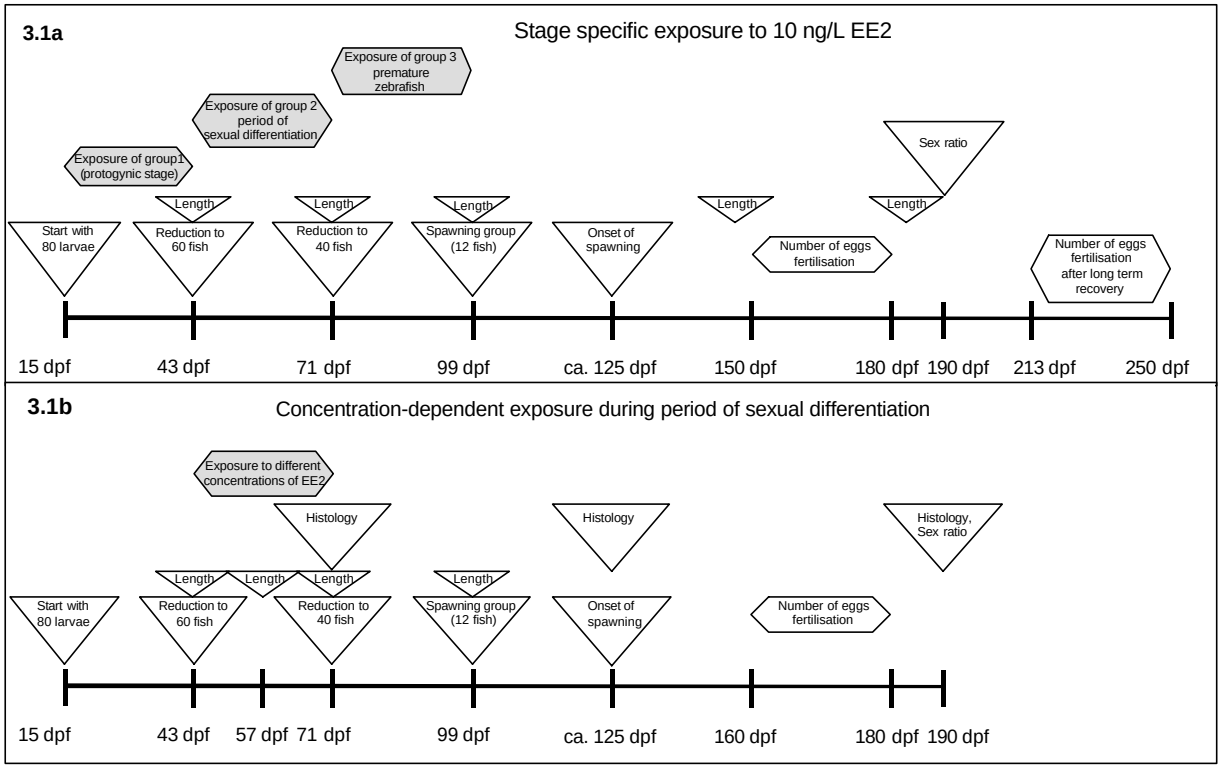

Figure 3.1a: Summary of the course of the stage-specific exposure experiment with the different endpoints, determined at different times. Fish exposed during the period of sexual differentiation (group 2) showed an exposure-related effect in the number of eggs and in fertilisation, when these parameters were measured between day 150 and 180. Hence these measurements were repeated when normal mating behaviour was observed.

Figure 3.1b: Summary of the course of exposure to graded EE2 concentrations during the period of sex differentiation with the different endpoints, determined at different times. (see text for more details).

Results

Ethynylestradiol concentrations in the experimental aquaria

The actual test concentrations of EE2 in the aquaria were in good agreement with the nominal concentrations. The measured values of EE2 varied between 80 % and 120 % of the nominal concentrations of 3 and 10 ng EE2/L, respectively (see Table 3.2). Only for the 1.67-ng EE2/L treatment did the average concentration of EE2 account for only 71 % (1.14 ng EE2/L) of the nominal concentration.

Table 3.1: Test concentration of ethynylestradiol given in ng/L. Analytical values (as mean measured values) at T = 0 and the percentage of the nominal concentration on T = 48 are listed for each concentration.

Nominal concentration [ng/L]	**T = 0h mean measured value (range) [ng/L]**	**T = 48h % of nominal concentration (range)**
10 (n = 10)	9.27 (7 – 11.6)	73.7 (0.15 – 98)
3 (n= 3)	2.77 (2.5 – 3.1)	80 (66.7 – 86.7)
1.67 (n=3)	1.14 (0.23 – 1.8)	49.7 (11.4 – 77.8)

Growth

Effect of developmental stage-specific exposure on body length

On day 43 pf there was no significant difference in body length between experimental treatments (fish exposed from day 14 to day 42 pf) and fish not treated with EE2 (Figure 3.2a). On day 67 pf the mean length of control fish was 15.9 ±0.19 mm, while fish exposed to 10 ng EE2/L between days 43 and 71 pf had a significantly reduced body length of 14.8 ± 0.17 mm. On day 99 pf those fish exposed to 10 ng EE2/L from day 72 to day 99 pf were 19.6 ± 0.19 mm long. This body length was significantly lower than the length of control fish (21.1 ± 0.19 mm). The significant growth reduction observed for fish exposed to 10 ng/L EE2 during days 72-99 pf was compensated during subsequent rearing in non-contaminated water, since the bodies of adult fish (185 dpf) did not differ significantly in length from those of the controls. However, fish exposed to EE2 between days 43 and 71 pf had a significantly lower body length than the controls, even after reaching the adult stage.

Effect of exposure to different EE2 (1.67 – 10 ng/L) concentrations during days 43-71 pf on body length

At the beginning of the exposure (day 43 pf) no significant difference of fish body length between the experimental aquaria was found (Figure 3.2b). At day 57 the length of fish in all EE2 treatments was reduced compared to controls, but the reduction was significant only for the 10 ng EE2/L concentration (mean body length of 14.5 ± 0.28 mm , compared to 16.29 ±

0.29 mm in controls). At the end of the exposure, on day 71 pf, a concentration-related effect of EE2 on body length was found. For all EE2 concentrations fish length was significantly lower than in the control (control: mean 19.38 ± 0,28 mm; 10 ng EE2/L: 16.79 ± 0.27 mm; 3 ng EE2/L: 17.63 ± 0.29 mm, 1.67 ng EE2/L: 18.03 ± 0,28 mm). At day 99 pf, after a month of recovery under control conditions, the length of the previously exposed fish was still reduced although the effect was no longer significant except for the concentration 10 ng EE2/L.

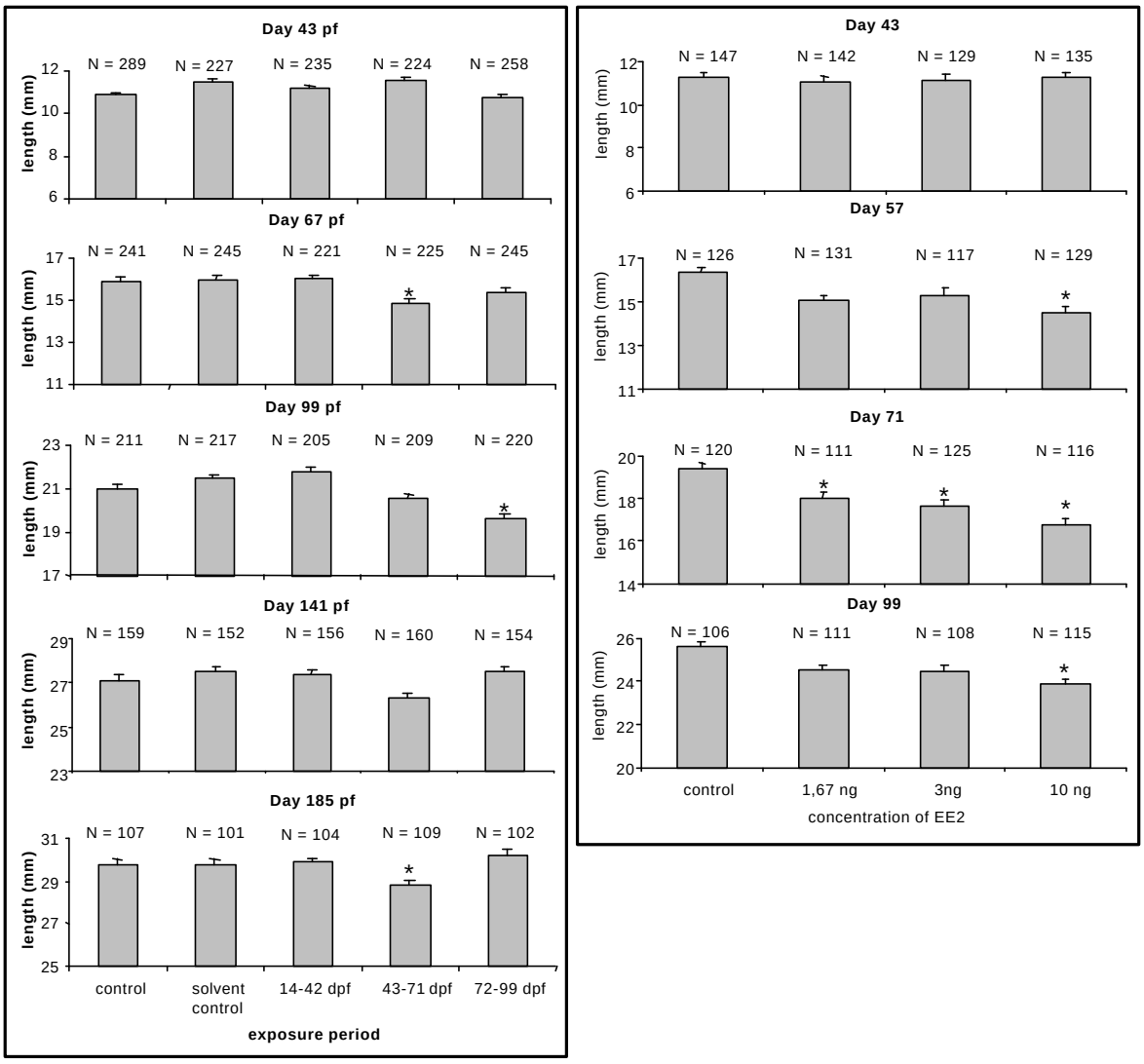

Figure 3.2a: Total length (mm) of zebrafish exposed to 10 ng EE2 during different developmental periods (left). Treatments are indicated on the x-axis: control, solvent control, exposure to 10 ng EE2/l between days 14-42 pf, exposure to 10 ng EE2/l between days 43-71 pf, or exposure to 10 ng EE2/l between days 72-99 pf Samples were taken at the end of each exposure period, only on day 67 pf were the samples taken three days before the end of the treatment. Further length measurements were made at day 141 pf and day 185 pf.
N: number of fishes.
*significant $p < 0.05$; Scheffle's Test; one way ANOVA

Figure 3.2b: Total length (mm) of zebrafish exposed from day 43-71 pf to different concentrations of EE2. (right) Samples were taken at the beginning of the exposure on day 43 pf; in the middle of the exposure period on day 57 pf; at the end of the exposure period on day 71 pf and on day 99pf, after one month of recovery under control conditions.
N: number of fishes.
*significant on the 95% level following Scheffle's test; one way ANOVA

Effect of developmental stage-specific exposure to 10 ng EE2/L on reproduction of adult fish

Fish groups exposed during different developmental periods took different amounts of time to begin mating behaviour and spawning. Whereas fish exposed from day 72 to day 99 pf started to spawn at the same time as the control group (day 138 pf), the onset of spawning of zebrafish exposed from day 43 to day 71 was delayed for 12 days to day 150 pf. Test fish exposed from day 15 to day 42 began spawning 12 days earlier than the control group, on day 126 pf (Fig. 3.3).

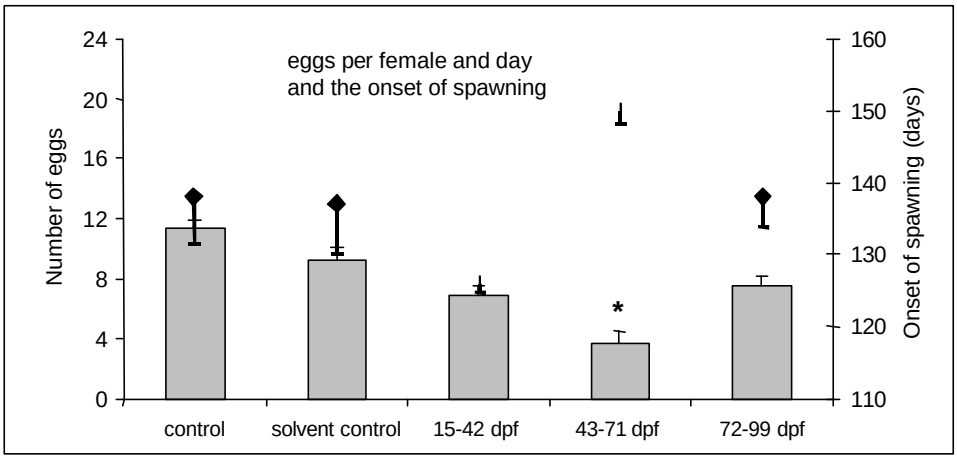

Figure 3.3: Mean number of eggs per female and day and the onset of spawning of zebrafish treated with 10 ng EE2 during different life stages. **Columns:** mean(± SE) number of eggs per female and day. Average of 25 daily counts.
Black rhombus: mean (± SE) onset of spawning in days post fertilisation
*significant $p < 0.05$; Scheffle's Test; one way ANOVA

Egg production per female and day is also shown in Figure 3.3. For all exposure periods a reduction of the daily egg production per female compared to control females was indicated. This reduction was significant ($p < 0.05$), but only for fish exposed from day 43 to day 71 pf. Similarly, fertilisation showed a tendency to decrease in all EE2 treatments, but the reduction was significant ($p < 0.05$) only for the group exposed between day 43 and day 71 pf (Figure 3.4).

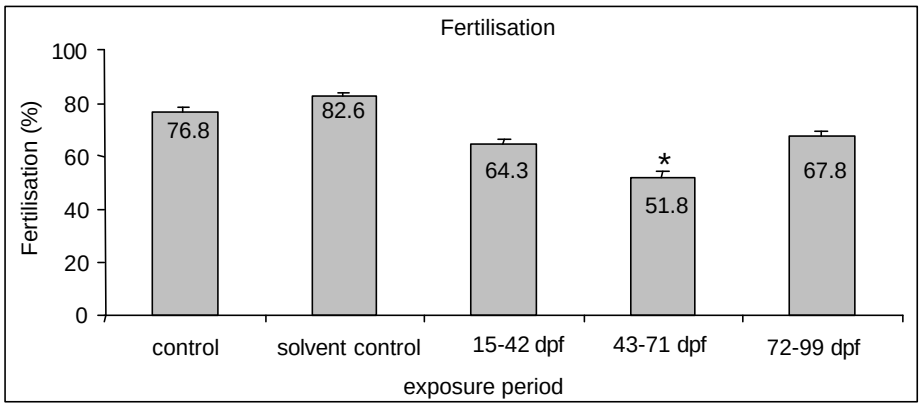

Figure 3.4: Fertilisation of zebrafish treated with 10 ng EE2 during different life stages. (*significant $p < 0.05$;Scheffle's Test; one way ANOVA)

To explore the possibility that reproductive parameters of fish exposed between days 43 and 71 pf may return to control levels later on during adult life, the fish were maintained in non-contaminated water up to day 213 pf. At this age, after 142 days in non-contaminated water, they showed normal mating behaviour, regular spawning, and female egg production at the same level as control fish (data not shown). The fertilisation, however, was still significantly lower than in controls. (Figure 3.5).

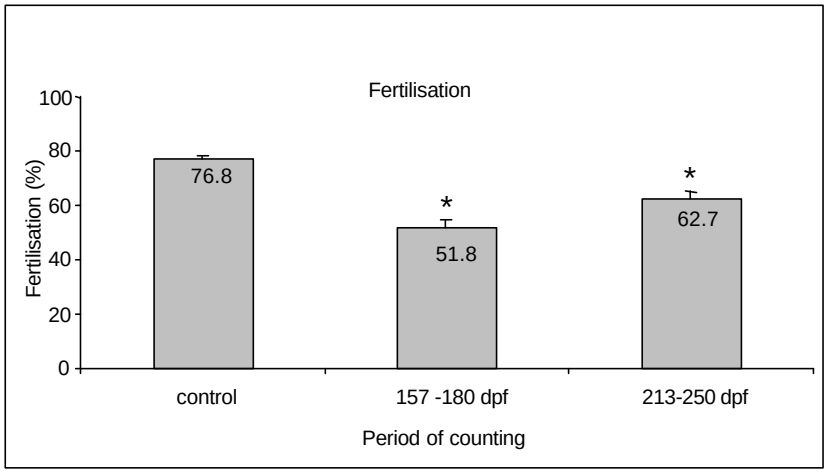

Figure 3.5: Fertilisation of zebrafish treated with 10 ng EE2 during day 43 -71 pf, at the beginning of spawning and after an additional month of recovery. (*significant $p < 0.05$; Scheffle's Test; one way ANOVA)

Effect of exposure to different EE2 (1.67 – 10 ng/L) concentrations during days 43-71 pf on reproduction of adult zebrafish

Fish exposed to 10 ng/L EE2 during days 43 to 71 pf showed a delayed onset of spawning: while control fish started to spawn on day 137 pf, the first spawning of fish exposed to 10 ng EE2/L was observed on day 180 pf. Fish exposed to lower EE2 concentrations started spawning at an earlier age than the control: those exposed to 1.67 ng EE2/L started on spawn at day 123 pf, those exposed to 3 ng EE2/L, on day 124 pf (Figure 3.6).

The number of eggs per female did not differ between the different EE2 treatments. The slight increase in the number of eggs laid per female in the exposed fish compared to control fish was statistically not significant (Figure 3.6).The effect of EE2 on fertilisation was concentration-dependent (Figure 3.7). Fertilisation success decreased to 88.2 % of the control value in fishes treated with 1.67 ng EE2/L, to 62 % in fish exposed to 3 ng EE2/L and to 49 % with 10 ng EE2/L.

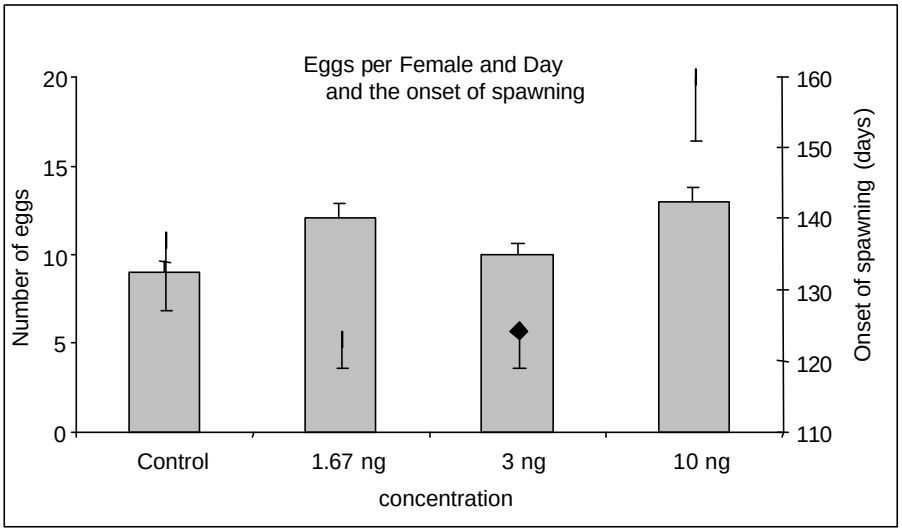

Figure 3.6: Mean number of fertilised eggs per female and day and the onset of spawning of zebrafish treated with different concentrations of EE2 during days 43 -71 pf
Columns: mean(± SE) number of eggs per female and day. Average of 25 daily counts
Black rhombus: mean (± SE) onset of spawning in days post fertilisation
*significant $p < 0.05$; Scheffle's Test; one way ANOVA

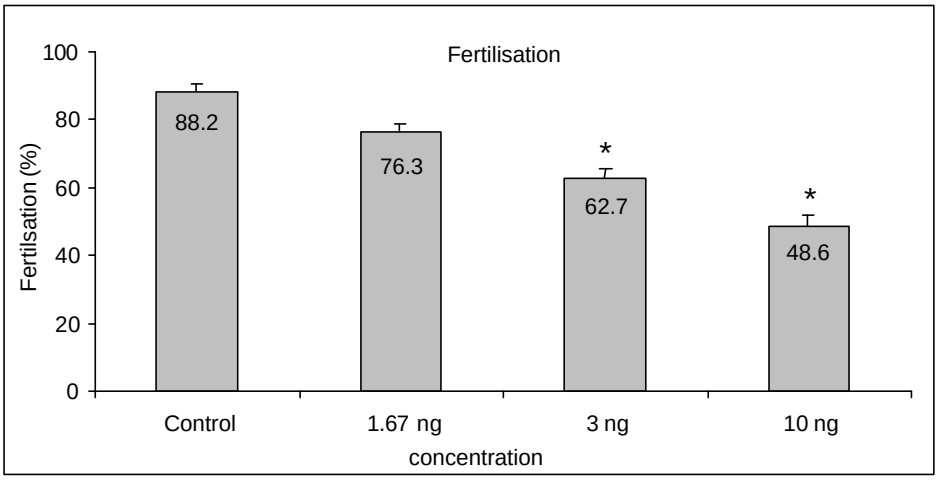

Figure 3.7: Fertilization of zebrafish treated with different concentrations of EE2 during days 43 -71 pf. (*significant $p < 0.05$, following Sheffle's Test; one way ANOVA)

Gonad histology of zebrafish exposed during the period of sexual differentiation

The ontogeny of gonad morphology in non-exposed zebrafish was described in detail in Chapter 1. Here we describe the alterations of gonad morphology at different ages, after exposure to EE2 from day 43 to day 71 pf. Fifty-five fish were sampled directly after termination of the exposure, another 53 shortly before the onset of spawning on day 140 pf, and 121 at the end of the experiment on day 190 pf.

OVARIAN DEVELOPMENT

71-DAY-OLD ZEBRAFISH

Ovaries of the control group:

In 71-day-old control fish, the ovaries contained previtellogenic oocytes but no more mature oocyte stages. Two major forms of ovarian morphology could be observed. In the first form the ovaries were composed almost exclusively of densely packed perinucleolar oocytes; only in the periphery of the gonadal tissue were a few nests of germ cells detectable (Figure 3.9 B).

The second form was characterised by alterations of oocyte morphology and tissue structure. In the altered areas, the oocytes decreased in size, and the shape of both the whole cell and the nucleus became irregular. The number of oocytes was declining. The close contact between the oocytes got lost so that empty spaces appeared between the oocytes. The proportion of stromal tissue in the gonads was increased. These stroma-rich gonads were usually much smaller in size than the oocyte-rich gonads .

One out of 13 examined ovarian-containing fishes of the control group displayed an ovary of the second morphological form while the remaining 12 individuals showed the first form.

Ovaries of exposed fish

In 71-day-old fish, exposed from day 43 pf to 71 pf to different concentrations of EE2, also the ovaries contained previtellogenic oocytes only. The fish showed a decreased frequency of the ovarian form with densely packed perinucleolar oocytes. As indicated in Table 3.2, this effect appeared to be concentration-dependent, although the low n-number does not allow a firm conclusion. In addition to the decreasing number of perinucleolar oocytes, also their size declined. These reduction of oocyte size was significant and it increased with increasing EE2 concentration (Table 3.2) In the control group a mean diameter of 37.6 ± 0.55 µm was measured whereas in the 1.67-ng group the mean oocyte diameter was 30.5 ± 0.43 µm, in the 3-ng group a diameter of 29.8 ± 0.4 µm was measured, and in the 10-ng group the oocyte diameter was 27.8 ± 0.37 µm (Figure 3.8a).

140-DAY-OLD ZEBRAFISH

As summarised in Table 3.2, a high percentage in all groups still contained immature ovaries.

Ovaries of the control group:

The mean diameter of the perinucleolar oocytes in control fish was 61.0 ± 0.89 µm.

The gonad of one female showed some morphological changes such as enlarged areas with rather heterogeneous size distribution of oocytes, resorbing oocytes, and increased frequency of non-germ cells areas (Figure 3.8b)

Figure 3.9: Ovaries of 71-dpf-old zebrafish, Page 100

The pictures show the wide range of ovarian development both in exposed and in unexposed zebrafish. Oocytes were staged according to Selman et al. (1993). For details see text and Chapter 1.

A - B: Ovaries from control fishes

A: This gonad is equivalent to one specification of a gonad with altered ovarian morphology, as described in detail in Chapter 1. The gonad wall is partly disintegrated. The oocytes **(do)** are degenerated. Somatic cells **(sc)** and cell fragments **(cf)** are visible. The germ cells **(gc)** are arranged in nests. They correspond with Type 1b germ cells, as described in Chapter 1 (5 µm-section).

B: Part of an ovary in an very early stage of maturation. Most oocytes are in the perinucleolar stage (**poc**). Only a few oocytes in the cortical alveolus stage (**coc**) are found. (2.5 µm-section).

C - E: Ovaries from zebrafish, exposed to 10 ng EE2 from day 43 to day 71

C: Ovary with perinucleolar oocytes (**poc**). In addition to the oocytes a huge area of non-germ tissue (**ngt**) inside the ovaryis visible, This tissue consists of darkly stained cells and less basophilic connective material

C1: Enlargement of C to demonstrate the transition from the oocyte-area of the ovary to the area with non-germ tissue (3 µm-section).

D: Part of an ovary with degenerating (**do**) and vaculolated (**vo**) oocytes as well as cell fragments (cf). A gonad wall is virtually no longer existent (2 µm-section)

E: Part of an ovary, degenerated to a lesser extent than the ovary shown in D. This gonad demonstrates the asynchronous grade of degeneration in the different regions of one and the same gonad.

E1: Enlargement of E to show a nearly completely resorbed former perinucleolar oocyte (arrow), whereas in other regions of the gonad virtually intact oocytes (**poc**) are found (2 µm-section).

Pay attention to the different scales

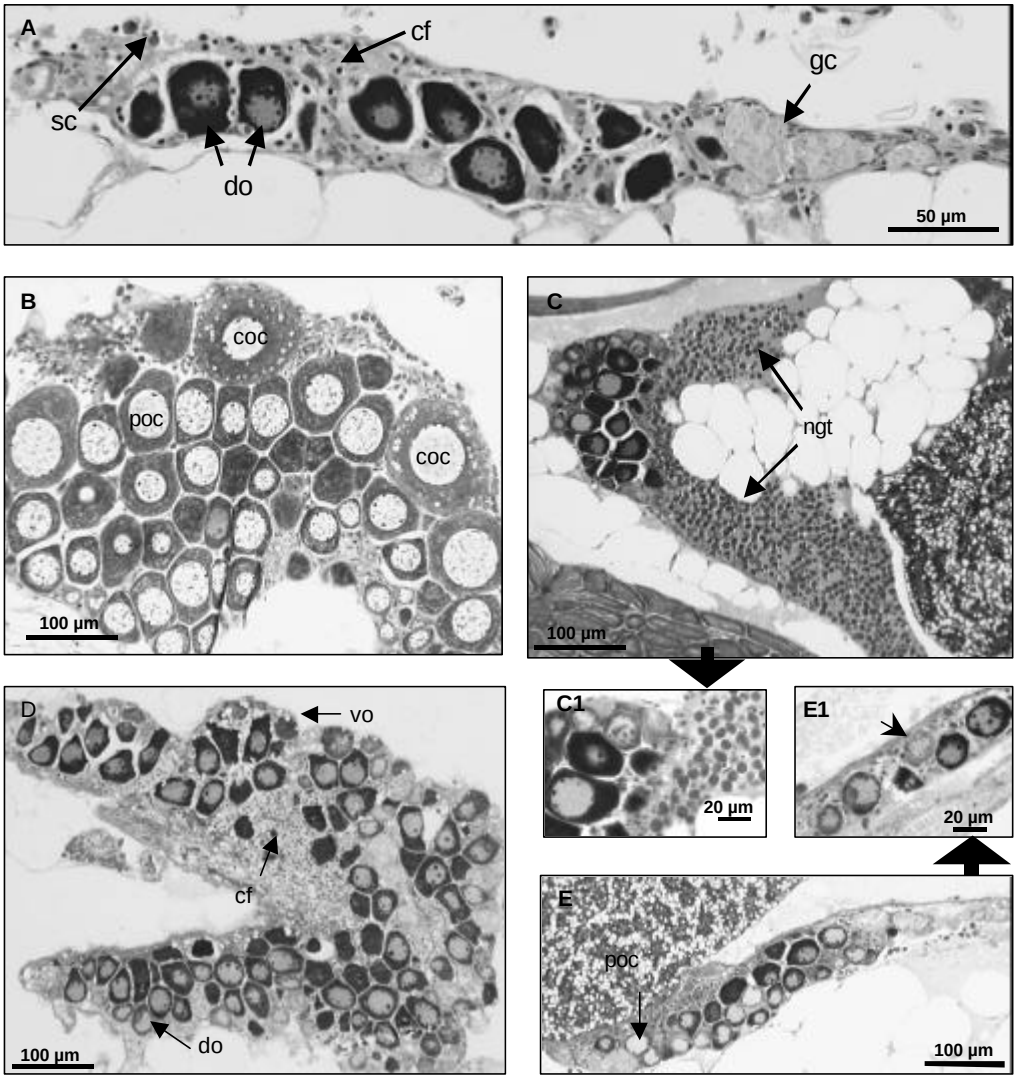

Figure 3.9: Legend see page 99

Ovaries of fish exposed to 1.67 ng EE2/L:

The mean diameter of the perinucleolar oocytes in fish exposed to 1.67 ng/L was 49.2 ± 0.85 µm. None of the 8 mature or maturing ovaries showed morphological changes.

Ovaries of fish exposed to 3 ng EE2/L:

The mean diameter of the perinucleolar oocytes in fish exposed to 3 ng/L was 47.7 ± 0.83 µm. In 1 out of 5 maturing or mature ovaries morphological changes were found.

Ovaries of fish exposed to 10 ng EE2/L:

Due to technical reasons, only two ovaries could be measured in the group exposed to 10 ng EE2; the results for this group were not reliable

190-DAY-OLD ZEBRAFISH

All females were reproductively active. At the end of the experiment on day 190 pf a concentration-related reduction of the oocyte diameter could no longer be measured (Figure 3.8c). The mean oocyte diameters of the exposed populations are slightly reduced in comparison to the control group, but this effect was not statistically significant.

As shown in Table 3.2, in the group exposed to 10 ng/L EE2, 6 out of 12 females had ovaries, at least some of them showed morphological alterations These alterations are illustrated in Figure 3.10 D-F, whereas in the control group 1 out of 12 fish expressed an ovary with morphologically alterations. In fish, exposed to 1.67 ng/L no ovaries were found with these alterations and fish exposed to 3 ng/L EE2 2 fish out of 13 displayed ovaries with the above described alterations.

Table 3.2: The morphological appearance of the ovaries of 140-dpf-old zebrafish exposed to graded concentrations of EE2 from day 43 to day 71 pf (Page 102).

[1]The immature ovaries with altered morphologically altered appearance correspond to the second form of ovarian morphology as described above and in detail in chapter 1.

[2]The fish with mature, but histologically altered ovaries, do not correspond to the immature gonads with altered morphology. The alterations of the mature ovaries are expressed by enlarged areas with rather heterogeneous size distribution of oocytes, resorbing oocytes, and an increased frequency of non-germ cells areas.

[3]The histological observations of the 190-dpf-old zebrafish were done with the spawning fish only, which had been disposed in groups of 4 females and 8 males per test vessel. Therefore no concentration related histological classification of gonad type ratio was possible.

Table 3.2: Legend, see page 101

Age	Concentration EE2/L	Fish with immature ovaries, containing perinucleolar oocytes only	Number of fish with maturing or mature ovaries	Immature ovaries with densely packed perinucleolar oocytes	Immature ovaries with altered morphologically altered appearance[1]	Fish with mature ovaries in normal morphological appearance	Fish with mature, but histologically altered ovaries[2]	Percentage of fish with ovarian-like gonads (Total number of examined fish)
71 dpf	**Control** (N =13)	13	0	12	1	--	--	52 % (25)
	1.67 ng (N =11)	11	0	8	3	--	--	85 % (13)
	3 ng (N = 15)	15	0	6	9	--	--	100 % (15)
	10 ng (N = 11)	11	0	5	6	--	--	100 % (11)
140 dpf	**Control** (N =6)	4	2	not measured.	not measured	2	0	60 % (10)
	1.67 ng (N =13)	5	8	not measured.	not measured.	6	2	68 % (19)
	3 ng (N = 13)	8	5	not measured.	not measured.	4	1	86 % (15)
	10 ng (N = 2)	1	1	not measured	not measured	0	1	12 % (9)
190 dpf	**Control** (N =13)	0	13	--	--	12	1	not measured.[3]
	1.67 ng (N =11)	0	11	--	--	11	0	not measured.[3]
	3 ng (N = 15)	0	15	--	--	13	2	not measured.[3]
	10 ng (N = 18)	0	18	--	--	12	6	not measured.[3]

Figure 3.10: Ovaries of 140-dpf and 190-dpf old zebrafish, exposed to EE2 from day 43 to day 71 pf, illustrating the range of states of the ovarian development. Page 104

A – C: Ovaries of 140 dpf-old fishes (7-µm section).

A: Part of a resting premature ovary exposed to 10 ng EE2 during days 42-71 pf. Except for two cortical alveolus stage oocytes (**coc**), only previtellogenic oocytes (**poc**) are found.

B: Part of an maturing ovary exposed to 3ng EE2. The most developed oocytes are vitellogenic oocytes (**voc**); cortical alveolus stage oocytes (**coc**) and previtellogenic oocytes (**poc**) are also present.

C: Part of a mature ovary, exposed to 3 ng EE2. This is a typical part of an reproductively active ovary, containing oocytes at all developmental stages, beginning from previtellogenic oocytes (**poc**) at the edge up to mature oocyte (**moc**).

D –F: Ovaries of 190-dpf-old fishes, exposed to 10 ng EE2 (7-µm section). These pictures are examples for impaired ovaries, as described in the text.

D: Part of an ovary with degenerated previtellogenic oocytes (**poc**), cortical alveolus stage oocytes (**coc**) vitellogenic oocytes (**voc**) at different points in the progress of resorption. In mature oocytes the follicle layer is dissolved as one of the first steps (**white arrow**). Additional ingrowing stroma and unstructured non-germ tissue (**ngt**) are found inside the ovary between the oocytes.

E: Part of an ovary with areas of intact oocytes and additionally an area with non-germ tissue (**ngt**). The oocytes in the latter area are degenerated (**do**). **poc**: perinucleolar oocyte, **coc:** cortical alveolus stage oocytes, **voc:** vitellogenic oocytes. **moc**: mature oocyte.

F: Enlargement of Figure E.

Pay attention to the different scales

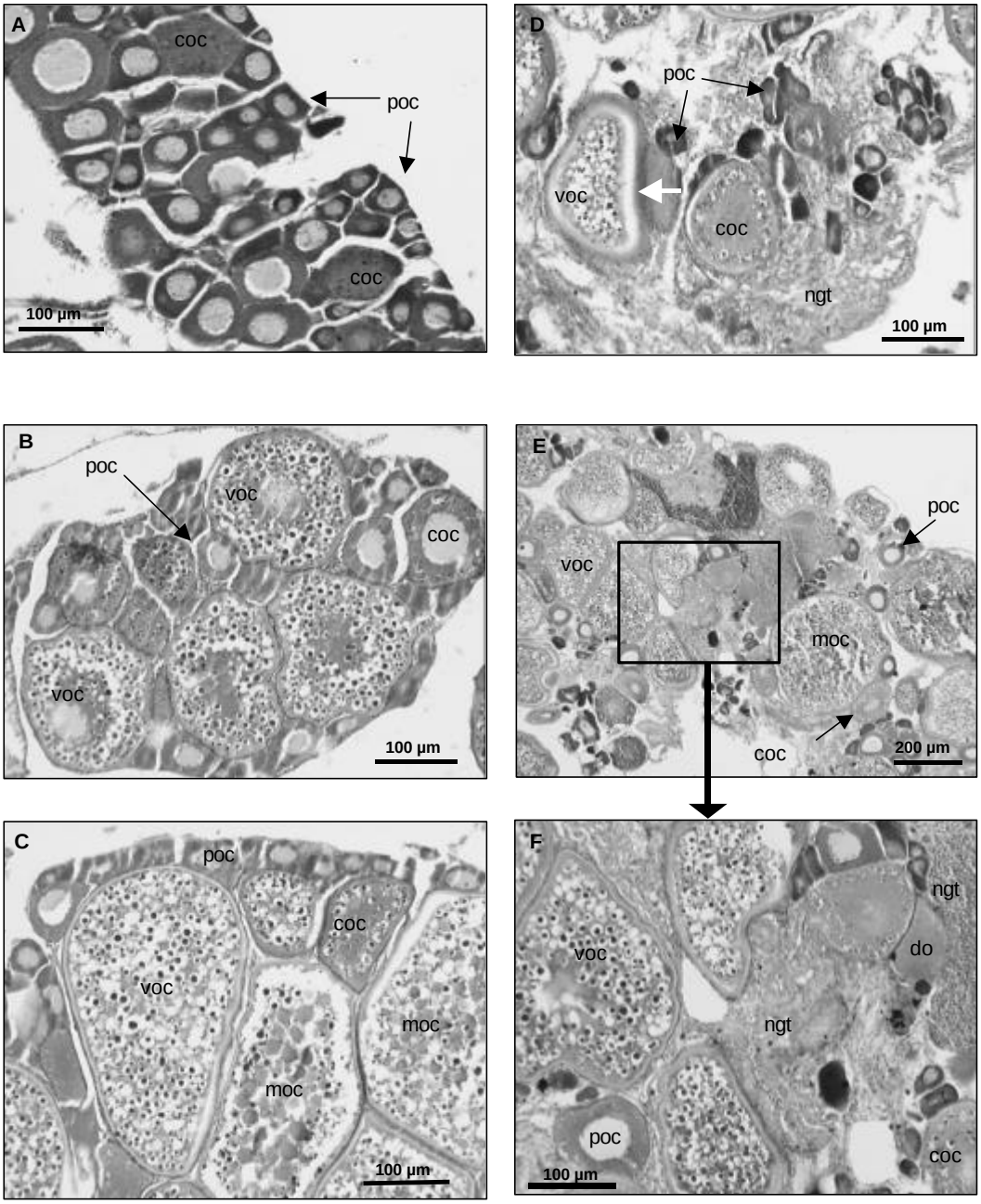

Figure 3.10: Legend see page 103

TESTES DEVELOPMENT IN ZEBARFISH EXPSOED TO DIFFERENT CONCENTRATIONS OF EE2 DURING DAYS 43-71 PF

71-DAY-OLD ZEBRAFISH

Immediately after the exposure, there was a statistically significant, concentration-dependent decrease in the frequency of testes-like gonads (chi²-test; $p < 0.05$) (Figure 3.12a). At the concentrations 3 and 10 ng EE2/L no testes-like gonads at all were found; all dissected fish had ovary-like gonads. In the 1.67-ng group, 11 out of 13 fish had ovary-like gonads and the other two contained testes-like gonads, whereas in the control group 8 fish with testes and 4 with ovaries were found. All testes (8 in the control group and 2 in the 1.67-ng/L EE2 exposure group) were immature, none of them containing sperm (Figure 3.11E).

140DAY-OLD ZEBRAFISH

Two month after termination of the exposure, there was no longer a detectable concentration-dependent alteration of gonad composition (Figure 3.12b).

Testes of control fish:

All 4 dissected males had sperm-containing testes

Testes of fish exposed to 1.67 and 3 ng/L EE2:

All 6 dissected males exposed to 1.67 ng /L EE2 and the one male exposed to 3 ng/L EE2 contained sperm. One fish that had been exposed to 3 ng EE2 had a gonad containing both maturing oocytes and spermatids. This was the only fish with mixed gonads found in the experiments.

Testes of fish exposed to 10 ng/L EE2:

2 out of 5 males exposed to 10 ng/L EE2 were unable to reproduce. No morphological difference between the mature testes and the testes of the control group could be found.

190-DAY-OLD ZEBRAFISH

The histological observations of the 190-dpf-old zebrafish were done with the spawning fish only, which had been disposed in groups of 4 females and 8 males per test vessel. Therefore no concentration dependent histological classification of gonad type ratio was possible.

On day 190 pf, at the end of the experiment, all testes contained sperm. No morphological difference could be found between testes of fish from the different exposure concentrations (Figures 3.11A and B).

In 5 out of 103 dissected testes single previtellogenic oocytes were found, distributed over all ages and concentrations, including control populations (Figures 11C and D). Table 3.3 summarises the maturity stages of the testes.

Table 3.3: The maturity stages of the testes of zebrafish exposed to graded concentrations of EE2 from day 43 to day 71 pf. An individual fish can contain both sperm and atretic oocytes in one and the same testis.

*The histological observations of the 190-dpf-old zebrafish were done with the spawning fish only, which had been disposed in groups of 4 females and 8 males per test vessel. Therefore no concentration related histological classification of gonad type ratio was possible.

Age	Concentration EE2/L	Immature testes	Sperm-containing testes	Testes containing oocytes	Percentage of testes-like gonads (Total number of examined fish)
71 dpf	**control** (N = 12)	12	0	1	48 % (25)
	1.67 ng (N = 2)	2	0	0	15 % (13)
	3 ng (no testes)	-	-	-	0 % (15)
	10 ng (no testes)	-	-	-	0 % (15)
140 dpf	**control** (N = 4)	0	4	0	40 % (10)
	1.67 ng (N = 6)	0	6	0	32 % (19)
	3 ng (N = 1)	0	1	0	1 % (15)
	10 ng (N = 7)	2	5	0	2 % (9)
190 dpf	**control** (N = 21)	0	21	0	not measured*
	1.67 ng (N = 22)	0	22	1	not measured*
	3 ng (N = 18)	0	18	1	not measured*
	10 ng (N = 14)	0	14	2	not measured*

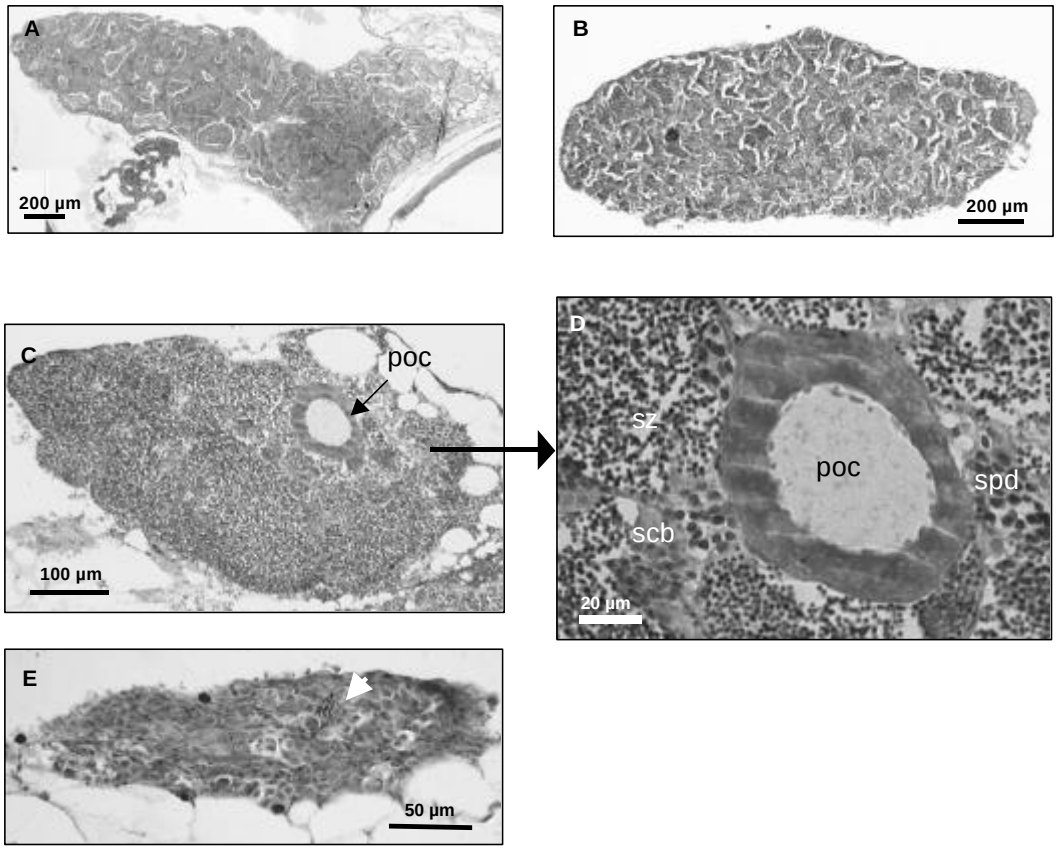

Figure 3.11: Zebrafish testes following exposure to different concentrations of EE2 .

A: **T**estis of a 190-dpf-old fish, grew under control conditions. (3-µm section). For more details see page 35 and page 36 (chapter 1).

B: Part of a testis of a 190-dpf-old fish, exposed to 10 ng EE2 from day 43 to day 71 to illustrate the histological appearance, which is not different from that under control conditions (7-µm section).

C: Part of a testis of a 190-dpf-old fish, exposed to 3 ng EE2 from day 43 to day 7 1, containing one perinucleolar oocyte (**poc**). Oocytes are found in testes in a minor percentage throughout all concentrations (7-µm section).

D: Enlargement of Fig. 3.10.C. The oocyte causes only slight alteration of the testis structure. The male cells, spermatozoa (**sz**); spermatids (**spd**) and spermatocytes B (**sc b**) next to the oocyte (**poc**) appear morphologically normal.

E: Immature testis of a 71-dpf-old zebrafish. A few spermatids (white arrow) are visible, as the most advanced germ cells (7-µm section).

Pay attention to the different scales

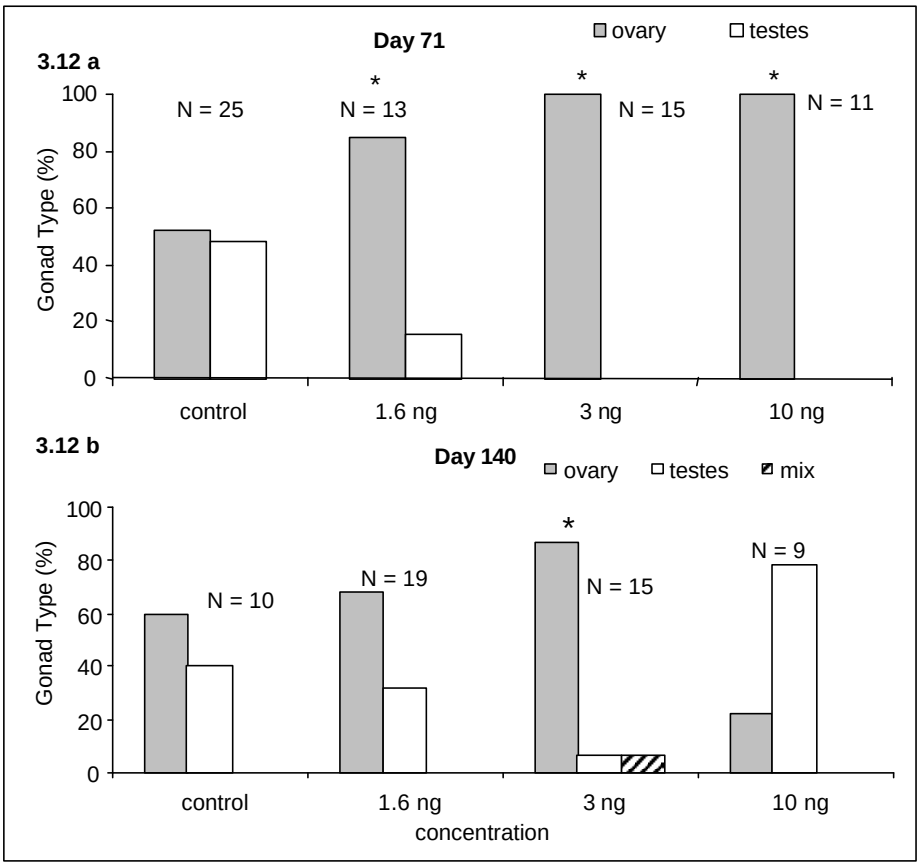

Figure 3.12: Proportions of the different gonad types in zebrafish after exposure to different concentrations of EE2 from day 43 to day 71 pf * significant ;chi²; $p < 0.05$

a: 71-dpf-old zebrafish

b: 140-dpf-old zebrafish

N: Number of individuals

Gonadal sex ratio of adult zebrafish at the end of the experiments

At the end of both experiments, the stage-specific exposure during development (experiment 1) and the exposure to different EE2 concentrations between days 43 and 71 pf (experiment 2), the fish in all test vessels were sexed by macroscopic examination of the gonads after dissection

Gonadal sex ratio of adult fish following stage-specific exposure during development (experiment 1)

The experimental cohort exposed to 10 ng EE2/L from day 15 to day 42 displayed a gonadal sex ratio significantly different from that of the control. This group was composed of 63 % males and 37 %. females, while the control comprised 49 % males and 51 % females. The proportions following exposure to 10 ng EE2/L from day 43 to day 71 or from day 43 to day 99 were 52 % males and 48 % females, and 47 % males and 53 % females, respectively (Figure 3.13a).

Gonadal sex ratio of adult fish exposed to different EE2 concentrations between days 43 and 71 pf (experiment 2)

The control group was composed of 60 % males and 40 % females. This ratio was significantly different from that for all exposed populations (chi^2; $p < 0.05$). The variation between the different concentrations was, however, marginal. They ranged from 52 % male and 48 % female for the 1.67-ng population to 45 % male and 55 % female, and 44 % male and 56 % female for the 3 ng and the 10 ng population, respectively (Figure 3.13b).

It has to be emphasised that the gonadal sex ratio also varies considerably even in the control groups. In the experiment on the stage-specific EE2 effects, the control gonadal sex ratio was 49 % males to 51 % females; in the experiment on the concentration dependency of the EE2 effects, gonadal sex ratio of the control group was 60 % males to 40 % females. These values differ significantly from one another (chi^2-test; $p < 0.05$).

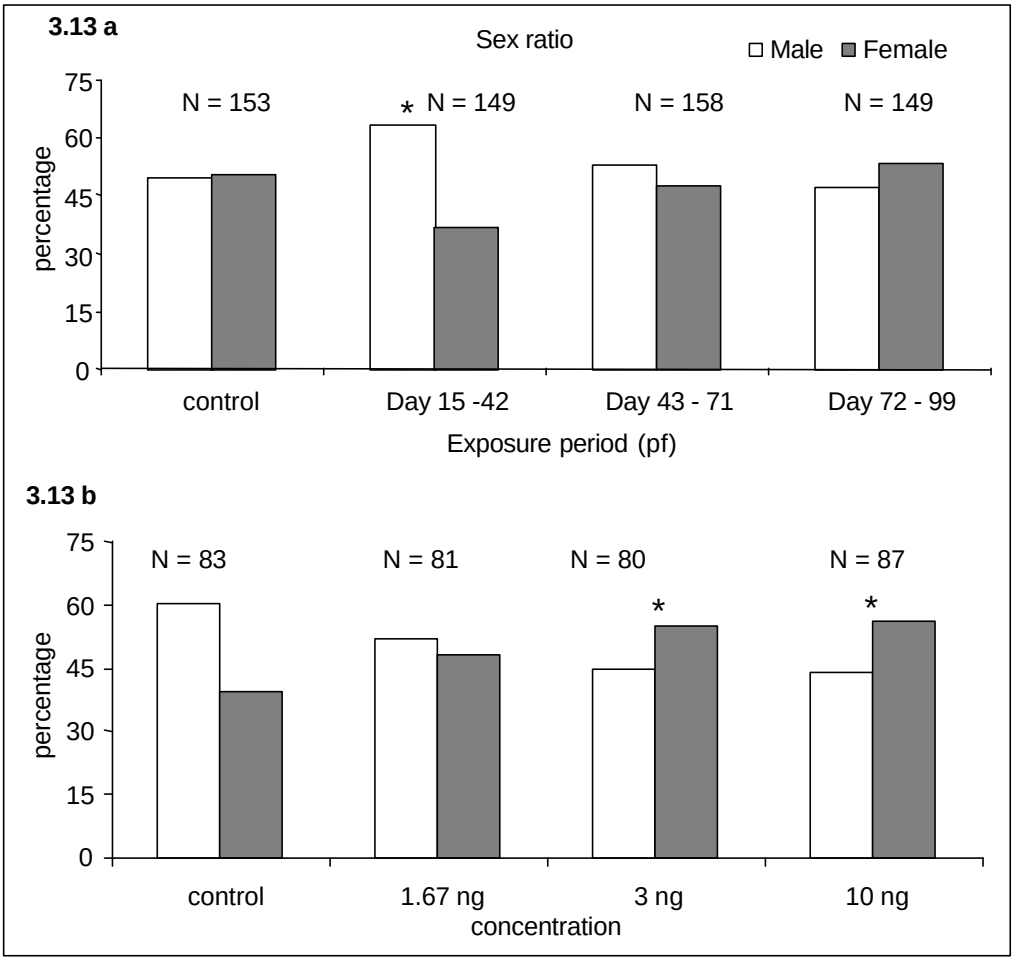

Figure 3.13: Sex Ratio of zebrafish. The sex ratio was determined by macroscopic identification the gonads on day 190 pf.
a: Zebrafish exposed to 10 ng/L EE2 in different developmental periods
b: Zebrafish exposed to different concentrations of EE2 from day 43 to day 71 pf
N: Number of fish; *significant to the respective control group; chi^2; $p < 0.05$

Discussion

The aim of this study was to learn whether there are particular periods of zebrafish development during which the fish are especially sensitive to estrogen exposure, so that effects on the population level are evident, and whether these effects are comparable to those of permanent exposure. Therefore we measured the effect of stage-specific developmental exposure to environmentally realistic concentrations of EE2 upon reproductive parameters and gonadal development in the zebrafish.

Growth

Zebrafish showed a reduced growth rate in terms of body length during exposure to EE2. The reduction of somatic growth can be a reaction of the individual fish to environmental stress (Smolders et al., 2002). In case of EE2 it might be speculated that the amount of energy necessary to metabolise the substance reduces the fraction of energy available for somatic growth. There are several reports in the literature documenting a reduction of somatic growth in estrogen-exposed fish (i.e. Örn et al., 1998, Scholz and Gutzeit 2000, Bjerselius et al., 2001). A growth-related effect on gonadal development and on reproduction could not excluded. The finding that the delay in growth had already been compensated by day 99 pf in all fish but those exposed to the highest concentration, before the onset of spawning of the control group, together with the observed alterations of gonad morphology and of reproduction even after recovery, indicate that the gonadal phenomena were not growth-related.

Reproduction

The onset of spawning was found to be delayed in the group exposed to 10 ng/L EE2 during the sex differentiation period. In zebrafish permanently exposed to EE2 Schäfers et al. (2002) found a delay in the onset of spawning at a concentration as low as 1.7 ng/L. Fish exposed to 10 ng/L did not spawn at all. This was also found for fish permanently exposed to 3 ng/L EE2 (Chapter 2). Histological examination revealed an absence of fish with testes following exposure to 3 ng/L as well as in the 10-ng group and also in fish permanently exposed to 3 ng/L or more. When the exposed fish were given the opportunity to recover under control conditions, all groups in all experiments started to spawn, indicating not a statistical error but an EE2-related retardation of sexual differentiation. The zebrafish is a juvenile hermaphrodite: that is, all zebrafish develop ovaries first, regardless of their genetic sex. The gonads of half of the fish are later transformed into testes, whereas the gonads of

the other half develop into mature ovaries. Exposure to estrogen could be an explanation for a retardation of the transformation process.

The number of eggs spawned per female is highly variable, even under control conditions (Nagel 1986). Besides the normal biological variability, one reason for this could be the number of fish in the individual aquaria. In a small group the reproductive success of a single fish is of greater importance than in a larger group, where a reproductive failure of a single fish can easily be masked by other individuals of the same sex. This study employed 4 females and 8 males as a spawning group, according to (Nagel 1986). This comparatively small group could explain the different results found in the different experiments. Whereas during the stage-specific exposure a significant reduction in the number of eggs laid by fish exposed during the period of sexual differentiation was seen, this was not evident in the second experiment, where the fish were exposed to the same concentration of EE2 during the same time period. Schäfers et al.(2002) found a reduction in fish permanently exposed to EE2 at a concentration of 1.7 ng/L, whereas with higher concentrations no spawning occurred at all, as mentioned above. As demonstrated in Chapter 2, stopping the exposure before the period of sexual differentiation had no effect on the number of eggs laid or on the onset of spawning.

Exposure to 3 ng/L EE2 during the period of sexual differentiation leads to a significant reduction in fertilisation, whereas exposure to 10 ng EE2/L during other periods had no effect on fertilisation. In fish permanently exposed to EE2 the effect is found at a concentration of only 1.7 ng/L (Schäfers et al. 2002), and permanent exposure to 3 ng/L EE2 leads to total reproductive failure (Chapter 2). Male mating behaviour is able to recover after a period without exposure, but fertilisation is evidently not. This result confirmed the experiment of Chapter 2 and of Schäfers et al. In all tests normal mating behaviour was observed following an appropriate recovery period, but in all experiments the fertilisation was still significantly reduced. The endpoints pre-adult growth, time until first reproduction, egg production and fertilisation were correlated. With respect to the results in this study and as confirmed by Schäfers et al. (2002) fertilisation seems to be the most relevant endpoint in terms of population dynamics, followed by the onset of spawning. Individual egg production is fo a parameter of low relevance to population dynamics This is also indicated by the population model developed by Oertel et al. (1991). Apparently a reduction of the number of eggs per female by about 50 % does not influence the survival of the model population. But an alteration in the time until sexual maturity and the production of offspring in a time window

not optimal for surviving will clearly affect wild populations, especially seasonal spawners in boreal regions. The mating is the cue for the female egg production and therefore the cue for the onset of spawning. Reduced fertilisation may have various causes: a direct effect of the substance on spermatogenesis, an effect on released sperm only, or on the eggs by changing the egg membrane so that penetration of intact spermatozoa is hindered or prevented (Bresch et al., 1990). This could not be clarified in our studies.

Gonadal development

The maturation stage of both female and male gametes corresponded to the reproductive findings. Not until the beginning of spawning were mature gonads found, and at the end of the experiments all zebrafish were reproductively active.

The normal phenotypic sex ratio for zebrafish populations is 1 male: 1 female, but with a high variability. For example, Andersen et al. (2000) reported a male to female ratio of 68: 32, Vaughan et al. (2001) a ratio of 56: 44 and (Zott, pers. comm.), 30: 70. This was confirmed by Schäfers (pers. comm.), who found in various life cycle experiments a variation in the male to female ratio between 60: 40 and 40: 60 in the control groups. In our experiments the phenotypic male: female ratios of the controls (determined by macroscopic identification of the gonadal sex at day 190 pf) were 49: 51 and 60: 40. Sex differentiation in fish is a highly labile process and exposure to estrogens or androgens during the critical period of the development can lead to complete sex reversal, as used frequently in aquaculture to produce the economically more valuable sex (Piferrer 2001), (Gimeno et al. 1998), (Baroiller et al. 1999). Temperature can also affect sexual differentiation in some species, as shown by (Fujioka 2001) for honmoroko (*Gnathopogon cacrulescens)* and by (Vaughan et al. 2001) for the zebrafish.

In 71-dpf-old fish examined directly after exposure to EE2 during the period of sexual differentiation, there was a significant difference in the percentage of fish containing ovaries compared to the control group. In the fish exposed to 3 ng/L and to 10 ng/L only ovaries were found. The same result was obtained in 57-dpf-old fish after an exposure to 10 ng/L from day 43 pf (data not shown), but not in 140-dpf-old fish after a two-month period of recovery. At that time no concentration-related difference in the gonad type was visible. These findings confirmed the results of Chapter 2, namely that no male zebrafish were found before stopping the exposure to 3 ng/L, and are in agreement with the studies of (Legler et al., 2001). Concentrations of 3 ng/L EE2 or higher are obviously able to substitute for the falling endogenous estrogen level in genetic males at the beginning of sexual differentiation, which extended the period of juvenile hermaphroditism for as long those fish are exposed. These

experiments indicated that exposure to environmentally realistic concentrations of EE2 did not change the sex ratio, after the exposed fish had been given an opportunity to recover under control conditions. No stage-specific effect of exposure to estrogens on adult sex ratio was found. This lack of effect was not due to temperature or other abiotic factors, since constant and identical regimes in temperature, oxygen, pH and light were maintained throughout all aquaria for all experiments.

This developmental retardation is also found in ovaries, as indicated by the diameter of the perinucleolar oocytes. Immediately after the exposure period a concentration-related reduction of the oocyte diameter could be seen, but not at the end of experiment. The oocyte diameter is a sign of maturing. As described in detail by Selman et al., (1993), the larger the perinucleolar oocytes are, the more developed the ovary is. In the protogynous stage it is impossible to distinguish between genetic females and genetic males. As indicated by the higher percentage of altered ovaries in exposed fish, it is quite possible that one reason for the concentration-dependent difference in the oocyte diameter found directly after termination of the exposure is that some of the oocytes being measured are those of protogynous males immediately before maturation, which are supposed to be smaller than oocytes in genetic females.

In some mature ovaries disturbed areas were found. The number of females containing ovaries with those impairments seemed to be correlated with the exposure concentration, but because so few fish were dissected, this effect could not be firmly established.

On day 140 pf all testes contained sperm, except those exposed to 10 ng/L EE2. This could be one reason for the delay of the onset of spawning in that group. At the end of the experiment all testes contained sperm. No morphological alterations in male zebrafish gonads could be detected, suggesting that if a genetic male is able to finish sexual differentiation, it will do so completely. This was in contrast to the report of van der Ven et al., (2001), who found a decrease in the diameter of the spermatocysts after a three-week exposure of adult zebrafish. Neither the findings of our study nor the results of (van der Ven et al. 2001) are an explanation of the reduced fertilisation. Single atretic perinucleolar oocytes were found in a small percentage (less than 5 %) in sperm-containing, reproductively active testes distributed over all ages and concentrations. This could be interpreted with reference to the genesis of the testis. As male zebrafish are going through the period of juvenile hermaphroditism, it is quite possible that a single oocyte is not absorbed completely for some reason.

Conclusions

It was shown that during the period of sexual differentiation, zebrafish are especially sensitive to exposure to estrogens. In our experiment this period falls between day 40 and day 70 pf. It seems that exposure to EE2 at concentrations of 3 ng/L during that period prolongs the period of juvenile hermaphroditism and leads to reversible alterations of the reproductive parameter. These reproductive effects were of the same quality as in permanently exposed fish, but are less strongly expressed and appear after exposure to higher concentrations only. The recovery time the exposed fish needed until the onset of spawning was shorter and fertilisation was not as much reduced as in permanently exposed fish. A concentration-related skewing of the gonadal sex ratio at the end of the experiments, after giving the fish the possibility to recover, could not be found. The effect of exposure to estrogen on the gonadal development seems to consist in a retardation of the testes development only and not in a permanent shift of the gonadal sex ratio.

Acknowledgement

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References

Andersen, L., Bengtsson, B.-E., Björk, M., Gessbo, A., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K. L., Lundgren, A., Petersen, G. I., Steinholz, A., and Örn, S., (2000). "Zebrafish for Testing Endocrine Disrupting Chemicals", Chemical Nordic council, Copenhagen. TemaNord **555**.

Baroiller, J. F., Guiguen, Y., and Fostier, A., (1999). "Endocrine and environmental aspects of sex differentiation in fish", *Cellular and Molecular Life Sciences* **55**, 910-931.

Bigsby, R., Chapin, R.E., Daton, G.P. Davis, B.J., Gorski, J., Gray, L.E., Howdeshell, K.L., Zoeller, R.T. and vom Saal, F.S. (1999). Evaluating the effects of endocrine disruptors on endocrine function during development. *Environ Health Persp* **107** (suppl 4):113-118.

Bjerselius, R., Lundstedt-Enkel, K., Olsen, H., Mayer, I., and Dimberg, K., (2001). "Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17 â-estradiol", *Aquatic Toxicology* **53** (2), 139-152.

Böhmer, W., Kurzawa, B.: Bestimmung von 17α-Ethinylestradiol mittels GC/MS/MS nach SPE-Extraktion an EMPORE disks, *GIT Labor-Fachzeitschrift*, 10/2000.

Bresch, H., Beck, Ehlermann, D., Schlazus, H., and Urbaneck, M., (1990). "A long-term Toxicity Test comprising reproduction and growth of Zebrafish with 4-Chloroaniline", *Arch. Environ. Contam. Toxicol.* **19**, 419-429.

Colborn, T. and Clement, C. (eds.) (1992). “Chemically induced alterations in sexual and functional development: the wildlife/human connection.”, Priceton Scientific Publishing, Princeton, New Jersey.

Desbrow, C., Routledge, E. J., Brighty, G. C., Sumpter, J. P., and Waldock, M., (1998). "Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening", *Environmental Science & Technology* **32** (11), 1549-1558.

Fujioka, Y., (2001). "Thermolabile sex determination in honmoroko", *Journal of Fish Biology* **59** (4), 851-861.

Gimeno, S., Komen, H., Gerritsen, A. G. M., and Bowmer, T., (1998). "Feminisation of young males of the common carp, *Cyprinus carpio*, exposed to 4-tert-pentylphenol during sexual differentiation", *Aquatic Toxicology* **43** (2-3), 77-92.

Giuellette, L.J, Crain D.A., Rooney, A.A. and Pickford, D.P. (1995). Organization versus activation: the role of endocrine-disrupting contaminants (EDCs) during embryonic development in wildlife. *Environ Health Persp* **103** (Suppl 7):157-164.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, M., Sumpter, J. P., Taylor, T., and Zaman, N., (1997). "Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout", *Environmental Toxicology and Chemistry* **16** (3), 534-542.

Jobling, S., Sheahan, D., Osborne, J. A., Matthiessen, P., and Sumpter, J. P., (1996). "Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to estrogenic alkylphenolic chemicals", Environmental Toxicology and Chemistry 15 (2), 194-202.

Kavlock, R. J., Daston, G. P., DeRosa, C., FennerCrisp, P., Gray, L. E., Kaattari, S., Lucier, G., Luster, M., Mac, M. J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D. M., Sinks, T., and Tilson, H. A., (1996). "Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the US EPA-sponsored workshop", *Environmental Health Perspectives* **104**, 715-740.

Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., and Sumpter, J. P., (2001). "Effects of the synthetic estrogen 17 -ethynylestrdiol on the life-cycle of the fathead minnow (*Pimephales promeales*)", *Environmental Toxicology and Chemistry* **20** (6), 1216-1227.

Legler, J., van der Zee, M., Lanser, P., Jonkers, C. C. H., van der Ven, L., Vethaak, A., van der Burg, B., and Murk, A. J., (2001). "Biological validation of the transgenic zebrafish assay: Effects of (xeno-)estrogens on sexual differentiation and male reproduction", in: Proceedings of the 22th annual meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, USA .

Nagel, R., (1986). "Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio*, Ham.-Buc.)", *Journal of Applied Ichthyology*, 173-181.

Nagel R. (1993). Fish and environmental chemicals – a critical evaluation of tests. In: Braunbeck, T, Hanke W, Segner H (eds. Fish ecotoxicology and ecophysiology. VCH, Weinheim. pp. 174-178.

Nagel R., Isberner K. (1998). “Testing of chemicals with fish – a critical evaluation of tests with special regard to zebrafish.” In: Braunbeck, T, Hinton D. E, Streit B (eds). Fish Ecotoxicology. Birkhäuser, Basel. pp. 337-352.

Oberdorster, E. and Cheek, A. O., (2001). "Gender benders at the beach: Endocrine disruption in marine and estuarine organisms", *Environmental Toxicology and Chemistry* **20** (1), 23-36.

OECD, Organisation for Economic Co-operation and Development, (1992a). "OECD guidelines for testing of chemicals, section 2, Guideline 203: Fish, acute toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992b). "OECD guidelines for testing of chemicals, section 2, Guideline 210: Fish, Early-life stage toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1998). "OECD guidelines for testing of chemicals, section 2, Guideline 212: Fish, Short-term test on embryo and sac-fry stages", Paris, France .

Oertel, D.; Schäfers, C.; Poetke, H.J.; Nagel, R. and Seitz, A (1991). “Simulation of the population dynamics of zebrafish in complex experimental system.” *Verh. Ges. Ökologie*. **20** (2), 865-869

Örn, S., Anderson, P. L., Förlin, L., Tysklind, M., and Norrgren, L., (1998). "The impact on reproduction of an orally administered mixture of selected PCBs in Zebrafish (*Danio rerio*)", *Arch.Environ.Contam.Toxicol.* **35**, 52-57.

Piferrer, F., (2001). "Endocrine sex control strategies for the feminization of teleost fish", *Aquaculture* **197** (1-4), 229-281.

Rodgers-Gray, T.P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M.J., Sumpter, J.P. and Tyler. C.R. (2001). Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces time- and dose-dependent and persistent disruption in gonadal duct development. *Environ Sci Technol* **35**:462-470.

Schäfers, C.; Böhmer, W.; Schmitz, A. and Wenzel, A. (2002). “The relevance of xenoestrogenic effects for fish populations: Full life cycle tests with zebrafish (*Danio rerio*).” *Environmental Toxicology and Chemistry*, in press

Scholz, S. and Gutzeit, H. O., (2000). "17-alpha-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (Oryzias latipes)", Aquatic Toxicology **50** (4), 363-373.

Selman, K., Wallace, R. A., Sarka, A., and Qi, Xiaoping, (1993). "Stages of oocyte development in the zebrafish *Brachydanio rerio*", *Journal of Morphology* **218**, 203-224.

Smolders, R., Bervoets, L., De Boeck, G., and Blust, R., (2002). "Integrated condition indices as a measure of whole effluent toxicity in zebrafish (*Danio rerio*)", *Environmental Toxicology and Chemistry* **21** (1), 87-93.

Stumpf, M., Ternes, T.A., Haberer, K. & Baumann, W. Nachweis von natürlichen und synthetischen Östrogenen in Kläranlagen und Fließgewässern (Determination of natural and synthetic estrogens in sewage plants and river water, German with abstract in English*). Vom Wasser* **87**, 251-261 (1996).

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R. D., and Servos, M., (1999). "Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany, Canada and Brazil", *Science of the Total Environment* **225** (1), 81-90.

Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J., Jegou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Muller, J., RajpertDeMeyts, E., Scheike, T., Sharpe, R., Sumpter, J., and Skakkebaek, N. E., (1996). "Male reproductive health and environmental xenoestrogens", *Environmental Health Perspectives* **104**, 741-803.

Tyler, C. R., Jobling, S., and Sumpter, J. P., (1998). "Endocrine disruption in wildlife: A critical review of the evidence", Critical Reviews in Toxicology **28** (4), 319-361.

van der Ven, L., van den Brandhof, E. J., Robinson, J., de Vlugt, S., and Wester, P. W., (2001). "Supplemental histological determents in the evaluation of endocrine disruption in zebrafish", in: Proceedings of the 11th annual meeting of the Society of Environmental Toxicology and Chemistry, Madrid, Spain .

Vaughan, M., van Egmond, R., and Tyler, C. R., (2001). "The effect of Temperature on Sexual Differentiation and Development in the Zebrafish, *Danio rerio*", in: Proceedings of the 11th annual meeting of the Society of Environmental Toxicology and Chemistry, Madrid, Spain .

General Discussion

Introduction

The present thesis investigated the estrogen-related alterations of gonadal histology and of reproductive parameters in the zebrafish (*Danio rerio*). In the first section normal gonadal development was described in detail, with special attention to the period of histological differentiation of the gonads. The results serve as a basis for comparison with gonadal histopathology following estrogen exposure. As shown in this thesis, the zebrafish is a juvenile hermaphrodite; that is, all individuals initially develop ovaries, and subsequently in half of the population the ovaries transform into testes, while the other half continues development into functional females. It is essential to keep this phenomenon in mind when using zebrafish as a model species in aquatic toxicology. Otherwise the normal ontogeny could be misinterpreted as a reaction to endocrine-altering chemicals.

The second approach was to compare developmental and reproductive disturbances resulting from chronic exposure to a potent synthetic estrogen (ethynylestradiol) with those caused by a weakly estrogenic chemical (bisphenol A). Effects of bisphenol A (BPA) on fertilisation were found only at concentrations much higher than usually present in surface waters. On the other hand, permanent exposure to environmentally realistic concentrations of ethynylestradiol (EE2) caused a total failure of reproduction and would lead to extinction of the population. Histological analysis indicated a suppression of the transformation from female to male gonads, related to the degree of exposure to EE2. Even after 50 days of recovery a significant proportion of zebrafish containing ovaries did not participate in reproduction. Morphological alterations in fertile testes were not observed.

The aim of the third thematic line of this thesis was to find a period in development during which the zebrafish is particularly sensitive to estrogen exposure. Therefore the fish were exposed to EE2 in different developmental periods. Following exposure during the period of sexual differentiation, zebrafish displayed effects on reproduction and gonadal histology that were comparable to those evoked by exposure throughout their life cycle. These lasting reproductive effects were not as strong as in permanently exposed fish, but impaired the population. This estrogen-sensitive period corresponded to the period in which the transitory gonads were found. Exposure during developmental stages other than the period of sexual differentiation did not have histopathological or reproductive effects.

In this chapter the gonadal development of the zebrafish is presented in the context of teleost gonadal differentiation in general. The results are compared with literature reports and possible physiological explanations for the findings are discussed. The possibilities of

comparing the results described in this thesis to other model species are explored. The limitations of an inter-species comparison, considering the different endpoints used in different species and in different reports, will be discussed. Finally, the adaptation of the results of this thesis to existing and impending test guidelines will be examined.

Gonad differentiation in teleost fish

Gonadal development varies considerably in fish. The majority of fish species are gonochoristic; that is, undifferentiated primordial germ cells (PGCs) develop into ovaries or testes during ontogenesis. PGCs develop at the dorsal wall of the coelomic cavity at the site of the future gonadal ridges. During the larval period, the gonad anlage gradually arises from the PGCs and surrounding somatic cells (Yamamoto 1969, Timmermans 1987), and grows into morphologically differentiated gonads during puberty. Such fish do not undergo a sex change. In addition to gonochorism, several forms of hermaphroditism are found, including sequential hermaphroditism (either protandry or protogyny) as well as simultaneous hermaphroditism. Protrandrous fishes develop first as males and then later change to females, whereas protogynous fishes develop first as females and then later become males. Simultaneous hermaphrodites are capable of releasing viable eggs or sperm during the same spawning (Sadovy and Shapiro 1987, Helfman et al. 1997, Nakamura et al. 1998). Protogyny is by far the most common form of hermaphroditism, probably reflecting the fact that most teleosts, including gonochoristic species, differentiate first as non-functional females as speculated by Helfman et al. (1997). As demonstrated in Chapter 1, the zebrafish is a juvenile protogynous hermaphrodite; that is, all zebrafish gonads differentiate first into ovaries and in half of the population the ovaries are transformed into testes. The period of sexual differentiation in the zebrafish starts around day 40 post-fertilization (pf) and is completed around day 70 pf.

Hormones

Sexual differentiation of developing gonads in fish is considered to be under the control of steroid hormones (Baroiller et al. 1999, Jalabert et al. 2000, Nagahama 2000, Piferer 2001). Yamamoto (1969) suggested in the late sixties that sex steroids are the natural inducers of sex in fish: generally, androgens induce phenotypic masculinization (except for cases of paradox feminization) and estrogens induce phenotypic feminization (Yamamoto 1969, Piferer 2001). Since then, numerous studies including recent molecular work (see Baroiller et al. 1999, Nagahama 2000) have corroborated and extended the original hypothesis of

Yamamoto (1969), although contradictory observations do exist (e.g., Kawahara and Yamashita 2000).

Hormones and their receptors are transcribed at precise times during development and within a specific dose range in order to activate specific target genes and to induce tissue differentiation. Environmental compounds that mimic estrogen action may become effective through the same signalling pathways as the endogenous hormone, but they are not under co-ordinate control as are the endogenous factors; therefore environmental estrogens may irreversibly disturb developmental processes and lead to phenotypic abnormalities.

In experiments with the medaka, *Oryzias latipes*, Yamamoto (1969) demonstrated that, independent of genetic sex determination, estrogen treatment leads to differentiation into the female phenotype, and androgen treatment leads to the male phenotype. Hormonal manipulation of sex is widely practised in aquaculture, where monosexual populations are often desired (Hunter and Donaldson, 1983). Depending on the species and on the economic requirements, all-male populations (i.e., of cichlids) or monosexual female stocks (i.e., salmonids) have been preferred. Successful masculinisation of a cichlid through androgen administration was first demonstrated by Eckstein and Spira (1965) on *Oreochromis aureus*. In the culture of salmonids, estrogen treatments have been successful in producing nearly 100 % females in several species (Hunter and Donaldson, 1983).

In the zebrafish, too, exposure to estrogens like EE2 influence gonadal development. In contrast to the above-mentioned salmonids, in the zebrafish the influence of environmentally realistic concentrations of estrogens appears to be reversible. The results of this investigation indicate that estrogens are able to suppress the transformation from ovaries into testes. It was shown in Chapters 2 and 3 that the development of the testes was retarded as long as the fish was exposed. Histological observations revealed that the gonadal development had been stopped in the protogynous stage. The same results were obtained when the fish were exposed only during the period of sexual development. As mating behaviour is the cue for oocyte maturation and oocytes remain in the developmental stage of perinucleolar oocytes until shortly before maturing (Selman et al. 1993), it is not possible to distinguish female zebrafish from protogynous males using histological analysis. Only after the genetic males have ceased to be exposed are they able to develop into phenotypically functional males. It may be speculated that the external estrogen level is able to substitute for the falling endogenous estrogen level, which normally allows the genetic male to develop testes. This inference was supported by Fenske (2002), who found a vitellogenin induction in exposed males but not in those that were no loner exposed. If the timing of the estrogen exposure was not exact, zebrafish were unaffected. As demonstrated

in Chapter 3, exposure to 3 ng/L EE2 during the period of sexual differentiation completely prevented the transformation from ovaries into testes, but exposure to a concentration more than three times higher during other periods had no discernible effects.

The brain-pituitary axis

The factors that trigger the onset of sex-specific steroidogenesis in undifferentiated gonads of fish remain unknown to date. The brain-pituitary-gonad (BPG) axis, which regulates gonadal steroidogenesis in the reproducing animal, is thought to be quiescent in pre-pubertal fish (Schulz and Goos 1999). The major anatomical structures in this axis, along with the hormones by means of which their influence is exerted, are largely conserved across vertebrate taxa (e.g. Demski, 1984). While the mechanisms that maintain the steady-state functioning of this axis are largely similar, marked differences exist in the manner by which the axis is generated (Francis 1992). In mammals the gonads differentiate early relative to other organs. Gonadal products determine the fate of the gonoducts, the relevant central nervous system structures and the pituitary. Francis (1992) postulated that in teleosts the polarity of these structures may be reversed. Instead of the gonads determining the fate of the relevant brain structures, events in the brain may determine the fate of the gonads. The brain must have developmental primacy in any sequential hermaphrodite in which sex change is under social control. The only way behaviour can affect the gonads is through the brain. If, in fact, the brain initiates sex differentiation in teleosts, this would of course markedly distinguish them from mammals, and might provide an explanation for the difference in sexual instability in the different taxa. The brain is more subject to environmental influence than are gonads themselves (Francis 1992).

Conclusions

This thesis demonstrated that the zebrafish is a protogynous hermaphrodite. Exposure to environmental concentrations of 17α-ethynylestradiol during the period of sexual differentiation appears to be able to suppress this transformation and to prolong the protogynous stage of gonadal development as long as the zebrafish is exposed. It could be speculated that external estrogen is able to substitute for the falling endogenous estrogen level. The suppression of the transformation is reversible once the external estrogen is removed. After termination of exposure the genetic males are able to develop functional testes, but with a reduced fertilization ability. It is necessary to take account of this kind of gonadal development, so that the gonad ontogenesis of the zebrafish will not be misinterpreted as a reaction to exposure regimes. Unlike the potent synthetic estrogen 17α-

ethynylestradiol, the weakly estrogenic chemical bisphenol A affects only fertilization. and only at environmentally unrealistic concentrations. One reason for the difference in estrogenic potency could be the difference in the molecular structures. As shown on pages 7 and 8, the chemical structures of 17α- ethynylestradiol and the natural estrogen 17â-estradiol are very similar, and quite different from that of bisphenol A.

Zebrafish in full-life-cycle tests

The hypothesis that wildlife may have been negatively affected as a consequence of exposure to chemicals that interact with the endocrine system has led to a surge in scientific effort to evaluate chemical pollutants for hormonal activity (Tyler et al., 1998). A wide range of comparable cheap and fast *in vitro* assays are now available for screening endocrine disruption (Zacharewski 1997). However, *in vitro* systems have little relevance in wildlife toxicology, as *in vitro* effects are not necessarily predictive of *in vivo* effects (Laws et al., 1996). In addition, *in vitro* systems are not suitable for population-relevant endpoints such as reproduction, fertilisation and hatching success, and cannot detect effects on the F1- or the F2-generation. One integrative approach for the assessment of ecologically relevant effects of chemicals in sublethal concentrations on growth and reproduction can be full-life-cycle or multi-generation tests. In a fish full-life-cycle or multi-generation test, the species concerned are exposed in a flow-through system or in semi-static conditions. Depending on the nature and solubility of the test substance, it is added either to the water or to the fish food. Exposure of the fish over the whole life span, including the reproductive period, guarantees that the chemical can act during a potentially unknown sensitive developmental stage and enables a determination of reproductive alterations. A fish used for a full-life-cycle experiment should be small, easy to handle and complete a life cycle in a few months only. The zebrafish meets these criteria. The life cycle is about 4 months, and, in the laboratory, the fish can be stimulated to breed throughout the year, which makes it appropriate for multi-generation tests and therefore suitable for assessing toxic effects of chemicals on development and reproduction (Nagel 1993, Nagel, 1994, Nagel and Isberner 1998, Andersen et al., 2000). Moreover, the zebrafish is one of the recommend fish species in several international guidelines for chemical toxicity assessments. It was shown in this thesis that the period of sexual differentiation is vulnerable to exposure to estrogens. Other species commonly used for fish full-life-cycle tests are the medaka (*Oyzias latipes*) and the fathead minnow (*Pimephales promelas)*. Both species are also recommended in the above-mentioned test guidelines. With respect to overall physiological sensitivity, zebrafish, medaka and fathead minnow seem not to differ substantially but the comparison is difficult, given the

differences in exposure protocols, endpoints etc between individual studies (Schäfers et al. 2002, Segner et al. 2002, Länge et al., 2001).

Fish full-life-cycle protocols for testing endocrine-active substances

Full-life -cycle tests are designed to assess chronic toxicity. For the assessment of endocrinologically active substances adequate parameters are lacking, and it is still not clear how the different model species react.

It is important to know the ontogeny of sexual differentiation of the model fish, so that the fish will not be exposed during a period of development in which it is insensitive to estrogen exposure. As demonstrated in Chapter 3 for the zebrafish, the most sensitive period for exposure to estrogens is the period of sexual differentiation between day 40 and day 70, whereas exposure during other developmental periods was ineffective. Gray et al. (1999) obtained similar results for the medaka, but the in the medaka this period of sexual differentiation is around day 3 post-hatching, and exposure during the period in which the zebrafish is sensitive would not have any effect (Gray et al. 1999). Furthermore, the affected parameters differ between species. For instance, sex ratio is a well-established endpoint in the fathead minnow and in the medaka. Male mature fathead minnows develop breeding tubercles, which makes it easy to distinguish between males and females (Miles-Richardson et al., 1999). And in the Japanese medaka monosex populations are available. Typically, white females (X^rX^r) and orange-red males (X^rY^R) were used to produce the test population. Mating a white female with an orange-red male results in a progeny composed of 50% white genetic females (X^rX^r) and 50% orange-red genetic males (X^rY^R) (Yamamoto 1958). In contrast, for the zebrafish the sex ratio is not a suitable endpoint. This species has a 1 male : 1 female ratio as seen in Chapter 1, where the gonadal sex of 153 individuals was histologically analysed; there was a high variability in the groups of control fish, ranging from 30% male: 70% female to 70% male: 30% female, o as demonstrated in Chapter 3 and in published reports (i.e. Andersen et al., 2000, Vaughan et al., 2001, Schäfers et al. 2002). Furthermore, external sexing is possible in adult zebrafish only at the end of the reproductive cycle.

Due to the different gonadal development of the three species (the fathead minnow and the medaka are gonochoristic fish, whereas the zebrafish is a protogynous juvenile hermaphrodite), estrogenic effects in zebrafish need to be evaluated and interpreted differently from those observed in gonochoristic species. As demonstrated in Chapters 2 and 3, a potent synthetic estrogen like EE2 is obviously able to suppress the transformation from protogynous ovary-containing fish to phenotypically functional males. This finding is

supported by Legler et al. (2001), who detected no males in a group of zebrafish as long as they were exposed to 10 nmol/L E2. After the exposure was terminated and an appropriate recovery time had elapsed, the group comprised only males with morphologically normal testes.

The different mating behaviour of the three species makes it difficult to compare the reproductive output. For the fathead minnow single pairs were transferred to breeding tanks (Sohoni et al., 2001), which makes it possible to measure the single reproductive output of the individual fish. To measure the reproductive performance of medaka, one male and two females were kept together (Shioda and Wakabayashi, 2000), which makes it impossible to determine the reproductive output of a single female. In the pair-breeding species the reproductive output of the individuals is difficult to extrapolate to the population level.

The zebrafish, in contrast, is a group spawner, so that up to 30 individuals were kept together for measuring reproduction. A reproductive failure of a single individual could easily be masked by other fish of the same sex. This spawning behaviour makes it easy to analyse the reproductive output and therefore the survival of the population (Oertel 1991) but impossible to find out how much a single fish contributes to the reproductive output of the group and how endocrine-active chemicals influence the reproductive system on the individual level, without histopathological analysis.

Although both the medaka and the fathead minnow are gonochoristic fish, they react differently to estrogen exposure, indicating different key physiological steps in regulation of this process (Baroiller and Guiguen 2001). Exposure of adult fathead minnow makes the males less fertile, as indicated by a reduction of spermatozoa and an increase in spermatogonia (Panter et al., 1998, Miles-Richardson et al., 1999). The same was found by Sohoni et al. (2001) following lifetime exposure to bisphenol A. Exposure to more potent estrogens such as EE2 leads to total absence of male fathead minnows (Länge et al., 2001). No intersex or testes-ovaries were found in the fathead minnow. In contrast, prolonged exposure of male medaka to an estrogen agonist beginning around the period of gonadal differentiation is most effective for the development of testes-ovaries, but this intersex condition can also induced when exposure begins at later life stages (Gray et al. 1999). Permanent male-to-female sex reversal can be induced through egg microinjection of o,p ' -DDT (Metcalfe et al., 2000) and following an injection of 0.5 or 2.5 ng EE2/egg (Papoulias et al., 2000). These authors did not find intersex. These results were supported by (Scholz and Gutzeit 2000), who found a complete sex reversal following exposure of juvenile medaka to 100 ng/l EE2. At lower EE2 concentrations, which did not result in sex reversal, no alteration of testicular structure was detected and male fertility appeared to be unchanged (Scholz and

Gutzeit 2000). This was explained by Shibata and Hamaguchi (1988) on the basis of the sexual bipotentiality of the spermatogonia. They exposed adult male medaka to estradiol and, depending on the exposure period, ovotestes or ovaries were found. These effects were reversible: after the fish had had an opportunity to recover in tap water, their testes reappeared.

However, the zebrafish has the advantage of a shorter test duration (the fathead minnow needs 5-6 months compared to 3-4 months in zebrafish to develop from the egg to the reproducing adult), which enables more sensitive evaluations in terms of more statistical power in interpreting an endpoint (decreased fertilisation despite normal egg numbers and mating behaviour) that is similar to that used for studies of estrogenic effects in mammals. Moreover, the relevance of decreased fertilisation for population dynamics is more easy to interpret quantitatively than the relevance of altered sex ratios (Schäfers et al. 2002).

Test systems and guidelines for testing endocrine-active chemicals in the aquatic environment

In laboratory experiments, short (acute) exposures are usually employed, yet the effects of hormone mimics may not become manifest immediately. In addition, certain life stages are more sensitive than others to exposure to endocrine modulators. As demonstrated in Chapter 3, zebrafish are particularly sensitive to estrogens during the period of sexual differentiation, whereas exposure during other periods has no effects.

It is evident that the suggestion of CSTE (1999) to use fish early-life-stage tests as an estrogenic screen is not suitable for zebrafish and many other fish. Similarly, the existing OECD-Test guidelines are not sufficient to detect possible endocrine effects (OECD 1984, OECD 1992a, OECD 1992b, OECD 1998, OECD 2000). Only in a fish full-life-cycle test are all developmental stages, including reproduction, of an individual included. Moreover, this is the only way to evaluate the potential impacts of chemicals on population level. A corresponding endocrine screening program has been developed by the US-EPA (Fenner-Crisp et al. 2000) and a new OECD Test guideline is in progress (Huet 2000). With a candidate pool of over 85,000 potential endocrine-active chemicals (EACs), a priority-setting process would be needed to sort this universe of chemicals and focus on identifying those substances which should go through the screening program in a reasonable timeframe (Fenner-Crisp et al., 2000). A hierarchical system has to be established, beginning with comparing the chemical structures of the potential EACs and validation by receptor assays. The first screening should be followed by an *in vivo* fish testing scheme, based on hierarchical tiers. It is still under discussion which and how many low-cost short-term

methods will be established, but it is generally accepted that the highest-priority test system has to be a fish full-life-cycle test (Ashby et al. 1997, Huet 2000).

In order to develop an *in vivo* screening assay, the results of the thesis can be used to support a test which includes an exposure of zebrafish from 3 weeks onwards until first reproduction (mating plus spawning). Endpoints for the estrogenic activity would mainly be "delay in first reproduction", "vitellogenin induction" and "histological analysis of gonad development". Vitellogenin induction is a rapid reaction to exposure to estrogens of both male and female zebrafish (Andersen et al. 2000, Fenske 2002) as well as other test species (Harries et al. 1997, Harries et al. 2000, Flammarion et al. 2000). In addition vitellogenin induction is detectable, without stopping the assay, far beyond the beginning of reproduction, and as shown by Fenske (2002) it is as sensitive as fertilization in the zebrafish. As also demonstrated by Fenske (2002), vitellogenin induction is reversible and usable only as an indicator of current estrogenic exposure, but not sufficient to predict reproductive effects. For the prediction and explanation of possible reproductive effects a histopathological analysis is necessary, although it is not as sensitive as vitellogenin induction and fertilization.

The suggested screening assay is less time- and work-effective than a full-life-cycle test because it starts with a life stage which does not need much care anymore, but it provides more reliable information on the estrogenic activity of the test compound. The test period fully covers the "sensitive window", i.e. the time of sexual differentiation in zebrafish development. By this means "organisational effects" of chemicals on zebrafish sexual development can be detected, even if these effects are not mediated through the estrogen receptor. If the test indicates an effect, it can easily be expanded to a life-cycle study, excluding only the first three weeks of development, which are evidently irrelevant to estrogenic effects (Chapter 2, Schäfers et al. 2002). The assay should eliminate false negative results, as the data in Chapters 2 and 3 indicate that a delay of a week or more until first reproduction is likely to indicate an estrogenic effect.

Conclusion

This work demonstrated the use of gonadal histopathology as a valuable parameter for full-life-cycle tests of zebrafish exposed to endocrine-active substances. Gonad histopathology reliably predicts reproductive effects at the population level before the onset of spawning. These estrogen-related effects could be detected only following exposure during the estrogen-sensitive period of sexual differentiation. This can minimise the effort required for a full-life-cycle test, because zebrafish at that age are not as sensitive to

handling stress as hatched fry. It should be kept in mind that gonad histopathology is not as sensitive as, i.e., vitellogenin induction and fertilization.

General conclusion

The present work presents a description of the morphology of gonad differentiation in developing zebrafish. The zebrafish is a protogynous hermaphrodite. Exposure to environmental concentrations of 17α-ethynylestradiol during the period of sexual differentiation appears to be able to suppress this transformation and to prolong the protogynous stage of gonadal development as long as the zebrafish is exposed. After termination of exposure the genetic males are able to develop functional testes, but with a reduced fertilization ability. This gonadal development of the zebrafish should be kept in mind when using the species as a test organism. Unlike the potent synthetic estrogen 17α-ethynylestradiol, the weakly estrogenic chemical bisphenol A causes effects in fertilization only, and at environmentally unrealistic concentrations. Exposure during different developmental periods revealed a stage-specific estrogen sensivity during the period of sexual differentiation. Exposure during that period delays testes differentiation and alters reproductive parameters. The effects are of the same quality but not as persistent as those of permanent exposure, and are visible at higher concentrations only.

This work demonstrated the use of gonad histopathology as a valuable parameter for full-life-cycle testing of zebrafish exposed to endocrine-active substances. Gonad histopathology reliably predicts reproductive effects at the population level before the onset of spawning. These estrogen-related effects were associated only with exposure during the estrogen-sensitive period of sexual differentiation. Hence the effort required for a full-life-cycle test can be minimised, because zebrafish at that age are not as sensitive to handling stress as hatched fry.

References

Andersen, L., Bengtsson, B.-E., Björk, M., Gessbo, A., Holbech, H., Hylland, K., Norrgren, L., Pedersen, K. L., Lundgren, A., Petersen, G. I., Steinholz, A., and Örn, S., (2000). "Zebrafish for Testing Endocrine Disrupting Chemicals", Chemical Nordic council, Copenhagen. *TemaNord* **555**.

Ashby, J., Houthoff, E., Kennedy, S. J., Stevens, J., Bars, R., Jekat, F. W., Campbell, P., VanMiller, J., Carpanini, F. M., and Randall, G. L. P., (1997). "The challenge posed by endocrine-disrupting chemicals", *Environmental Health Perspectives* **105** (2), 164-169.

Baroiller, J.F., Guguin, Y. and Fostier, A. (1999). Endocrine and environmental aspects of sex differentiation in fish. *Cell Molec Life Sci* **55**:910-931.

Baroiller, J. F. and Guiguen, Y., (2001). "Endocrine and environmental aspects of sex differentiation in gonochoristic fish", in: Scherer, G. and Schmid M. Eds. Genes and Mechanisms in Vertebrate Sex Determination.

CSTE (European Committee on Toxicity, Ecotoxicity and Environment) (1999). “Opinion on human and wildlife health effects of endocrine disrupting chemicals, with emphasis on wildlife and ecotoxicology test methods.” Brussels. 96 pp.

Demski, L.S., (1984). “The evolution of neuroanatomical substrates of reproductive behaviour: sex steroid and LHRH-specific pathways including the terminal nerve”, *Am. Zool*. **24**, 809-830.

Donaldson, E. M. and Hunter, J. R., (1982). "Sex control in fish with particular reference to salmonids", *Can.J.Fish.Aquat.Sci*. **39**, 99-110.

Eckstein, B. and Spira, M. (1965). “Effect of sex hormones on the gonadal differentiation in a cichlid, *Tilapia aurea*”. *Biol. Bull.*, **129**, 482-489.

Fenner-Crisp, P. A., Maciorowski, A. F., and Timm, G. E., (2000). "The endocrine disruptor screening program developed by the US Environmental Protection Agency", *Ecotoxicology* **9** (1-2), 85-91.

Fenske, M. (2002). “Developmental exposure of zebrafish (*Danio rerio* Hamilton-Buchanan, 1822) to endocrine active substances: Impact on VTG, aromatase and sexual differentiation” PhD-thesis at the University of Halle-Wittenberg. Germany

Flammarion, P., Brion, F., Babut, M., Garric, J., Migeon, B., Noury, P., Thybaud, E., Tyler, C. R., Palazzi, X., and Tyler, C. R., (2000). "Induction of fish vitellogenin and alterations in testicular structure: Preliminary results of estrogenic effects in chub (*Leuciscus cephalus*)", *Ecotoxicology* **9** (1-2), 127-135.

Francis, R. C., (1992). "Sexual lability in teleosts: developmental factors", *The Quarterly Review of Biology* **67** (1), 1-18.

Gray, M. A., Teather, K. L., and Metcalfe, C. D., (1999). "Reproductive success and behavior of Japanese medaka (Oryzias latipes) exposed to 4-tert-octylphenol", *Environmental Toxicology and Chemistry* **18** (11), 2587-2594.

Harries, J. E., Runnalls, T., Hill, E., Harris, C. A., Maddix, S., Sumpter, J. P., and Tyler, C. R., (2000). "Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*)", *Environmental Science & Technology* **34** (14), 3003-3011.

Harries, J. E., Sheahan, D. A., Jobling, S., Matthiessen, P., Neall, M., Sumpter, J. P., Taylor, T., and Zaman, N., (1997). "Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout", *Environmental Toxicology and Chemistry* **16** (3), 534-542.

Helfman, G. S., Colltte, B. B. & Facey, D. E. (1997). *The diversity of fishes.* Blackwell Science.

Huet, M. C., (2000). "OECD activity on endocrine disrupters test guidelines development", *Ecotoxicology* **9** (1-2), 77-84.

Hunter, G.A. and Donaldson, E.M. (1983). “Hormonal sex control and its application to fish culture”. In Hoar, W.S., Randell, D.J. and Donaldson, E.M. (eds.), “Fish Physiology”, Vol. 9B, pp. 223-303, Academic Press , New York.

Jalabert,B., Baroiller, J.F., Breton, B., Fostier, A., Le Gac, F., Guiguen, Y. and Monod, G. (2000). Main neuro-endocrine, endocrine and paracrine regulations of fish reproduction, and vulnerability to xenobiotics. *Ecotoxicology* **9**:25-40.

Kawahara, T., Yamashita, I. (2000) Estrogen-independent ovary formation in the medaka fish, *Oryzias latipes*. *Zool Sci* **17**:65-68.

Länge, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G. H., and Sumpter, J. P., (2001). "Effects of the synthetic estrogen 17a-ethynylestrdiol on the life-cycle of the fathead minnow (*Pimephales promeales*)", *Environmental Toxicology and Chemistry* **20** (6), 1216-1227.

Laws, S. C., Carey, S. A., Kelce, W. R., Cooper, R. L., and Gray, L. E., (1996). "Vinclozolin does not alter progesterone receptor (PR) function in vivo despite inhibition of PR binding by its metabolites in vitro", *Toxicology* **112** (3), 173-182.

Legler, J., van der Zee, M., Lanser, P., Jonkers, C. C. H., van der Ven, L., Vethaak, A., van der Burg, B., and Murk, A. J., (2001). "Biological validation of the transgenic zebrafish asay: Effects of (xeno-)estrogens on sexual differentiation and male reproduction", in: *Proceedings of the 22th annual meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, USA* .

Metcalfe, T. L., Metcalfe, C. D., Kiparissis, Y., Niimi, A. J., Foran, C. M., and Benson, W. H., (2000). "Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*)

exposed to o,p '-DDT in water or through maternal transfer", *Environmental Toxicology and Chemistry* **19** (7), 1893-1900.

Miles-Richardson, S. R., Kramer, V. J. , Fitzgerald, S. D., Render, J. A., Yamini, B., Barbee, S. J., and Giesy, J. P., (1999). "Effects of waterborne exposure of 17 beta-estradiol on secondary sex characteristics and gonads of fathead minnows (Pimephales promelas)", *Aquatic Toxicology* **47** (2), 129-145.

Nagahama, Y. (2000). Gonadal steroid hormones: major regulators of gonadal sex differentiation and gametogenesis in fish. In: Norberg B et al. (eds). Reproductive physiology of fish. University of Bergen Press, Bergen. pp. 211-222.

Nagel R. (1993). Fish and environmental chemicals – a critical evaluation of tests. In: Braunbeck, T, Hanke W, Segner H (eds. Fish Ecotoxicology and Ecophysiology. VCH, Weinheim. pp. 174-178.

Nagel, R., (1994). "Complete life cycle tests with zebrafish- a critical assessment of the results", in: Müller, R. and Lloyd, R. Sublethal and chronic effects of pollutants on Freshwater Fish. FAO Fishing New Book, Blackwell Science, 371 pages , 188-195.

Nagel R., Isberner K. (1998). “Testing of chemicals with fish – a critical evaluation of tests with special regard to zebrafish.” In: Braunbeck, T, Hinton D. E, Streit B (eds). Fish ecotoxicology. Birkhäuser, Basel. pp. 337-352.

Nakamura, M. and Kobayashi. T., Chang XT, Nagahama Y (1998). “Gonadal sex differentiation in teleost fish.” *J Exp Zool* **281**, 362-372.

OECD, Organisation for Economic Co-operation and Development, (1984). "OECD guidelines for testing of chemicals, section 2, Guideline 204: Fish, prolonged toxicity test: 14-day Study", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992a). "OECD guidelines for testing of chemicals, section 2, Guideline 203: Fish, acute toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1992b). "OECD guidelines for testing of chemicals, section 2, Guideline 210: Fish, Early-life stage toxicity test", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (1998). "OECD guidelines for testing of chemicals, section 2, Guideline 212: Fish, Short-term test on embryo and sac-fry stages", Paris, France .

OECD, Organisation for Economic Co-operation and Development, (2000). "OECD guidelines for testing of chemicals, section 2, Guideline 215: Fish, Juvenile Growth Test", Paris, France .

Oertel, D.; Schäfers, C.; Poetke, H.J.; Nagel, R. and Seitz, A (1991). “Simulation of the population dynamics of zebrafish in complex experimental system.” *Verh. Ges. Ökologie*. **20** (2), 865-869

Panter, G. H., Thompson, R. S., and Sumpter, J. P., (1998). "Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oestrone", *Aquatic Toxicology* **42** (4), 243-253.

Papoulias, D. M., Noltie, D. B., and Tillitt, D. E., (2000). "An in vivo model fish system to test chemical effects on sexual differentiation and development: exposure to ethynylestradiol", *Aquatic Toxicology* **48** (1), 37-50.

Piferer, F. (2001). Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture* **197**:229-281.

Piferrer, F. and Donaldson, E. M., (1992). "The comparative effectiveness of the natural and synthetic estrogen for the direct feminization of cinook salmon (*Oncorhynchus tshawytscha*)", *Aquaculture* **106**, 183-193.

Ross, R.M. (1978). “Reproductive behaviour of the anemonefish *Amphiprion melanopus* on Guam”, *Copeia*, 103-107

Sadovy, Y. and Sharpiro, D. Y., (1987). "Criteria for the diagnosis of Hermaphroditism in Fishes", *Copeia* **1**, 136-156.

Schäfers, C.; Böhmer, W.; Schmitz, A. and Wenzel, A. (2002). “The relevance of xenoestrogenic effects for fish populations: Full life cycle tests with zebrafish (*Danio rerio*).” *Environmental Toxicology and Chemistry*, in press

Scholz, S. and Gutzeit, H. O., (2000). "17-alpha-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (Oryzias latipes)", *Aquatic Toxicology* **50** (4), 363-373.

Schulz, R.W. and Goos, H.J.Th. (1999). Puberty in male fish: concepts and recent developments with special reference to the African catfish (*Clarias gariepinus*). *Aquaculture* **177**:5-12.

Segner, H., Fenske, M., Maack, G., Schäfers, C. and Wenzel, A. 2001: “Life-time exposure to ethynylestradiol induces irreversible infertility in the zebrafish *Danio rerio*” in prep.

Selman, K., Wallace, R. A., Sarka, A. & Qi, X. (1993). “Stages of oocyte development in the zebrafish *Brachydanio rerio*.” *Journal of Morphology* **218**, 203-224.

Shibata, N. and Hamaguchi, S., (1988). "Evidence for the Sexual bipotentiality of spermatogonia in the fish, Oryzias latipes", *Journal of Experimental Zoology* **245**, 71-77.

Shioda, T. and Wakabayashi, M., (2000). "Effect of certain chemicals on the reproduction of medaka (*Oryzias latipes*)", *Chemosphere* **40** (3), 239-243.

Sohoni, P., Tyler, C. R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C. , Evans, M., Toy, R., Gargas, M., and Sumpter, J. P., (2001). "Reproductive effects of long-term exposure to

bisphenol a in the fathead minnow (Pimephales promelas)", *Environmental Science & Technology* **35** (14), 2917-2925.

Timmermanns, L. P. M., (1987). "Early development and differentiation in fish", *Sarsia* **72**, 231-339

Tyler, C. R., Jobling, S., and Sumpter, J. P., (1998). "Endocrine disruption in wildlife: A critical review of the evidence", *Critical Reviews in Toxicology* **28** (4), 319-361.

Vaughan, M., van Egmond, R., and Tyler, C. R., (2001). "The effect of Temperature on Sexual Differentiation and Development in the Zebrafish, *Danio rerio*", in: *Proceedings of the 11th annual meeting of the Society of Environmental Toxicology and Chemistry*, Madrid, Spain .

Yamamoto, T., (1958). "Artificial induction of functional sex-reversal in genotypic females of the medaka (*Orizias latipes*)", *Journal of Fish Biology* **50** , 150-156.

Yamamoto, T. (1969), "Sex differentiation", in W.S. Hoar and D.J. Randall (eds.) Fish Physiology, Vol 3, 117-175, Academic Press, New York.

Zacharewski, T., (1997). "In vitro bioassays for assessing estrogenic substances", *Environmental Science & Technology* **31** (3), 613-623.

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List of Publications

Manuscripts independent of this thesis

Maack, G. and George, M.R. 1999: "Contributions to the Reproductive Cycle of Encrasicholina punctifer Fowler, 1938 (Engraulidae) from West - Sumatra, Indonesia", *Fisheries Research* 44, 113-120

Fenske M., Maack G., Ensenbach U. and Segner H. 1999: "Assessment of estrogenic effects in the zebrafish, *Danio rerio*" International Symposium on Reproductive Phsyiology of Fish, Bergen Norway

Maack, G. 2000: "Perkembangan Histologi Oocyte Ikan Teri Putih (Encrasicholina punctifer, Fowler, 1938) di Sumatra Barat – Indonesia- The Oocyte development of Encrasicholina punctifer (Ikan Teri putih) Fowler, 1938 from West - Sumatra, Indonesia; a histological description" Journal Terubuk, Indonesia

Schäfers, C., Wenzel A. and Maack G. 2001: "Ethynylestradiol – a contrceptive for fish?" Nauyn-Schmiedebergs' *Archives for Pharmacology,* 363 (4): 668 Suppl. S

Maack, G. Fenske, M. Schäfers, C. Schmitz, A. and Segner, H. 1999: "Gonad development of zebrafish exposed to low levels of enthynylestradiol during different life stages. *Journal of Fish Biology* 55, Suppl. A: 245-246

Segner, H., Caroll, K., Fenske, M. Janssen, C.R., Maack, G. Pascoe, D. Schäfers, C., Vandenbergh, G.F., Watts, M. and Wenzel, A. 2001: "Identification of endocrine disrupting effects inaquatic organisms: the project "IDEA" *Ecotoxicology and Environmental Safety*, in press

Segner, H., Fenske, M., Maack, G., Schäfers, C. and Wenzel, A. 2002: "Life-time exposure to Ethynylestradiol induces irreversible infertility in the zebrafish *Danio rerio*" in prep.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ich habe mich in früherer Zeit noch nicht um den Doktorgrad beworben

Leipzig, den 15.03.2002