Effects of UV filters (benzophenones and octocrylene) and mifepristone on different life stages of zebrafish (*Danio rerio*)

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(Dekan)

«Nur wer sein Ziel kennt, findet den Weg.»

Laozi

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Zusammenfassung

In dieser Dissertation werden ökotoxikologische Wirkungen von Chemikalien, die in Körperpflegeprodukten (Benzophenone und Octocrylen) und als Pharmazeutika (Mifepriston und Progesteron) Anwendung finden, bei Zebrafischen (*Danio rerio*) untersucht. Pharmazeutika werden bei Mensch und Tier für den Schutz der Gesundheit und zur Vermeidung und Behandlung von Krankheiten verwendet. Körperpflegeprodukte wie Kosmetika und Sonnencrèmes dienen der Hautpflege und dem Schutz vor schädlicher UV-Strahlung. Sonnenschutzmittel und viele Kosmetika enthalten organische UV-Licht absorbierende Chemikalien, so genannte UV-Filter. Solche Chemikalien werden auch in vielen Produkten verwendet um ihre Stabilität zu gewährleisten und den Abbau durch UV-Strahlung zu verhindern. Bei einigen dieser Körperpflegeprodukte sind Risiken für Mensch und Umwelt nicht auszuschliessen, aufgrund ihrer hormonellen Aktivität und Stabilität in der Umwelt. Auch gewisse Pharmazeutika wie synthetische Steroidhormone zeigen Umweltrisiken. Steroidhormone werden in der Empfängnisverhütung, Hormonbehandlung und bei Schwangerschaftsabbruch (Mifepriston) eingesetzt. Beide Stoffgruppen gelangen aus industriellen und kommunalen Abwässern sowie durch Freizeitaktivitäten (insbesondere UV-Filter) in Oberflächengewässer. Zudem werden UV-Filter und Pharmazeutika in Abwasserreinigungsanlagen nicht komplett entfernt.

In Oberflächengewässern liegen die Konzentrationen von UV-Filtern und Pharmazeutika gewöhnlich im tieferen ng/L- bis µg/L-Bereich. Im Fettgewebe von Fischen und Muscheln erreichen diese Stoffgruppen jedoch Konzentrationen von mehreren zehn bis hundert ng/g. Generell ist sehr wenig bekannt über die Wirkungen und die Wirkungsmechanismen von UV-Filter-Rückständen auf Fische. Zudem ist die Auswirkung von Rückständen an Progesteron (P4) und dem Antiprogestin Mifepriston (MIF, RU486) in Gewässerorganismen weitgehend unerforscht. Es ist sehr wenig bekannt über die Wirkung solcher Steroidhormone und inwieweit sie ein Risiko für die Reproduktion von Fischen darstellen, da Wasserlebewesen diesen Stoffen dauerhaft ausgesetzt sind.

Das Ziel dieser Doktorarbeit ist es, Wirkungen der häufig verwendeten UV-Filter Benzophenon-3 (BP-3), Benzophenon-4 (BP-4) und Octocrylen (OC) bei Zebrafischen zu untersuchen. Im Weiteren soll die Wirkung von tiefen Konzentrationen von Mifepriston

(MIF, RU486) und Progesteron (P4) in verschiedenen Entwicklungsstadien von Zebrafischen untersucht werden. Dabei sollen auch Wirkungsmechanismen mit Hilfe von Genexpressionsanalysen (mittels RT-qPCR und Microarrays) untersucht werden. Weiterhin soll auch untersucht werden, ob Genexpressionsänderungen mit physiologischen Wirkungen in Zusammenhang gebracht werden können. Besonderes Interesse gilt dabei der Expressionsänderung von Genen, die im Hormonsystem (z.B. Steroidhormon Rezeptoren) und in der Steroidhormon-Synthese beteiligt sind. Dies wird im Gehirn, der Leber und in den Gonaden von adulten Zebrafischen und zusätzlich in Embryonen untersucht.

Der UV-Filter BP-3 zeigt bei 2 - 312 µg/L anti-östrogene und anti-androgene Aktivität in männlichen Zebrafischen und Embryonen. Dies zeigt sich in einer verminderten Transkription von Östrogen- (*esr1*) und Androgen- (*ar*) Rezeptoren sowie von Enzymen, die an der Steroidhormon-Synthese beteiligt sind (P450 Aromatase B (*cyp19b*), 3β-Hydroxysteroid Dehydrogenase (*hsd3b*), 17β-Hydroxysteroid Dehydrogenase Typ 3 (*hsd17b3*), 11β-Hydroxylase (*cyp11b*) und 11β-Hydroxysteroid Dehydrogenase Typ 2 (*hsd11b2*)). Diese Daten bestätigen frühere *In vitro*-Resultate. Die Microarray Analyse in der Leber wies auf keine klar identifizierbaren zusätzlichen Wirkmechanismen hin. BP-3 führte auch nicht zu einer Induktion von Vitellogenin (auf mRNA- (*vtg1*) und Protein-Ebene (VTG)). Damit schliesst dieser Biomarker für östrogene Aktivität von Fremdstoffen eine solche Wirkung von BP-3 in Fischen aus. Die Gonaden wiesen keine durch BP-3 induzierten histologischen Veränderungen auf. Die chemische Analyse des Expositionswassers und Fischgewebe zeigten, dass BP-3 teilweise zum östrogenen Benzophenon-1 (BP-1) metabolisiert wird, ähnlich wie im Menschen. Die Daten weisen darauf hin, dass BP-3 in umweltrelevanten Konzentrationen für Fische keine toxikologisch relevanten Wirkungen hat.

Eine ähnlich zielgerichtete Genexpressions-Analyse in verschiedenen Geweben wurde bei der Untersuchung des UV-Filters BP-4 angewendet. In adulten männlichen Zebrafischen und in Embryonen zeigte sich bei 2782 µg/L BP-4 eine reduzierte Transkription von Östrogen- (*esr1*, *esr2b*) und Androgen- (*ar*) Rezeptoren, Vitellogenin (*vtg1*, *vtg3*) und verschiedener Enzyme der Steroidhormon-Synthese (*hsd17b3*, P450 Aromatase A (*cyp19a*)). Dies weist auf eine anti-östrogene und anti-androgene Aktivität von BP-4 im Konzentrationsbereich von 28 - 2782 µg/L hin. Auch in diesem Fall bestätigen die *In vivo*-Resultate frühere *In vitro*-Daten. Basierend auf Transkriptionsänderungen bestimmter Marker-Gene in der

Schilddrüsenentwicklung (Hematopoietically-expressed Homeobox Protein (*hhex*) und Paired Box 8 (*pax8*)) werden auch mögliche Effekte auf das Schilddrüsenhormon-System vermutet.

Der häufig verwendete UV-Filter OC zeigte hingegen in adulten männlichen Zebrafischen und Embryonen keine hormonellen Aktivitäten bei 22 - 383 µg/L. Dies wird aus der Transkriptionsanalyse und der unveränderten Konzentration an Sexualhormonenen im Blutplasma geschlossen. In diesem Fall bestätigen die *In vivo*-Daten die früheren *In vitro*-Daten nicht. Hingegen zeigen die Microarray-Analysen, dass OC andere, unerwartete Wirkungen auf die Genexpression hat. So zeigt sich eine Beeinflussung der Blutzell- und Blutgefässbildung, Blutzirkulation, Blutbildung und Organentwicklung. Die Expressionsänderungen variierten mit der Expositionsdauer, sie waren aber bei Embryonen und adulten Fischen sehr ähnlich. Weitere Experimente sind notwendig um zu zeigen, welche physiologischen Auswirkungen diese Änderungen in der Genexpression in den Fischen haben.

Die Wirkungen von MIF und P4 wurden in einer Reproduktionsstudie mit Brutpaaren untersucht. Dabei wurden adulte Zebrafische nach einer Vorexpositions-Phase (ohne Substanzapplikation) für 21 Tage bei tiefen Konzentrationen von 5 - 77 ng/L MIF und 25 ng/L P4 exponiert. Die befruchteten Eier wurden dann während der Embryonalentwicklung für 96 beziehungsweise 120 Stunden zu denselben Konzentrationen exponiert (F1 Generation). In den adulten Fischen wurden physiologische Parameter (Eier-Produktion, Sexualhormon Konzentrationen, Histologie) sowie die Genexpression untersucht. Zudem wurden MIF und P4 in rekombinanten Hefesystemen untersucht, um die *In vitro*-Aktivität zu ermitteln. Zebrafische die zu 5 und 77 ng/L MIF exponiert wurden, zeigten eine erhöhte Eierproduktion während der 21 Tage-Exposition. Hingegen hatten 39 ng/L MIF und 25 ng/L P4 keinen Einfluss auf die Fertilität. Die Reproduktionseffekte von MIF spiegelten sich auch in histopathologischen Beobachtungen in den weiblichen Geschlechtsorganen wieder. Die Ovarien exponierter Weibchen zeigten post-ovulatorische und atretische Follikel sowie eine Ansammlung von Protein-Flüssigkeit. Die Blutplasmakonzentrationen der Sexualhormone (11-Ketotestosteron und 17β-Östradiol) waren aber unverändert. Die Befruchtungsrate der abgelegten Eier, Schlupfrate und Überlebensrate der Embryonen (F1 Generation) wurde durch MIF und P4 nicht beeinflusst. Zudem wurden Transkriptionsänderungen bestimmter Gene des Hormonsystems (*esr1*, *ar*, Progesteron Rezeptor (*pgr*), Glukokortikoid Rezeptor

(*gr*) und *vtg1*) und der Steroidhormon-Synthese (*hsd17b3, hsd3b*, *cyp19a* und *cyp11b*) gefunden. Jedoch erklären die Genexpressionsänderungen nur zum Teil die gefundenen Effekte auf die Eierproduktion. Die Genexpressionsänderungen waren in adulten (F0 Generation, nach 21 Tagen) und embryonalen (F1 Generation, nach 120 Stunden) Zebrafischen ähnlich, wobei jene der Steroidhormon Rezeptoren (*esr1*, *ar*, *pgr* und *gr*) markanter in den Embryonen waren. Die beobachteten MIF- und P4-induzierten Expressionsänderungen spiegelten sich teilweise in ähnlichen *In vitro*-Aktivitäten wieder. Eine anti-progestogene Aktivität von MIF konnte im Hefe-basierten *In vitro* System jedoch nicht gezeigt werden.

Zusammengefasst zeigen die Resultate dieser Doktorarbeit, dass Genexpressionsanalysen (Microarrays und RT-qPCR) und *In vitro* Systeme wichtige Techniken sind, um hormonelle Aktivitäten und/oder andere unbekannte Wirkmechanismen von Umweltchemikalien zu ermitteln. Die Daten zeigen, dass es wichtig ist Expressionsdaten mit physiologischen Parametern zu kombinieren um ein toxikologisches Profil von Chemikalien erstellen zu können. Natürlich hat jede dieser Techniken Limitierungen, was es schwierig macht, abschliessende Aussagen über das Umweltrisiko von BP-3, BP-4, OC, MIF und P4 zu machen. Allerdings geben die Daten erste wichtige Einblicke in die Wirkmechanismen beziehungsweise Reproduktionseffekte dieser Chemikalien. Aufbauend auf dieser Arbeit sind weitere Studien nötig um Schlüsse über mögliche Umweltrisiken dieser UV-Filter, MIF und P4 zu ziehen.

Summary

This thesis addresses effects of personal care products (PPCPs) and pharmaceuticals to zebrafish (*Danio rerio*). In human and veterinary medicine pharmaceuticals are used for health protection, prevention and treatment of diseases. Personal care products including cosmetics and sun screens are used for human welfare and contain organic UV absorbing chemicals, so-called UV filters to protect the human skin from UV irradiation. Such compounds are also used to prevent degradation of consumable products by absorption of harmful UV irradiation. Some of the PPCPs may be of environmental concern, because of their endocrine activity, persistence and adverse effects to biota. They enter the aquatic environment either directly from industrial and urban discharges and recreational activities, or indirectly via discharges of wastewater resulting from incomplete removal in wastewater treatment plants (WWTPs). Depending on their physico-chemical properties (usually small organic molecules, $\log K_{OW} > 3$) these chemicals bear the potential for bioaccumulation.

In surface waters concentrations of UV filters and pharmaceuticals are usually in the lower ng/L to µg/L range, and reach up to ten to hundred ng/g lipids in fish or mussels. Some PPCPs including progestins, which are used in contraception and hormone replacement therapies, result in adverse effects at such low concentrations in fish. Effects of anti-progestins, such as mifepristone, which is used for pregnancy termination, lack attention so far. On the other hand, some UV filters have been shown to cause feminization of male fish, although at concentrations higher than encountered in the environment. In general little is known about the molecular mechanism behind potential effects of PPCPs, as well as which environmental risks they pose for chronic exposure of aquatic wildlife at low concentrations.

Thus, this thesis addresses the effects of PPCPs including the UV filters, benzophenone-3 (BP-3), benzophenone-4 (BP-4) and octocrylene (OC), as well as the anti-progestin mifepristone (MIF, RU486) and progesterone (P4) on different developmental stages of zebrafish. We focused on transcriptomics, using microarrays and RT-qPCR, to elucidate the modes of action of these chemicals and tried to link them to physiological changes in fish. Thereby we investigated alterations of mRNA expression of genes involved in the endocrine

system (steroid hormone receptors) and steroidogenesis (steroidogenic enzymes) in multiple tissues.

In adult zebrafish males and embryos the UV filter BP-3 indicated an antiestrogenic and antiandrogenic activity at 2 - 312 µg/L based on transcriptional down-regulation of steroid hormone receptors (estrogen receptor (*ers1*) and androgen receptor (*ar*)) and several steroidogenic enzymes (P450 aromatase B (*cyp19b*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), 11β-hydroxylase (*cyp11b*) and 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*)). This indicates a similar activity as previously reported *in vitro*. Microarray analysis in liver of adult fish did not reveal novel modes of action of BP-3 at 312 µg/L. Vitellogenin, the biomarker for estrogenicity, was not induced at transcriptional (*vtg1*) and protein level (VTG), and no histological changes were observed in testes. We found that BP-3 is partly metabolized to the estrogenic benzophenone-1 (BP-1), similar to humans, and that the metabolite may contribute to the observed effects of BP-3.

A similar targeted gene approach was applied in multiple tissues of adult zebrafish males and embryos exposed to BP-4. Based on transcriptional down-regulation of steroid hormone receptors (*esr1, esr2b*, *ar*), vitellogenin (*vtg1*, *vtg3*) and some steroidogenic enzymes (*hsd17b3*, P450 aromatase A (*cyp19a*)), we hypothesized an antiestrogenic and antiandrogenic activity of BP-4 at 28 - 2782 µg/L, similarly to previously reported *in vitro* activities. Potential effects on the thyroid hormone synthesis were hypothesized due to transcriptional alterations of some marker genes (hematopoietically-expressed homeobox protein (*hhex*) and paired box 8 (*pax8*)) in the developing thyroid.

In contrast, the frequently used UV filter OC did not show any hormonal activities in adult zebrafish males and embryos at 22 - 383 µg/L, based on gene transcription analysis and plasma sex steroid hormone levels. This is in contrast to previously reported *in vitro* activities. The global transcriptomics analysis by microarrays indicated other unexpected modes of action of OC, such as interference with blood cell/vessel formation, blood circulation, haematopoeisis and organ development. The expressional changes of transcripts involved in these pathways at 22 - 383 µg/L were similar in adult males and embryos. They varied in time and indicate a similar mode of action at different life stages. Further

experiments are necessary to show as to what extent and how these molecular mechanisms of OC result in physiological changes in zebrafish.

Reproductive effects of MIF and P4 were addressed in a reproduction assay, where breeding groups of zebrafish were exposed to 5 - 77 ng/L MIF and 25 ng/L P4, respectively. In addition, embryos (F1 generation) were exposed to the same concentrations for 96 - 120 hours. In this study physiological endpoints including egg reproduction, plasma sex hormone levels and gonad histology were compared to molecular (transcriptomics) and *in vitro* effects of MIF and P4 to get first insights into their potential environmental risks. Reproductive performance (egg production) was increased after a 21 days exposure of adult zebrafish to 5 and 77 ng/L MIF, but was not affected by 39 ng/L MIF and 25 ng/L P4. Reproductive effects were reflected in histopathological observations in ovaries of females, showing post-ovulatory follicles, atretic follicles and proteinaceous fluid. The levels of plasma sex steroid hormone levels (11-ketotestosterone and 17β-estradiol) were not affected in adult fish. Furthermore, the fertility of laid eggs, hatching and survival of embryos (F1 generation) was not affected by MIF and P4. Reproductive effects could only partly be linked to transcriptional alterations of target genes involved in the endocrine system (*esr1*, *ar*, progesterone receptor (*pgr*), glucocorticoid receptor (*gr*) and *vtg1*) and steroidogenesis (*hsd17b3, hsd3b*, *cyp19a* and *cyp11b*). The alterations were similar in adult (F0 generation, after 21 days) and embryonic (F1 generation, after 120 hours) zebrafish. The responsiveness of steroid hormone receptors (*esr1*, *ar*, *pgr* and *gr*) was stronger in embryos (F1 generation) than in adult fish. The observed expressional alterations of MIF and P4 *in vivo* were reflected partially by *in vitro* activities, but the antiprogestogenic activity of MIF was lacking in the yeast-based system.

In conclusion, the results of this thesis indicate that transcriptomics (microarrays and RT-qPCR) and *in vitro* assays are important tools to elucidate hormonal activities and/or other unknown modes of action of chemicals in the environmental. The data demonstrate that it is important to include physiological parameters in addition to expressional data to elucidate and characterize the toxicological profile of a given chemical. However, each of these techniques has its own limitations. Thus, final conclusions on the environmental risks of BP-3, BP-4, OC, MIF and P4 cannot be drawn based on the results presented in this thesis alone. Nevertheless, they provide first important insights into modes of action and

reproductive effects of these chemicals. Further investigations are necessary to conclude potential environmental risks of these PPCPs.

Related Publications

Blüthgen, N., Meili, N., Chew, G., Odermatt, A., Fent, K., **2014**. Accumulation and effects of the UV-filter octocrylene in adult and embryonic zebrafish (*Danio rerio*). Science of The Total Environment 476-477, 207-217.

Blüthgen, N., Castiglioni, S., Sumpter, J.P., Fent, K., **2013**. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 1. Reproductive and early developmental effects. Aquatic Toxicology 144-145, 83-95.

Blüthgen, N., Sumpter, J.P., Odermatt, A., Fent, K., **2013**. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 2. Gene expression analysis and *in vitro* activity. Aquatic Toxicology 144-145, 96-104.

Blüthgen, N., Zucchi, S., Fent, K., **2012**. Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 263, 184–194.

Zucchi, S., **Blüthgen, N.**, Ieronimo, A., Fent, K., **2011**. The UV-absorber benzophenone-4 alters transcripts of genes involved in hormonal pathways in zebrafish (*Danio rerio*) eleuthero-embryos and adult males. Toxicology and Applied Pharmacology 250 (2), 137-146.

Related Scientific Communications and Visits

Conference Activities:

2013 Oral presentation at conference PRIMO 17 in Faro, Portugal, Title: Toxicological profile of the UV filter octocrylene in adult and embryonic zebrafish revealed by transcriptomics, Student Award.

2012 Poster presentation at 6th SETAC World congress in Berlin, Title: Effects of the UV filter benzophenone-3 (BP-3) at low concentrations in different developmental stages of zebrafish.

2011 Oral presentation at conference PRIMO 16 in Long Beach, California, USA, Title: Changes in global gene expression by the UV filter benzophenone-3 in zebrafish (*Danio rerio*).

Research Visits:

02/2013 - 08/2013 Research Visit at Brunel University, Institute for the Environment, London, U.K., supervised by Prof. John P. Sumpter, funded by SNF Mobility Grant.

03/2013 (1 week) Mario Negri Institute, Milan, Italy, supervised by Dr. Sara Castiglioni, collaboration for chemical analysis, funded by ProDoc.

Abbreviations

ar	Androgen receptor
3-BC	3-benzylidene camphor
BP-1	Benzophenone-1
BP-2	Benzophenone-2
BP-3	Benzophenone-3
BP-4	Benzophenone-4
cDNA	Complementary deoxyribonucleic acid (DNA)
cRNA	Complementary ribonucleic acid (RNA)
cyp1a1	Cytochrome P450 family 1
cyp11a1	Cytochrome P450 family 11
cyp11b	11β-hydroxylase
cyp17a	Cytochrome P450 family 17
cyp19a	Cytochrome P450 aromatase A
cyp19b	Cytochrome P450 aromatase B
DHP	17α,20β-dihydroxy-4-pregnen-3-one
E2	17β-estradiol
EDCs	Endocrine disrupting chemicals
EE2	17α-ethinylestradiol
EHMC	2-ethylhexyl-4-methoxycinnamate
ELISA	Enzyme-linked immunosorbent assay
esr1	Estrogen receptor
FSH	Follicle stimulating hormone
hAR	Human androgen receptor
hER	Human estrogen receptor
hhex	Hematopoietically-expressed homeobox protein
hpf	Hours post fertilization
HPG	Hypothalamus-pituitary-gonadal axis
hsd3b	3β-hydroxysteroid dehydrogenase
hsd11b2	11β-hydroxysteroid dehydrogenase type 2

hsd17b1	17β-hydroxysteroid dehydrogenase type 1
hsd17b3	17β-hydroxysteroid dehydrogenase type 3
hsd20b	20β-hydroxysteroid dehydrogenase
K_{OW}	Octanol-Water Partitioning coefficient
11-KT	11-ketotestosterone
LH	Luteinizing hormone
4-MBC	4-methylbenzylidene camphor
MIF	Mifepristone (RU486)
MIH	Maturation inducing hormone
MOA	Mode of action
mRNA	Messenger ribonucleic acid (RNA)
OC	Octocrylene
OD-PABA	Octyldimethyl-*p*-aminobenzoate
P4	Progesterone
pax8	Paired box 8
pgr	Progesterone receptor
PPCPs	Pharmaceuticals and personal care products
PR	human progesterone receptor
RIA	Radioimmunoassay
RT-qPCR	Real-time quantitative polymerase chain reaction (PCR)
RU486	Mifepristone (MIF)
StAR	Steroidogenic acute regulatory protein
T	Testosterone
T3	Triiodothyronine
T4	Thyroxine
THP	17α,20β,21-trihydroxy-4-pregnen-3-one
UV-A	Ultraviolet light 315 - 400 nm wavelength
UV-B	Ultraviolet light 280 - 315 nm wavelength
UV filter	Ultraviolet filter
VTG	Vitellogenin (protein), egg yolk sac precursor
vtg1	Vitellogenin 1 (gene)
WWTPs	Wastewater treatment plants

YAS	Recombinant yeast expressing the human androgen receptor
YES	Recombinant yeast expressing the human estrogen receptor
YPS	Recombinant yeast expressing the human progesterone receptor

Chapter 1

Introduction

1.1 Structure of this Thesis

This Thesis consists of five main sections (Chapters 2 - 6), reporting the main results obtained. They have been previously published in peer-reviewed journals. These publications are implemented by a general introduction to the topic of the dissertation and a general discussion and conclusions to the results obtained in the individual studies. The chapters presented are briefly described here.

Chapter 1

Chapter 1 provides a general introduction to the topic of the dissertation, including chemicals tested and their environmental fate, zebrafish as model organism and a short characterization of the technology used for transcriptomics analysis of the tissue samples. The last section of this chapter describes the objectives of the thesis.

Chapter 2

Chapter 2 provides results on the effects to adult male zebrafish and zebrafish embryos after exposure to low concentrations of the UV filter benzophenone-3 (BP-3). Similar exposure concentrations were chosen for both *in vivo* experiments, adult fish (2 - 312 µg/L) and embryos (8 - 438 µg/L), and compared afterwards. Molecular effects of BP-3 were assessed by microarray analysis in liver samples of adult fish and a targeted gene expression approach with focus on the endocrine system and steroidogenesis in different tissues of adult fish and embryos. Metabolism and transformation products of BP-3 were considered in the chemical analysis of exposure water and adult fish. Physiological effects of BP-3 were assessed by measuring protein levels of vitellogenin (VTG) and histological alterations in testes of adult fish.

Chapter 3

In Chapter 3 effects of the UV filter benzophenone-4 (BP-4) on adult male and embryonic zebrafish were investigated. Similar exposure concentrations of BP-4 (28 - 2782 µg/L) were

used in the *in vivo* experiments with adults and embryos. Molecular effects and thus potential effects on the estrogen and androgen signaling, on steroidogenesis and on early development of the thyroid hormone system were addressed by transcriptional analysis of a targeted gene approach in different tissues of adult fish and embryos.

Chapter 4

In Chapter 4 effects of the UV filter octocrylene (OC) were investigated on adult male and embryonic zebrafish. Adult zebrafish were exposed to similar concentrations (22 - 383 µg/L) as embryos (69 - 925 µg/L). Sample collection at different exposure times was considered in both exposure studies. Microarray analysis of different tissues of adult fish was performed to elucidate potential modes of action and affected biological pathways by OC. A targeted gene expression approach was used to validate the microarray data and to compare the concentration-dependent and exposure-time-dependent transcript alterations in different tissues and different developmental stages. Potential hormonal activities of OC were addressed by transcriptomics and measurement of sex steroid hormone levels in males. Bioaccumulation of OC in adult fish was assessed by chemical analysis.

Chapter 5

In Chapter 5 results are provided of a two-generation reproduction assay using adult zebrafish males and females exposed to the anti-progestin mifepristone (MIF) (5 - 77 ng/L) and the progestin progesterone (P4) (25 ng/L). Embryos (F1 generation) were further exposed to similar chemical concentrations (3 - 26 ng/L MIF and 254 ng/L P4, respectively). Reproductive effects, including egg production, spawning frequency and fertility of laid eggs were assessed as well as hatchability and survival of the F1 generation (embryos). Sex steroid hormone concentrations were measured in blood plasma of adult fish and histological alteration in ovaries of females and testes of males were assessed.

Chapter 6

Chapter 6 represents further results of the two-generation reproduction assay on MIF and P4 described in Chapter 5. Molecular effects based on transcriptomics analysis in adult tissues and embryos (F1 generation) are linked to the reproductive performance of adult fish. In addition, *in vitro* hormonal activities of MIF and P4 are assessed, to explain and compare the hormonal activities and modes of action observed *in vivo*.

Chapter 7

Chapter 7 comprises a general discussion and conclusions on the observed effects in zebrafish after exposure to UV filters and synthetic (anti-) progestins. The obtained results are critically discussed in a broader context. Overall conclusions are drawn on the studies performed and limitations of the performed research and used technologies are discussed. Further improvements of this type of studies and an outlook for further research are provided.

Appendices

In the Appendices the supporting information corresponding to the publications of Chapter 2 to 5 are provided.

1.2. General introduction to pharmaceuticals and personal care products (PPCPs) in the environment

Pharmaceuticals and personal care products (PPCPs) play an essential role for human and animal health and welfare. Humans and veterinarians use a variety of many different PPCPs for health protection, prevention and treatment of diseases and welfare, of which a proportion reaches the aquatic environment. Due to an increasing use and high requirements of general public for safe, stable and long-lasting (personal care) products, this issue is very relevant and its concern is likely to rise. Everybody is aware that taking pharmaceuticals may pose unwanted side-effects, unraveled in human toxicology, and humans decide for it on purpose. In contrast, ecotoxicology addresses effects of these anthropogenic chemicals in non-target organism, which are exposed chronically to mixtures of various PPCPs in the environment. The issue of PPCPs in the environment is not only relevant for the wildlife and populations, and hence the great natural diversity in ecosystems, it is also of concern for humans, as they represent the end of the food chain. Thus, contamination of the environment, in particular watercourses, does not only pose threat to the aquatic wildlife, flora and fauna, but also to drinking water.

Human pharmaceuticals are usually biologically active, and it is reasonable that similar effects occur also in non-target organisms, such as fish, due to their phylogenetic conservation of targets of these drugs (Gunnarsson et al. 2008). For example, the synthetic estrogen 17α-ethinylestradiol (EE2) binds to the estrogen receptor in fish as it does in woman, and causes estrogenic effects, including adversely affecting reproduction at concentrations below 1 ng/L (Länge et al. 2001). More recently, synthetic progestogens, which are mainly used in contraception and hormone replacement therapies, have been shown to cause adverse reproductive effects in fish at environmental relevant concentrations, in the ng/L range (Runnalls et al. 2013, DeQuattro et al. 2012, Paulos et al. 2010, Zeilinger et al. 2009). The same applies for selected glucocorticoids (e.g. beclomethasone dipropionate) (Kugathas et al. 2011) and beta-blocker (e.g. propranolol) (Huggett et al. 2002), which are human pharmaceuticals used to treat a variety of inflammatory and cardiac system diseases, respectively. Other drugs, such as trenbolone, used as growth promotor in beef, cause androgenic activities in fish, including irreversible masculinization and reduced fecundity at low ng/L concentrations (Morthorst et al. 2010,

Jensen et al. 2006, Ankley et al. 2003). The anti-inflammatory drug diclofenac causes renal lesions and gill alterations in rainbow trout at low environmental concentrations (µg/L) (Schwaiger et al. 2004, Triebskorn et al. 2004). As reported in Fent et al. (2006), these are only few examples showing that human pharmaceuticals can induce adverse effects to fish.

Personal care products, including UV filter compounds, are usually used for human welfare and are considered in ecotoxicology as some of these chemicals show endocrine activities and thus may affect fish reproduction. For example, estrogenic and reproductive effects have been shown for some UV filters in fish (Coronado et al. 2008, Fent et al. 2008, Weisbrod et al. 2007, Kunz et al. 2006a) at concentrations higher than encountered in the environment.

1.3. Environmental fate of PPCPs

Hundreds of human pharmaceuticals are consumed by the Swiss population in considerable amounts, of which a proportion reaches the environment, mainly through effluents from wastewater treatment plants (WWTP) or directly as discharges of PPCPs industries and urban runoffs. Veterinary pharmaceuticals enter the environment either directly (e.g. from aquacultures) or indirectly via application of manure to crop fields and thus runoff and drainage of contaminated soils (Ternes et al. 2002). Personal care products, including UV filters, enter the aquatic environment (e.g. lakes, rivers) either directly, as a consequence of bathing or other recreational activities, in particular in warm summer months, or indirectly from discharge of WWTP, as a result of the use of cosmetics, showering, washing activities, excretion and industrial waste. Thus PPCPs may threat the aquatic wildlife, flora and fauna by contamination of surface and ground water. In addition, human health is also affected by PPCPs due to contaminated drinking waters and fish. Figure 1 illustrates the here described main exposure routes of PPCPs into the aquatic environment.

Environmental concentrations of pharmaceuticals and personal care products (PPCPs) in surface waters are usually in the range of ng/L to µg/L (Huerta et al. 2013, Maggi et al. 2013, Gros et al. 2007), with usually higher concentrations in highly populated urban areas,

industrial and coastal sites or during summer months for UV filters. Although our current WWTP in Switzerland (and Europe) are working on high technological standards, some of them start to use ozonation, activated charcoal, catalysis or membrane filtration, they are not efficient enough to remove the variety of PPCPs (Gros et al. 2007, Esplugas et al. 2007, Ternes et al. 2002). In particular smaller and persistent molecules pass through WWTP, or chemicals are incompletely degraded and thus transformation products, which may possess biological activities, enter the aquatic environment.

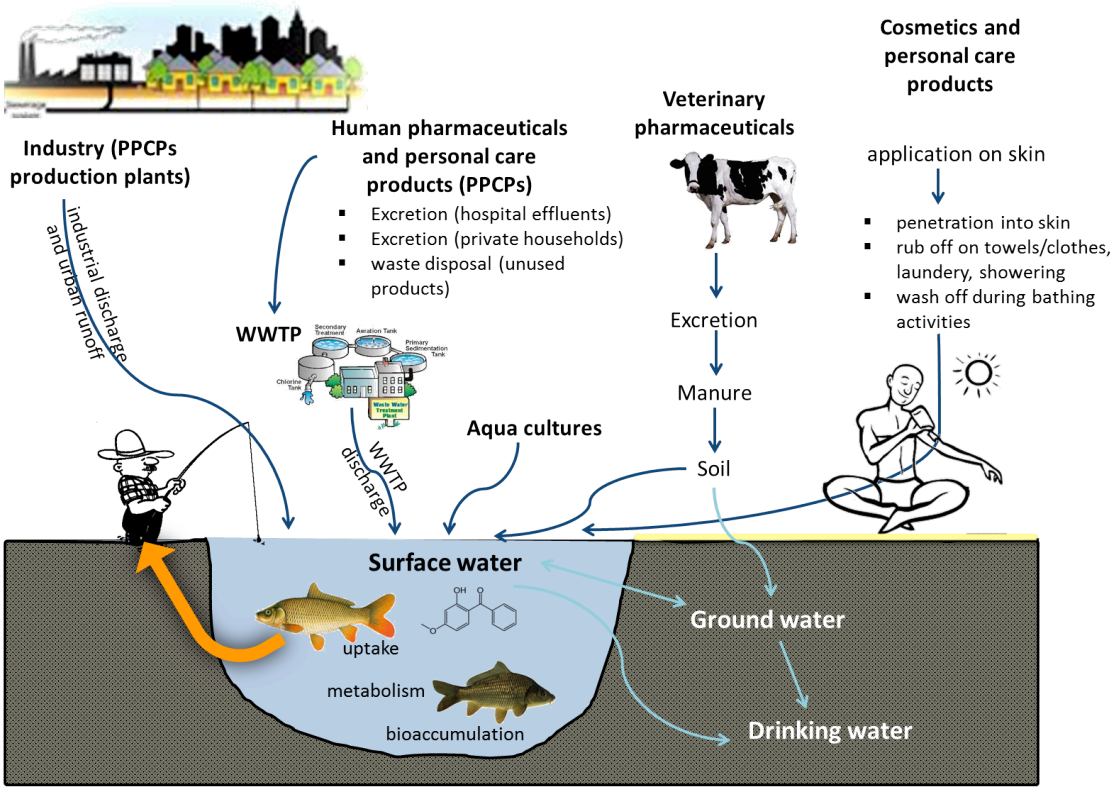


Figure 1: Major exposure routes of PPCPs into the aquatic environment.
WWTP: wastewater treatment plants, PPCPs: pharmaceuticals and personal care products

Once these chemicals enter the aquatic environment, they are taken up by aquatic organism (e.g. fish), depending on their physico-chemical properties (e.g. molecular sizes, $\log K_{OW}$, partitioning properties, solubility and bioavailability), and thus are bioconcentrated in tissues and potentially biomagnified along the food chain. Besides uptake, the mode of action of many PPCPs is mainly responsible for posing a threat to aquatic organism (Fent et al. 2006).

Regarding fish, it seems likely that similar effects occur to those in humans taking these pharmaceuticals, due to the phylogenetic conservation of the targets of these drugs, e.g. receptors and enzymes (Christen et al. 2010, Gunnarsson et al. 2008, Fent et al. 2006). Fish have a similar variety of metabolizing enzymes (Phase I and Phase II) as mammals, and thus it is important to consider biotransformation processes of chemicals (hydrolysis, oxidation, reduction and conjugation) for the environmental fate of PPCPs.

Finally, it is likely that environmental concentrations of PPCPs are increasing in the future due to increasing consumption by humans and discharge of PPCPs into rivers and lakes. As a consequence, the environmental fate of these chemicals, considering the chemical properties, biotransformation, persistence, bioaccumulation and toxicity, is of increasing concern as these PPCPs may pose potential threats to aquatic organism.

1.4. UV filters

UV filters are ingredients in many personal care products, for example in sunscreens, creams, lipstick, shampoos, hair sprays and perfumes (Hauri et al. 2003). They are also ingredients in food contact materials, such as plastics and cartons, or textiles, to protect our products from degradation (Muncke 2010). They are small and mainly organic molecules, comprising conjugated pi-systems to absorb the harmful energy-rich UV irradiation from sun light and thus to protect the human skin or products from damage and degradation. The inorganic UV filters (e.g. titanium dioxide and zinc oxide) protect the skin by reflecting and scattering the UV irradiation. For best protection against the whole UV spectrum, commercial formulations contain mainly mixtures of UV-A (315-400 nm) and UV-B (280-315 nm) filter compounds. The use of UV filters in cosmetics in Europe is regulated by the EU cosmetics directive, which lists currently 28 allowed UV filter compounds and a maximum of 10 % per organic chemical (Europa cosmetics regulation, http://ec.europa.eu/consumers/cosmetics/cosing/). Their physico-chemical properties are mainly responsible for their environmental fate. Most of the organic UV filters are lipophilic ($\log K_{OW} > 3$) and persistent due to their chemical structure and designed water-resistance in personal care products, and thus exhibit a potential for bioconcentration in fish and humans.

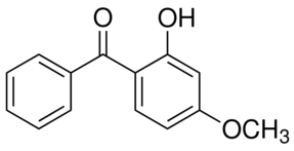
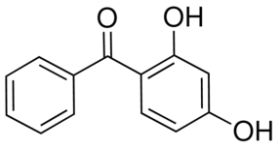
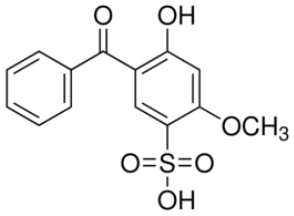
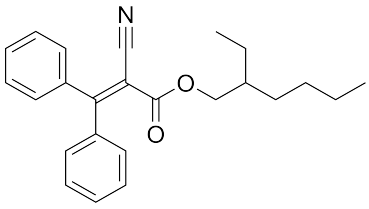
UV filters are frequently found in various biota and environmental concentrations are reported in ng/L to µg/L in surface waters (rivers, lakes) (Fent et al. 2010, Balmer et al. 2005), in lower ng/g dw in sediments (Rodil et al. 2008), up to several thousand ng/g lipids in mussels and fish (Gago-Ferrero et al. 2012, Buser et al. 2006), and ten to hundreds ng/g lipids in crustaceans and mollusks (Fent et al. 2010). Depending on their $\log K_{OW}$ and bioavailability, many UV filters adsorb to particles, and thus end up in sewage sludge during WWTP and accumulate in sediments and biota. Table 1 summarizes the chemical structures and physico-chemical properties of UV filters investigated in this thesis.

UV filters are considered as emerging and potential endocrine disrupting chemicals. Some of them pose estrogenic hormonal activity, in particular 4-methylbenzylidene camphor (4-MBC), 2-ethylhexyl-4-methoxycinnamate (EHMC), benzophenone-1 (BP-1) and benzophenone-2 (BP-2). Some UV filters, for example BP-1, BP-2, benzophenone-3 (BP-3) and 3-benzylidene camphor (3-BC), have been shown to cause adverse reproductive effects, including induction of the egg yolk sac precursor vitellogenin (VTG), gonad histology alterations, inhibition of oocyte production in female fish and spermatogenesis in male fish, loss of secondary sexual characteristics and loss of gender-specific mating behavior (Fent et al. 2008, Kunz et al. 2006a, Coronado et al. 2008, Weisbrod et al. 2007). EHMC, the most frequently detected UV filter, BP-3 and octyldimethyl-*p*-aminobenzoate (OD-PABA) have been shown to inhibit algal growth at concentrations up to 0.76 µg/L (Rodil et al. 2009), and very high concentrations of EHMC exhibit toxic reproductive effects on snails (Kaiser et al. 2012). Toxic effects on reproduction of crustaceans have been reported for BP-1, BP-3 and benzophenone-4 (BP-4) (Fent et al. 2010, Kusk et al. 2011). It is also reported that UV filters increase virus production in marine bacterioplankton which results in rapid and complete coral bleaching at low concentrations (Danovaro et al. 2008, Danovaro and Corinaldesi 2003).

For most of the UV filters it is poorly understood how they act and what their main target organs might be. *In vitro* data provide first insights into hormonal activities of UV filters (Kunz et al. 2006b, Kunz and Fent 2006), but these may differ to hormonal effects *in vivo*, as shown for some compounds (Kunz et al. 2006b). As mixtures, UV filters act additively or even synergistically *in vitro* (Kunz and Fent 2006b), and additive to antagonistic *in vivo* (Kunz and Fent 2009). Very little is known about their mode of action and alteration of transcription

profile of genes belonging to the endocrine system and steroidogenesis at low concentrations (Zucchi et al. 2011). It remains elusive how alterations on the molecular level can be translated to physiological and biological responses in fish. The identification of modes of action of UV filters in aquatic organism, such as fish, and linkage to physiological changes would help to elucidate their toxicity and to reveal their potential environmental risk.

Table 1: Chemical structures and physico-chemical properties of UV filters investigated in this Thesis.

UV filter	Chemical structure	MW [g/mol]	$logK_{OW}$
Benzophenone-3 (BP-3)		228.2	3.8
Benzophenone-1 (BP-1)		214.2	3.2
Benzophenone-4 (BP-4)		308.3	0.99
Octocrylene (OC)		361.5	6.9

Although reproductive effects mentioned above were obtained in laboratory studies at concentrations higher than those found in the environment, these first insights of endocrine disrupting activity of UV filters are alarming, considering the propensity of rapid accumulation and chronic exposure to mixtures of several UV filters and other chemicals in the environment and thus indicate a need for further studies.

1.5. Pharmaceuticals: progestins and anti-progestins

Regarding human pharmaceuticals, this thesis focuses on progesterone (P4) and the anti-progestin mifepristone (MIF, RU486), organic molecules with steroid hormone structures (see Table 2). Synthetic progestins are on sale since the 1950s and are used as single drug (so called "mini-pills") or in combination with estrogens in oral contraceptives (to block ovulation) and in (menopausal) hormone replacement therapies to treat for example abnormal uterine bleeding, endometriosis and breast, kidney or uterine cancer. Synthetic anti-progestins, such as MIF are used as an effective non-invasive method for medical termination of pregnancy up to 49 days of pregnancy, mostly in combination with prostaglandins, such as misoprostol. MIF is clinically used also for treatment of labor induction, menstrual regulation, endometriosis, contraception, Cushing Syndrome and steroid receptor containing tumors (e.g. in breast, ovary, prostate) among others, although its main application is late termination of pregnancy (Schaff 2010, Spitz 2003, Spitz et al. 1996). After daily administrations of µg to mg doses to humans, small proportions (in the ng/L range) enter the aquatic environment continuously, due to incomplete removal during WWTP (Ternes et al. 2002). MIF was detected in wastewater and hospital effluents at concentrations of 195 ng/L (Liu et al. 2011, Liu et al. 2010), and surface water concentrations have not yet been reported thus far, but are expected in the lower ng/L-range similar to other pharmaceuticals (Huerta et al. 2013, Gros et al. 2007). Environmental concentrations of P4 are reported in similar ranges with 0.3-1.4 ng/L in rivers in Spain (Kuster et al. 2008) and 14-27 ng/L (Pal et al. 2010) in freshwater rivers in the U.S.A. Thus, synthetic progestins and anti-progestins are relevant for aquatic organisms, considering that they may cause

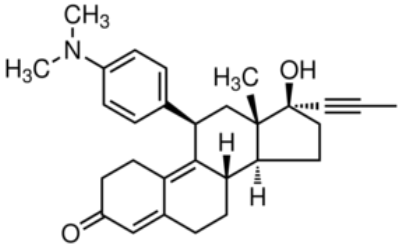
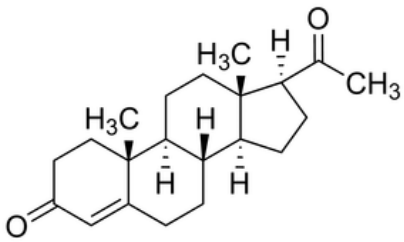
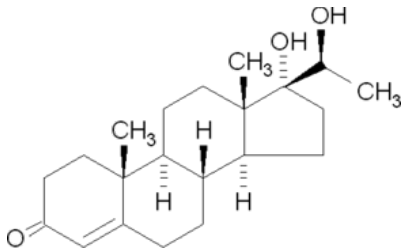
similar effects to those in humans due to the phylogenetic conservation of drug targets (Christen et al. 2010, Gunnarsson et al. 2008, Fent et al. 2006).

Reproduction in fish is mainly driven by gonadotropins, which are released from the pituitary, and are analogous to the mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH). They are responsible for the stimulated synthesis of sex steroids (androgens, estrogens and progestins), which act in turn on target tissues to regulate spermatogenesis, oogenesis, reproduction, sexual phenotype and behavioral characteristics (Arukwe 2001, Patiño and Thomas 1990). Progestins are important in stimulation of oocyte maturation, ovulation in females, stimulation of spermiation and sperm motility in males, initiation of meiosis in both sexes, and in some species also act as sex pheromones (Thomas 2012, Tokarz et al. 2013). In fish, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (chemical structure is given in Table 2) and 17α,20β,21-trihydroxy-4-pregnen-3-one (THP) have been identified as the major maturation inducing steroids (MIS) (Thomas 2012, Scott et al. 2010, Canario and Scott 1990). These two progestins induce oocyte maturation in teleosts by activation of nuclear and membrane-bound progesterone receptors (mPR), depending on the season, spermiation, time of the day and the hormonal stimulation (Thomas 2012, Scott et al. 2010). This is contrary to the situation in humans, where the natural progesterone (P4) is an important regulator during oocyte maturation and to maintain pregnancy via progesterone receptor binding. All progestins bind to the progesterone receptor (PR), but depending on their parent structure (progesterone, testosterone or spironolactone), also to other steroid hormone receptors (e.g. androgen, estrogen, glucocorticoid and mineralcorticoid receptors). Thus, many progestins exhibit multiple hormonal activities.

Ecotoxicological research on synthetic progestins and anti-progestins is very recent and focused mainly on reproductive effects of synthetic progestins in fish, but anti-progestins lack attention by now. For example, levonorgestrel (Runnalls et al. 2013, Svensson et al. 2013, Zeilinger et al. 2009), norethindrone (Paulos et al. 2010), drospirenone, gestodene and desogestrel (Runnalls et al. 2013) showed reduced or even stopped egg production in fish at low ng/L concentrations, but also P4 (DeQuattro et al. 2012) at higher concentrations. Some of these synthetic progestins pose androgenic activities *in vivo* and *in vitro* (Runnalls et al. 2013). On a molecular level, MIF and P4 have recently been demonstrated to affect gene

expression in zebrafish embryos, indicating androgenic and (anti-) progestogenic activities (Zucchi et al. 2012).

Table 2: Chemical structures and molecular weight of progestins and anti-progestin investigated in this thesis.

Progestin / anti-progestin	Chemical structure	MW [g/mol]
Mifepristone (MIF, RU486)		429.6
Progesterone (P4)		314.5
17α,20β-dihydroxy-progesterone (DHP)		332.5

Assuming that the annual consumption of these human pharmaceuticals is increasing in the future (Runnalls et al. 2010) and thus also the environmental concentrations are likely to rise, more research is required in this field. It is important to investigate which synthetic (anti-) progestins and at what concentrations they pose an environmental risk to aquatic

organism by affecting their reproductive behavior. This is particularly of concern as reproductive effects, which are the most sensitive endpoints for maintenance and striving of populations, occur at very low concentrations. Additive effects may occur in the environment due to its presence as mixtures. Very little is known about the mode of action of these pharmaceuticals and how transcriptional alterations can be linked to physiological and reproductive effects. The so far reported effects of (anti-) progestins are alarming, and with the current knowledge, it is very challenging to predict which of these compounds pose an environmental risk and threats to aquatic wildlife. Thus further research on effects of these steroid pharmaceuticals is very important.

1.6. Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*) are tropical freshwater fish belonging to the family of *Cyprinidae* and are native in river basins of India, Burma, Bangladesh, Myanmar, Pakistan and Nepal. It is one of the most important vertebrate model organisms in developmental biology, genetics, neurophysiology and biomedicine (Spence et al. 2008, Lawrence 2007). Zebrafish has a number of attributes that make it particularly attractive for research. They are small and robust fish, and large numbers of both genders can be kept easily and cheaply in the laboratory with low space requirements. They are asynchronous group spawners, and females are capable of spawning several hundreds of eggs almost every to several days all year round. Zebrafish have a short reproduction cycle of about 3 - 4 months (from fertilization to sexual maturity), and the embryonic development, including most organs, is fast (within 5 days after fertilization) (Perry et al. 2010). The different developmental stages are well characterized (Kimmel et al. 1995) and the translucent embryos are well suited for experimental manipulations and microinjections. Figure 2 illustrates adult zebrafish (male and female) and developing embryos. The small embryos are perfect for small-compound screenings (e.g. in 96-well plates), and ecotoxicological research. Zebrafish are frequently employed in development biology, and even used as vertebrate models for human diseases and screening of therapeutic drugs (Löhr and Hammerschmidt 2011). The complete sequenced genome of zebrafish (http://www.zfin.org) finds its application in genetic

research (using of morpholinos to knock-out genes) and transcriptomics, to study modes of action of chemicals, and to potentially link them to similar modes of actions in humans.

Figure 2: Adult zebrafish male (**A**) and female (**B**), showing the species characteristic longitudinal stripes. Developing zebrafish embryos (**C**), after about (left to right) 12, 24 and 72 hours post fertilization (according to Kimmel et al. 1995) (photo sources: internet, unknown photographers).

Zebrafish are used in ecotoxicological studies in order to elucidate endocrine disrupting chemicals (EDCs) (Scholz and Mayer 2008, Sanderson 2006). This often includes the investigation of biosynthesis of steroid hormones (steroidogenesis) as sexual hormones are considered to be the driving force for impacts of chemicals on the endocrine system and reproduction. The postulated pathway of steroidogenesis in zebrafish is shown in Figure 3 (Tokarz et al. 2013, Dietrich and Krieger 2009). The major sites of steroidogenesis, the interrenal, gonads and brain, synthesize the steroid hormones under control of the hypothalamus and the hypothalamus-pituitary-gonadal axis (HPG) (Tokarz et al. 2013, Arukwe 2001). The biosynthesis of all steroid hormones starts with the rate-limiting transport of cholesterol into mitochondria mediated by steroidogenic acute regulatory protein (StAR) and subsequently a series of enzymes modify the steroid backbone by adding or modification of functional groups.

In this thesis we focused on mRNA expression levels of some of these enzymes, which are named in Figure 3, although evidence of functionality is not given for all of them (Tokarz et al. 2013). The major steroid hormones in zebrafish are 17β-estradiol (E2), 11-ketotestosterone (11-KT) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) (highlighted in grey in Figure 3) and their role in reproduction is similar to mammals. E2 regulates reproduction in ovaries and acts on brain and pituitary to control vitellogenesis and yolk

formation in the liver (Arukwe 2001). 11-KT is the main androgen in zebrafish and induces spermatogenesis (DeWaal et al. 2008). Both hormones, E2 and 11-KT, are also involved in influencing the behavior of fish, for example by acting as sex pheromones (Scott et al. 2010, Stacey 2003). DHP, known as the maturation inducing hormone (MIH) in zebrafish, induces oocyte maturation and initiates meiosis (Scott et al. 2010, Canario and Scott 1990).

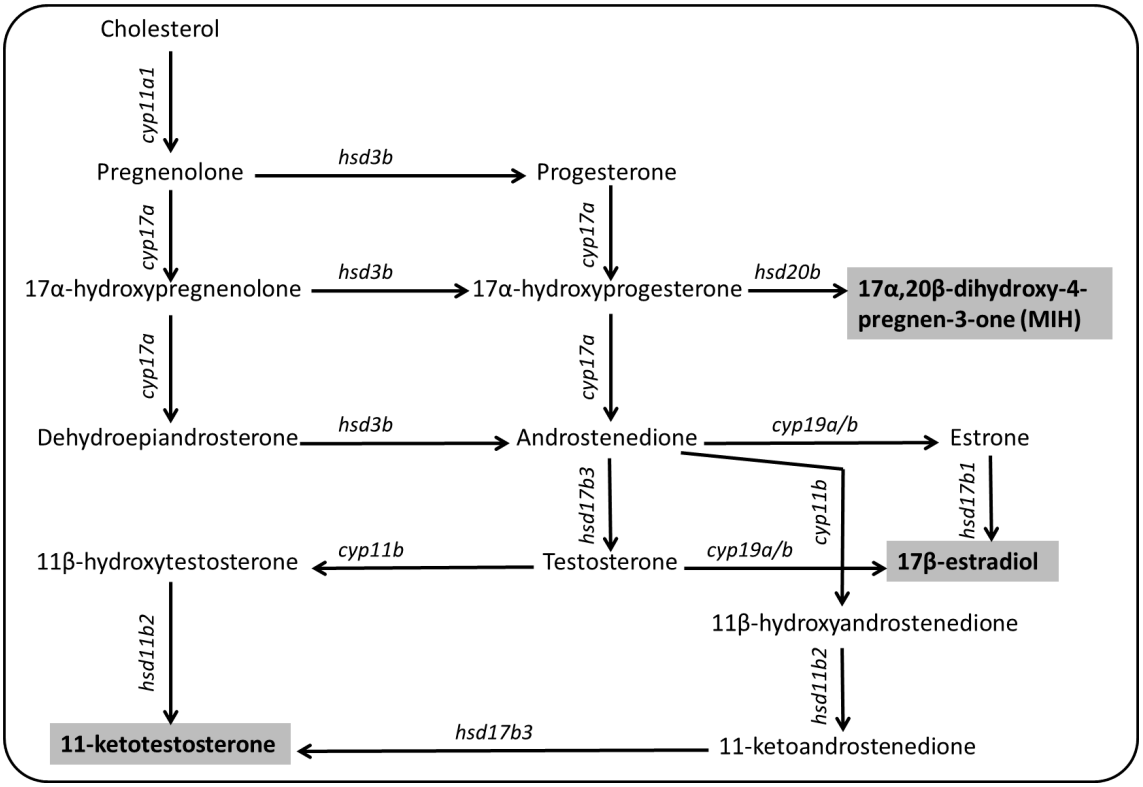


Figure 3: Postulated pathways of steroidogenesis in zebrafish. Gene names of supposed enzymes are denoted on each arrow (adapted from Tokarz et al. 2013, Dietrich and Krieger 2009). Gene names: *cyp11a1*: Cytochrome P450 family 11; *cyp17a*: Cytochrome P450 family 17; *hsd3b*: 3β-hydroxysteroid dehydrogenase; *hsd20b*: 20β-hydroxysteroid dehydrogenase; *hsd17b3*/*hsd17b1*: 17β-hydroxysteroid dehydrogenase type 3/type 1; *cyp19a*/*b*: Cytochrome P450 aromatase A/B; *cyp11b*: 11β-hydroxylase; *hsd11b2*: 11β-hydroxysteroid dehydrogenase type 2.

1.7. Transcriptomics

Transcriptomic studies can help in understanding of how chemicals act in organisms (e.g. fish) on a molecular level, what are the target organs of the chemicals and which effects they may cause, by measuring the expression levels of genes. In the last years, microarray technology has become an excellent but expensive tool in ecotoxicology to study transcriptional alterations of thousands of genes, or even the whole genome at once, whereas with quantitative real-time PCR (RT-qPCR) only expression levels of selected target genes can be investigated. Assuming that the alteration of genes transcription represents the primary action between chemicals (xenobiotics) and biota and thus the first action prior to physiological and biological changes, transcriptomics are used in order to show whether transcriptional alterations are indicative for physiological changes. Huge data of expressional changes of the whole genome after chemical exposure, associated with the discovery of completely unexpected biological pathways and mechanisms of action, are one of the greatest advantages of microarrays. Transcriptomics can be applied to individual tissues, to investigate the specific target organ of the chemical, or in whole bodies (e.g. fish homogenate) to get the whole picture of potential effects of the chemical (Zucchi et al. 2011, Lam et al. 2008, Hoffmann et al. 2008). Considering that chemicals usually affect several different genes, pathways and exhibit multiple modes of actions (MOA), microarrays represent a straightforward screening technique to get first insights into MOA of an individual chemical or mixtures.

One of the ambitious ideas of microarrays is to unravel specific expression profiles of a chemical, which will result in a transcriptional fingerprint of the compound, and thus represent the basis for the identification of compounds with similar MOA and to identify new biomarkers for adverse effects of a chemical (Fent and Sumpter 2011). Nevertheless, these technologies have their disadvantages, as microarrays are very expensive and the data interpretation is very complex and challenging and requires not only biological, physiological and bioinformatics skills. As Fent and Sumpter (2011) further noted, many genes, for example in zebrafish, are not annotated and their functions are not known, which makes the data interpretation even more difficult. It should also be considered that transcription levels of genes may be influenced by several environmental factors (e.g. temperature, pH, oxygen, stress, feeding, light-dark rhythm, circadian rhythm) and vary in time and exposure chemical

concentration, and hence transcriptomics represent only a "snapshot" and other important time points may be missed. Another limitation of transcriptomics is that the baseline for expression levels is unknown so far, although expressional changes are always compared to appropriate controls for each experiment (Fent and Sumpter 2011).

But for an overall conclusion on MOA and effects of a chemical on an organism (e.g. fish), one needs to know more about the variability of expression levels for individual fish, depending on their developmental stage, gender, maturation stage, between different species and how transcription levels vary due to environmental conditions (e.g. stress, feeding, circadian rhythm etc.). Nevertheless, transcriptomics in combination with well experimental designs are very useful tools in ecotoxicology to unravel MOA and potential (adverse) effects of chemicals of concern for the environment. Usually, combinations of transcriptomic technologies are used (Yuen et al. 2002), with microarrays as a challenging first technique and secondly, RT-qPCR is used for validation of selected target genes, which can also be easily extended to investigate time- and concentration dependence of the chemical of interest due to lower costs.

Figure 4 illustrates the typical work-flow of sample preparation for transcriptomics analysis, which was also followed in this thesis. The sample preparation for both technologies, microarrays and RT-qPCR, starts with extraction of the total RNA from exposed fish (whole body homogenates or tissues), which is subsequently reverse transcribed into cDNA. Following RT-qPCR analysis the cDNA is used for amplification and quantification of selected target genes. For microarray analysis, the cDNA is further reverse transcribed into cRNA and labeled with fluorescent cyanine 3 (Cy3) for one-color arrays. Afterwards, the Cy3-labeled cRNA is hybridized to the microarray chip, which consists of a glass slide on which each gene of an appropriate organism is represented by several copies of a certain oligonucleotide fragment (e.g. Agilent one-color microarray for zebrafish: 36'760 transcripts). Each sample (control and exposed fish) is hybridized on an individual array. After washing to remove unbound cRNA, the arrays are scanned and signal intensities of each transcript are compared between controls and treated samples (represents different expression). The analysis includes several quality control steps and requires special software tools. Finally, the obtained list contains all differentially expressed genes between controls and exposed

samples and can provide information on the modes of action and affected biological processes by the chemical.

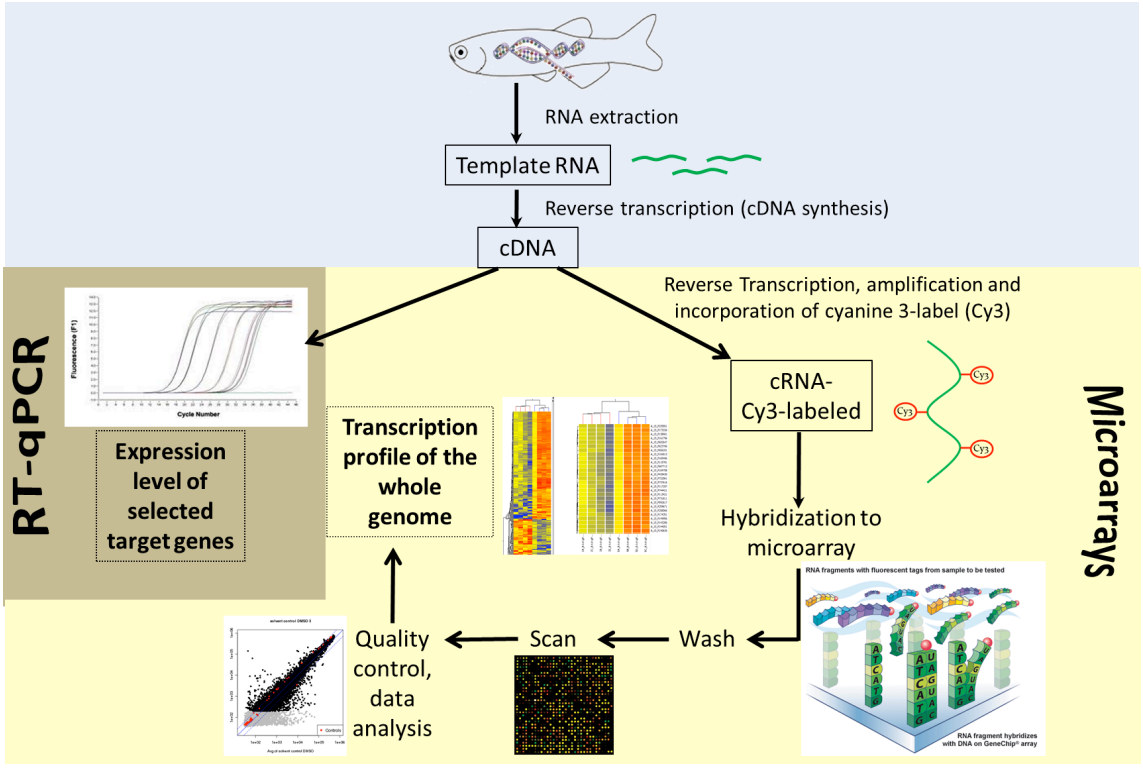


Figure 4: Typical workflow of sample preparation and transcriptomics analysis using zebrafish.

1.8. Objectives of this Thesis

The objectives of this thesis can be resumed in two parts that are closely related and are presented below.

1.8.1. Effects of UV filters on different developmental stages of zebrafish

The first part (Chapter 2 - 4) consists of *in vivo* exposure studies, which were performed to investigate potential endocrine effects of selected UV filters on different developmental stages of zebrafish. The UV filters (BP-3, BP-4 and OC) were selected based on their application and high usage (as they are some of the most frequently used UV filter in personal care products), and due to the knowledge gap regarding their mode of action in fish. The exposure concentrations were chosen from environmental to high concentrations, the latter to elucidate potential activities. The main objective of these studies was to unravel their modes of action, and which biological pathways they may affect, based on transcriptomics analysis. We were interested in transcription profiles of selected target genes in different developmental stages and at different exposure times, and how these alterations are related to physiological effects. The overall aim was to understand how these UV filters act on the endocrine system of zebrafish and whether they may be harmful for aquatic organism at environmental relevant concentrations. In addition, metabolism and potential transformation products, bioaccumulation and physiological alterations induced by these UV filters were investigated in the *in vivo* studies.

Thereby, we tested the following hypotheses described here point by point:

- The selected UV filters BP-3, BP-4 and OC exhibit hormonal activities (e.g. estrogenicity) *in vivo*, and thus may pose a risk to fish reproduction, similar to other UV filters.
- These UV filters do not exclusively act on the endocrine system and thus show also other (unexpected) modes of action.
- Microarrays are a useful technology to decipher new modes of action of UV filters.
- Transcriptional alterations can be translated into physiological and biological effects.

- Similar effects occur at different developmental stages.
- Similar metabolism and transformation products of BP-3 can be found in zebrafish to those in humans.

1.8.2. Reproductive effects of (anti-) progestins in zebrafish

The second part (Chapter 5 - 6) consists of results derived from a two-generation *in vivo* study performed to investigate reproductive effects of the synthetic progestin P4 and the anti-progestin MIF in zebrafish. This study was split into two parts (publications). The first publication (Chapter 5) describes reproductive effects of MIF and P4 derived from a reproduction assay. This included the reproductive performance of the breeding groups (number of eggs and frequency of spawning), fertility of laid eggs, hatching rate of embryos and survival of larvae after ongoing chemical exposure (F1 generation). Additionally, histological alterations in gonads of females and males and potential effects on sex steroid hormone levels are reported. In the second publication (Chapter 6) reproductive effects are related to transcriptional alterations of selected transcripts involved in the endocrine system and steroidogenesis in adult and embryonic zebrafish. In addition, transcriptional alterations of the F0 (adult fish) and F1 (embryos) generation are compared. To compare *in vivo* transcriptional alterations with *in vitro* hormonal activities of MIF and P4, activities in recombinant yeast-screens were assessed. This study was designed to combine physiological (egg reproduction, hormone level, histology), molecular (transcriptomics) and *in vitro* effects of MIF and P4, and to get first insights into their potential environmental risks.

Thereby, we tested the following hypotheses, which are given point by point:

- The synthetic anti-progestin MIF and the progestin P4 affect reproduction of zebrafish.
- Adverse reproductive effects are also reflected in histological alteration in ovaries of females and testes of males, as well as in changes in sex steroid hormone levels (17β-estradiol in females and 11-ketotestosterone in males).

- The fertility off laid eggs and hatch of embryos is affected by MIF and P4.
- Reproductive effects are reflected in transcriptional alterations of specific target genes involved in the endocrine system and steroidogenesis.
- A similar response in transcriptional alterations is observed in the F1 generation (embryos) compared to adult fish.
- Hormonal activities of MIF and P4 observed *in vitro* will predict effects on reproduction and the sex hormone system in zebrafish *in vivo*.

Chapter 2

Effects of the UV-filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*)

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2.1 Abstract

Organic UV filters including benzophenone-3 (BP-3) are widely used to protect humans and materials from damage by UV irradiation. Despite the environmental occurrence of BP-3 in the aquatic environment, little is known about its effects and modes of action. In the present study we assess molecular and physiological effects of BP-3 in adult male zebrafish (*Danio rerio*) and in eleuthero-embryos by a targeted gene expression approach focusing on the sex hormone system. Fish and embryos are exposed for 14 days and 120 hours post fertilization, respectively, to 2.4 - 312 µg/L and 8.2 - 438 µg/L BP-3. Chemical analysis of water and fish demonstrates that BP-3 is partly transformed to benzophenone-1 (BP-1) and both compounds are accumulated in adult fish. Biotransformation to BP-1 is absent in eleuthero-embryos. BP-3 exposure leads to similar alterations of gene expression in both adult fish and eleuthero-embryos. In the brain of adult males *esr1*, *ar* and *cyp19b* are down-regulated at 84 µg/L BP-3. There is no induction of vitellogenin expression by BP-3, both at the transcriptional and protein level. An overall down-regulation of the *hsd3b, hsd17b3, hsd11b2* and *cyp11b2* transcripts is observed in the testes, suggesting an antiandrogenic activity. No histological changes were observed in the testes after BP-3 treatment. The study leads to the conclusion that low concentrations of BP-3 exhibit similar multiple hormonal activities at the transcription level in two different life-stages of zebrafish. Forthcoming studies should show whether this translates to physiological effects.

Keywords: UV filter, benzophenone-3, benzophenone-1, metabolism, bioconcentration, gene expression, gonad-histology

2.2. Introduction

Organic UV-absorbing chemicals (UV filters) are an important class of emerging contaminants. They are increasingly used in personal care products (PCPs), in particular as light-filters to protect the human skin from harmful exposure to UV-irradiation. Many UV filters are aromatic organic chemicals comprising of conjugated pi-systems (chromophores) for light absorption. Currently, 28 UV filters are listed in the EU Cosmetics Directive for commercial cosmetic products containing up to 10% active ingredients (25% for titanium dioxide) (Zenker et al., 2008). UV filters are not only employed in sunscreens, but also in numerous cosmetics including creams, lotions, lipsticks, perfumes, shower bath, hair sprays and shampoos comprise these chemicals in their composition. Additionally, they find application for product safety in transparent packing materials to protect from degradation by UV irradiation (Hauri et al., 2003), but also in food-contact materials, such as plastics and cartons, from which they may migrate into foodstuff (Muncke, 2010).

UV filters can be released into the aquatic environment by wash-off during bathing, or indirectly via wastewater, which is a significant input source year round (Fent et al., 2010). They have been detected in all aquatic compartments of fresh and seawater, and some of them accumulate in fish (Fent et al., 2010; Balmer et al., 2005). Benzophenone-3 (BP-3) is one of the commonly used UV filter and was detected in lakes and rivers up to 125 ng/L in Switzerland (Fent et al., 2010; Poiger et al., 2004), and in Japan (Kameda et al., 2011). In raw and treated wastewater BP-3 occurs at concentrations up to 7.8 µg/L and 700 ng/L, respectively (Fent et al., 2008). Up to 4.9 µg/L BP-3 was detected in effluents from washing activities in households (Hernández-Leal et al., 2010) and it accumulates in sewage sludge (Gago-Ferrero et al., 2011; Rodil et al., 2009a). River sediments in U.S.A. (Schlenk et al., 2005) and China contain up to 13 ng/g dw (dry weight) BP-3 (Zhang et al., 2011). The lipophilicity of BP-3 ($\log K_{OW}$ =3.6) gives rise to accumulation in fish, where up to 151 ng/g lw (lipid weight) was detected (Fent et al., 2010). BP-3 seems to be transformed to different metabolites including benzophenone-1 (BP-1) and benzophenone-2 (BP-2), as detected in the urine of rats and humans (Kunisue et al., 2012; Diaz-Cruz et al., 2008). BP-3 and BP-1 were detected at 52 and 37 ng/L in river water in Spain, respectively (Negreira et al., 2009). BP-1 and BP-2 are also used as UV filters in product safety (Hauri et al., 2003) and display estrogenicity in fish (Weisbrod et al., 2007; Kunz and Fent, 2006b).

The presence of UV filters in the environment and their potential effects are of concern due to their hormonal activities in fish (Christen et al., 2011; Zucchi et al., 2011a,b; Coronado et al., 2008). BP-3 showed slight estrogenic, and both strong antiestrogenic and antiandrogenic activity *in vitro* (Kunz and Fent, 2006b). In Japanese medaka and rainbow trout BP-3 led to induction of vitellogenin (VTG) at 620 and 749 µg/L, respectively, as well as a reduction in hatching rates (Coronado et al., 2008). However, in transgenic zebrafish (Schreurs et al., 2002), and in fathead minnows (Kunz et al., 2006b) exposed to high concentrations of BP-3, VTG induction was not significant. Also BP-1 is estrogenic in fish, it shows multiple hormonal activities *in vitro* (Kunz and Fent, 2006b; Kunz et al., 2006b), and interferes with hormone action by inhibiting the activity of the testosterone-forming enzyme 17β-hydroxysteroid dehydrogenase-3 (*17bHSD3*) (Nashev et al., 2010).

To date, the modes of action of BP-3 are unknown and effects at environmental concentrations remain elusive. The fact that this UV filter is highly used, shows accumulation in the human body and in aquatic biota makes effect studies necessary. The aim of our present work was to elucidate in more detail the molecular and physiological effects of BP-3 in zebrafish (*Danio rerio*), and to compare transcriptional alterations in adult male fish and eleuthero-embryos. By following a targeted gene expression approach using qRT-PCR, we focused on expression of genes involved in hormonal pathways and steroidogenesis in different organs such as brain, liver and testes of male fish, and in eleuthero-embryos. Nuclear sex-steroid hormone receptors (estrogen receptor alpha, androgen receptor), estrogen related genes (vitellogenin, P450aromatase A and B) and genes involved in steroidogenesis (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase type 3, 11β-hydroxylase, 11β-hydroxysteroid dehydrogenase type 2) were assessed, as well as the expression of *cyp1a1,* which play a critical role in xenobiotic metabolism. VTG was analyzed in blood plasma and potential alterations in gonadal histology were investigated in adult male zebrafish. Furthermore, we assessed for possible transformation of BP-3 to other benzophenone-type metabolites by chemical analysis. By combining effects on the molecular and physiological level with BP-3 metabolism and bioconcentration, we provide a more complete toxicological profile of this important UV filter.

2.3. Materials and Methods

2.3.1. Chemicals. Benzophenone-3 (BP-3, 2-Hydroxy-4-methoxybenzophenone, Oxybenzone, CAS no: 131-57-7, purity ≥ 98%), benzophenone-1 (BP-1, 2,4-Dihydroxybenzophenone, CAS no: 131-56-6, purity ≥ 99%), benzophenone-2 (BP-2, 2,2',4,4'-Tetrahydroxybenzophenone, CAS no: 131-55-5, purity 97%), 4,4'-Dihydroxybenzophenone (4,4'-DHB, CAS no: 611-99-4, purity ≥ 95%), 4-Hydroxybenzophenone (4-HB, CAS no: 1137-42-4, purity ≥ 99%), Benzophenone-d_{10} (d_{10}-BP, CAS no: 22583-75-1, 98% D), 17α-ethinylestradiol (EE2, CAS no: 57-63-6, purity > 98%) were purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland). The solvents used, dimethylsulfoxide (DMSO), acetonitrile, methanol, ethanol, diethyl ether (anhydrous) were of HPLC grade and obtained from J.T. Baker (Stehelin AG, Basel, Switzerland), as well as hydrochloric acid (2M). Pentane and formic acid (98%) were purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland). All water used for sample preparation and HPLC was of HPLC grade and in-house prepared (Nanopure Diamond, Barnstead, Switzerland). The SPE columns used for water analysis, Strata-X (60 mg/3 mL), were purchased from Phenomenex (Brechbühler AG, Schlieren, Switzerland). Syringe filters Titan 2 HPLC Filter (17 mm, 0.45 um, PVDF membrane) were purchased from ThermoScientific (US). Bouin's solution (HT10132) and Roti Histofix 10% Formalin (A146.5), used for fixation, were obtained from Sigma-Aldrich (Buchs, Switzerland) and Roth (Arlesheim, Switzerland), respectively. Xylol and Hematoxylin Mayer were purchased from Biosystems (Nunningen, Switzerland) and Alcoholic Eosin-Y from Zeiss (Switzerland). Heparin ammonium salt (100 KU) was obtained from Sigma-Aldrich (Buchs, Switzerland), and BD Micro-Fine+Innen sterile insulin syringes (0.5mL, 0.33 mm (29G) x 12.7 mm) from Becton Dickinson (Allschwil, Switzerland). KoiMed Sleep (Ethylenglycolmonophenylether), used for stress-free euthanization of fish, was purchased from KOI&BONSAI Zimmermann (Bühlertann, Switzerland). The Kits used for RNA extraction (RNeasy Mini Kit 74104 and RNase-Free DNase Set 79254) and cDNA synthesis (iScript™ cDNA synthesis Kit 170-8890) were purchased from Qiagen (Basel, Switzerland) and Bio-Rad (Reinach, Switzerland), respectively.

2.3.2. Maintenance of adult zebrafish. Adult zebrafish (*Danio rerio*) (about 11 months old) were obtained from a professional local dealer (Harlan Laboratories, Inc., Itingen,

Switzerland), transferred to culture tanks (300 L) and acclimatized for 2 months in our laboratory prior to the experiment. Fish of both sexes were held in reconstituted deionized water (salt concentrations: $CaCl_2x2H_2O$ 147.0 mg/L, KCl 2.9 mg/L, $MgSO_4x7H_2O$ 61.6 mg/L, $NaHCO_3$ 32.4 mg/L) with a conductivity of 470-480 µS/cm, and applying a static-water renewal weekly. The water temperature was held constant at 27 ± 1 °C and the photoperiod was set at 16:8 h light/dark. Fish were fed twice daily with a combination of frozen brine shrimps (*Artemia salina*), white mosquito larvae and Daphnia magna (local supplier). Water parameters, such as nitrate, nitrite and pH were controlled regularly using Test strips (Easy Test, JBL) and oxygen supply was ensured (> 80 %).

2.3.3. Exposure experiments (study design).

Exposure of adult males. Adult male zebrafish were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated reconstituted water (12 fish / tank). The experimental setup consisted of six groups with 5 replicates each. The groups composed of water control, solvent control (0.01 % DMSO), the positive control 17α-ethinylestradiol (EE2, 5 ng/L), and three BP-3 concentrations. The lowest concentration of 10 µg/L BP-3 was assumed as a worst-case environmentally relevant concentration, 200 and 600 µg/L BP-3, respectively, were used for studying the modes of action. The quality of exposure water was continuously monitored by measuring oxygen concentration (> 80 %), pH value (6.4-6.8), nitrate and nitrite concentration (0 mg/L) and temperature (27 ± 1 °C). During the experiment, appearance, mortality and abnormal behavior of fish were recorded daily and death fish were removed from the tanks. The fish were held under certain light cycle of 16:8 h light/dark and were fed twice daily as described above. The study was conducted on the basis of the OECD guideline 204 and approved by the local cantonal veterinary office Basel (Switzerland).

Male fish were exposed for 14 days to BP-3, EE2 and the controls after an acclimatization of 4 days using reconstituted water only. A static-water renewal procedure was applied with complete water exchange every 48 h, meaning that fish were transferred to new tanks containing the appropriate BP-3 concentrations and water of the controls, respectively.

During the whole exposure special care was taken to avoid any (cross-) contamination of exposure water by wearing gloves due to ubiquitous presence of UV filter compounds.

At the end of the exposure experiment, fish were anesthetized in KoiMed Sleep (1.5 mL/1 L water). The total body length and body weight were measured for each individual fish. Five fish of every replicate were dissected immediately and brain, liver and testes were collected in RNA*later* and stored at -80 °C for subsequent RNA extraction. The blood was taken and collected separately from four of the dissected fish using heparinized needles (Becton Dickinson, Allschwil, Switzerland). After centrifugation the blood plasma was stored at -80 °C for vitellogenin (VTG) protein analysis. The liver samples were collected individually, whereas brain and testes were pooled for each replicate (n=4 fish per pool). One fish per replicate was fixed in Bouin's after opening of the abdominal part for histological measurements. The remaining fish were frozen in liquid nitrogen after sex determination and stored at -80 °C for subsequent body analysis.

Exposure of eleuthero-embryos. Zebrafish eleuthero-embryos were obtained by in-house breeding using unexposed males and females from our culture tanks. Immediately after fertilization eleuthero-embryos were examined under a stereomicroscope (Zeiss, DV4) and subsequently maintained at 27 ± 1 °C in autoclaved reconstituted water. All eleuthero-embryos at approximately 2-4 h post fertilization (*hpf*) (60 blastula-stage), were collected and placed into covered glass beakers containing 500 mL of reconstituted water and the appropriate concentration of BP-3 and DMSO (0.01 %, solvent control). The experimental setup for the eleuthero-embryo study consisted of 5 groups of exposure with 4 replicates each (100 fertilized eggs/replicate). The groups were chosen similar to the adult study including a water control, solvent control and nominal concentrations of 10, 200 and 600 µg/L BP-3. A static-water renewal rate was applied with complete water exchange every 24 h, by transferring the eleuthero-embryos to new beakers containing reconstituted water, the solvent and the appropriate BP-3 concentrations. The eleuthero-embryos were continuously exposed for 120 *hpf* at 27 ± 1 °C in well-aerated reconstituted water. The eleuthero-embryos were held under photoperiod 16:8 h light/dark and not fed during exposure. The appearance, mortality and abnormal behavior of the eleuthero-embryos were recorded daily.

The quality of the exposure water was monitored by measuring the oxygen concentration (> 80 %), pH value (6.4-6.8), and temperature (27 ± 1 °C). At the end of the exposure (120 *hpf*) 19-24 eleuthero-embryos per replicate were pooled and collected in RNA*later* and stored at -80 °C for subsequent RNA extraction. Care was taken during the whole exposure time to avoid any (cross-) contamination of exposure water by wearing gloves.

2.3.4. Analytical chemistry.

2.3.4.1. Quality assurance.

Water analysis. For water analysis two spike samples were prepared for each workup series and run in parallel to the exposure water samples (*n*=2 per treatment). The spike samples consisted of reconstituted water (25 mL each) which was spiked with known amounts of the analytes BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB. The concentrations for the spike samples were chosen according to nominal exposure concentrations of BP-3 (10, 200 and 600 µg/L). The spike samples served as quality control of the extraction method and were quantified similarly to the exposure water samples. All analytes were detected simultaneously by LC-MS/MS (adult study) and LC-DAD (at 290 nm) (embryo study) respectively, but quantified individually using an external calibration for each analyte. A linear regression model was applied within a concentration range of 0.05 - 5 µg/mL. In addition, the instrumental repeatability was assessed by six times injections of a standard mixture concentration (0.5 µg/mL) and is expressed as standard deviation of system suitability (SST).

Fish analysis. For the fish analysis d_{10}-BP was used as an internal extraction control standard. Therefore, 500 ng d_{10}-BP were added to each exposed and homogenized fish sample. Additionally, two spike samples were prepared for each workup series and run in parallel to the exposure fish samples. The spike samples consisted of an unexposed zebrafish which was spiked with 500 ng of each of the analytes BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP. The unexposed spike samples served as additionally quality control of the extraction method and were quantified similarly to the exposed fish samples. Detection, quantification and instrumental repeatability was done similarly to water analysis described above.

2.3.4.2. Analytical methods.

Water. Exposure water samples were taken three times randomly from every group at the beginning (0 h), after 24 h and prior to water renewal (48 h) for adults, from different replicate tanks. Water sample collection took place after 1, 7 and 11 days of exposure during the adult zebrafish study, and after 1, 3 and 4 days of exposure during the eleuthero-embryo study. The samples were protected from light and stored at -20 °C until analysis. The water samples were worked up in different series including samples of one exposure group per series (*n*=9 for adult study and *n*=6 for eleuthero-embryo study). Special care was taken during sampling and sample preparation to avoid any (cross-) contamination of samples due to the ubiquitous presence of UV filters. All material was rinsed several times with nanopure water and ethanol and glass ware was heated-up at 500 °C (for 3 h) prior to use. Moreover, the whole extraction procedure was performed in the dark to avoid possibly degradation of the UV filter compounds. The here developed analytical method allowed the simultaneous detection of BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB in water samples.

Stock standard solutions of BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB were prepared separately in ethanol containing 2000 µg/mL each. From these stock solutions, 7 working standard mixtures in ethanol containing each compound in a concentration range of 0.05 to 5 µg/mL were prepared. Working extraction control standard solutions containing each analyte were similarly prepared in ethanol. All standard and working solutions were stored in the dark at 4 °C prior to use.

A sample aliquot of 25 mL was subjected to solid-phase-extraction (SPE) using polymeric reversed-phase sorbent columns, Strata-X (60 mg/ 3 mL) (Phenomenex, Brechbühler AG, Schlieren, Switzerland). The columns were equilibrated subsequently with 2 mL methanol, HPLC-water and reconstituted water. After sample load (25 mL) the columns were washed with 2 mL HPLC-water and 5% methanol and subsequently dried under vacuum for about 30 minutes. After clean up the analytes were eluted and collected using 2 mL methanol/acetonitrile (90+10 v/v). Samples were dried under gentle stream of nitrogen at 30 °C and resuspended in ethanol and stored at -20°C until LC-MS/MS analysis. Samples of higher exposure groups were diluted into calibration range with ethanol.

Exposure water samples of the eleuthero-embryo study were prepared similarly with exceptions that based on water results of the adult study, spiked samples and calibration standards contained only BP-3 and BP-1 and the analyte detection was performed by LC-DAD (at 290 nm) with six calibration standards in the range of 0.1 to 5 µg/mL.

Fish. Fish were sampled at the end of exposure after 14 days and stored at -80 °C until analysis. The extraction of fish samples was performed using liquid-liquid extraction without complex cleanup procedure. The here developed analytical method allowed simultaneous detection of BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP (extraction control standard) in fish samples. The fish samples were worked up in different series containing samples of one exposure group per series and including two fish per replicate tank (*n*=10 samples per group). Precautions and pre-treatment of materials used was similar as described for water analysis.

Stock standard solutions of BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP were prepared separately in ethanol containing 1000 µg/mL each. From these stock solutions, 7 working standard mixtures in ethanol containing each compound in a concentration range of 0.01 to 3 µg/mL were prepared. Working extraction control standard solutions, containing only d_{10}-BP (internal extraction control standard for exposed fish samples) and a mixture containing each analyte (for spiked samples) were similarly prepared in ethanol.

The individual fish samples were homogenized with 4 mL HPLC water using an Ultra-Turrax mixer (IKA Ultra-Turrax T8 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany). Subsequently, the exposed fish samples were spiked with 500 ng of d_{10}-BP extraction control standard solution and the unexposed fish were spiked with 500 ng of the mixture standard solution containing all analytes. The samples were stabilized by adding 2M HCl (pH 1). The extraction was performed stepwise by adding first 4 mL diethyl ether and 10 % NaCl solution (pH 1). Next, 4 mL pentane was added and shaked again. Subsequently 4 mL of acetonitrile were added and the mixture was shaken vigorously for about 1 min. After centrifugation at 5000 rpm for 15 min at 4 °C the organic phases were collected. The extraction was repeated by using fresh pentane and acetonitrile. The organic extracts were combined and the volume was adjusted to about 20 mL with diethyl ether. An aliquot of 3 mL extract was filtrated through a pentane-purged 0.45 µm PVDF syringe filter (Titan 2 HPLC Filter, Thermoscientific, US). The aliquot solvents were evaporated to dryness using a gentle stream of nitrogen at

30 °C and the analytes were resuspended in ethanol. As a last purification step, the resultant residues were centrifuged at 13'300 rpm for 3 min at 4 °C and consequently, the supernatant was taken for LC-MS/MS analysis. The fish samples of higher exposure groups were diluted within the calibration range with ethanol.

LC-MS/MS and LC-DAD analysis. The analytes BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP were determined by LC-MS/MS (Agilent 1200) and LC-DAD respectively (Agilent 1100) in water and fish samples. The LC-MS/MS system consisted of an Agilent 1200 HPLC system (DAD at 290 nm) coupled to an Ion-Trap equipped with an ESI interface (Agilent Technologies). The software used for quantification was the Agilent ChemStation. The chromatographic separation was achieved by using a Zorbax SB-C18 column (150 mm x 3.0 mm, 3.5 µm particle size) and a C-18 guard column (4.6 mm x 12.5 mm, 5 µm particle size), both from Agilent Technologies (Basel, Switzerland) at column temperature of 40 °C. The gradient mixture consisting of HPLC grade water + 0.1 % formic acid and acetonitrile + 0.1 % formic acid was used at a flow rate of 0.5 mL/min. The elution gradient started with a mixture of 90 % water and 10 % of acetonitrile, the latter increased to 90 % within 15 minutes and was kept for 5 min, before the system was set back to initial conditions within 2.5 min. The total run time of 25 min included a re-equilibration of 2.5 min prior the next injection. The single sample injection volume was set to 3 µL for each sample using an autosampler. A summary of detected analytes including physicochemical properties, detected ions and retention times for the LC-MS/MS analysis is shown in the SI (Table S1). The setup used for MS parameters was similar to previous studies (Zenker et al., 2008).

The water samples of the eleuthero-embryo study were analyzed using an Agilent 1100 HPLC system, LC-DAD (Agilent Technologies) at 290 nm. The parameters used for chromatographic separation were similar to those described above for LC-MS/MS detection, but adapted for detection of less analytes. The elution gradient started with a mixture of 90 % water and 10 % of acetonitrile, the latter increased to 90 % within 10 min and afterwards, the system was set back to initial conditions within 2.5 min. The total run time of 14 min included a re-equilibration of 2.5 min prior the next injection. Samples were injected in duplicate with injection volume set to 10 µL.

2.3.5. Molecular und biochemical analyses.

RNA isolation. Total RNA was extracted from adult zebrafish tissues and pooled eleuthero-embryos (*n*=19-24 per pool and replicate) using the RN*easy* Mini Kit (Qiagen, Basel, Switzerland). In adult fish, liver was taken from individual animal, while brain and testes were pooled (*n*=4 per pool and replicate). All samples were further treated with RNase-Free DNase set (Qiagen, Basel, Switzerland) to purify the RNA from DNA contamination and to remove DNase and divalent cations from the samples. RNA concentration and quality was analyzed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington DE, USA) by measuring the absorbance at different wavelengths. The purity of every RNA sample was between 1.8 and 2.0 for both ratios 260/280 nm and 260/230 nm. Additionally, the purity of RNA samples was approved by electrophoresis obtaining two sharp bands for the large and small subunit ribosomal RNAs (rRNA) (intensity ratio for 28S/18S about 2:1) (Taylor et al., 2010). The RNA samples were stored at -80 °C for subsequent qRT-PCR analysis.

qRT-PCR analysis. The total RNA was isolated as described above from individual zebrafish liver, pooled adult brain and testes and pooled eleuthero-embryos. In total, the number of samples used for subsequent qRT-PCR were *n*=5 (5 replicates per dose group) for adult tissue samples, and *n*=4 (4 replicates per dose group) for eleuthero-embryos. 1 µg of the total RNA template was reverse-transcribed using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad, Reinach, Switzerland) in the presence of a modified MMLV-derived reverse transcriptase and a blend of oligo(dT) and random hexamer primers. The complete reaction mix was incubated for 5 min at 25 °C, following 30 min at 42 °C. The reaction was stopped by heating at 85 °C for 5 min. The cDNA was stored at -20 °C.

50 ng cDNA per reaction was used to perform qRT-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). The following real-time PCR profile reaction was used: enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (10 s), 57-60 °C (60 s) depending on the target transcript as shown in Table 1, followed by a melting curve analysis post run (65-95 °C) which confirmed the specificity of the chosen primers as well as the absence of primer dimers.

Gene-specific primers from published zebrafish primer sequences (Table 1) were obtained from Microsynth (Basel, Switzerland), and analyzed specifically in the following tissues. Vitellogenin 1 (*vtg1*) was determined in the adult liver, estrogen receptor alpha (*esr1*), androgen receptor (*ar*), P450aromataseB (*cyp19b*) and P450 enzyme (*cyp1a1*) in the adult brain, 3β-hydroxysteroid dehydrogenase (*hsd3b*), 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), P450aromataseA (*cyp19a*), 11β-hydroxylase (*cyp11b2*), 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*) in adult testes. The same target gene approach was used for the eleuthero-embryos. The ribosomal protein L13a (*RpL13a*) was selected as housekeeping gene for normalization, because its expression profile did not vary either under the experimental conditions, nor in different tissues analyzed. PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled eleuthero-embryo cDNA (R^2 > 0.98 for all primers). Due to the high efficiency, no corrections in calculations of the ΔCt values were necessary (Taylor et al., 2010). No template control (NTC) reaction was included in every run for each primer pair, to test for potential reagent contaminations and to evaluate presence of primer-dimers (Taylor et al., 2010).

Table 1: Primer sequences for quantitative RT-PCR analysis and sequence sources: vitellogenin 1 (*vtg1*), P450 enzyme (*cyp1a1*), estrogen receptor alpha (*esr1*), androgen receptor (*ar*), P450aromataseB (*cyp19b*), P450aromataseA (*cyp19a*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), 11β-hydroxylase (*cyp11b2*), 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*), ribosomal protein L13a (*RpL13a*).

Target gene	GenBank number	Sense primer (5' - 3')	Antisense primer (5' - 3')	Product size (bp)	Annealing temperature [°C]
vtg1 [a]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94	57
cyp1a1 [b]	AF210727	AATCCCAGACGGGCTACA	CCGGGCCATAGCACTTAC	122	60
esr1 [c]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163	60
ar [d]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237	60
cyp19b [b]	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94	58.5
cyp19a [b]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92	58.5
hsd3b [e]	AY279108	GCAACTCTGGTTTTCCACACTG	CAGCAGGAGCCGTGTAGCTT	102	60
hsd17b3 [e]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121	57
cyp11b2 [f,g]	NM_001080204	GCTCATGCACATTCTGAGGA	TGTGCTGAAGGTGATTCTCG	115	60
hsd11b2 [f,g]	NM_212720	TGCTGCTGGCTGTACTTCAC	TGCATCCAACTTCTTTGCTG	123	60
RpL13a [h]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100	57- 60

Data sources: [a] (Hoffmann et al., 2006), [b] (Arukwe et al., 2008), [c] (Martyniuk et al., 2007), [d] (Hossain et al., 2008), [e] (Hoffmann et al., 2008), [f] (Fuzzen et al., 2011), [g] (Alsop and Vijayan, 2008), [h] (Oggier et al., 2010).

All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). For calculating expression levels, normalization was performed by subtracting the mean threshold cycle (Ct) value for the housekeeping gene, which served as internal control, from the mean Ct value of the target gene (ΔCt value). The ΔCt values of treated samples were calibrated against the untreated (control) ΔCt values for all target genes. The relative amount of target molecules relative to the control was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The mRNA expression level of the different target genes are expressed as fold change (log2) according to the formula: $2^{-(\Delta Ct(\text{treated sample})-\Delta Ct(\text{untreated sample}))}$ and are referred to solvent control.

Vitellogenin protein analysis. The blood from four individual zebrafish per replicate tank was taken immediately after anesthesia, by cardiac puncture using heparinized insulin syringes (BD Micro-Fine+Innen sterile Insulin syringes, 0.5 mL, 0.33 mm (29G) x 12.7 mm (Becton Dickinson)). The sterile syringes were heparinized with ice-cold heparin solution (1000 Units heparin/mL) prepared with 0.85 % saline solution. The blood samples were centrifuged for 5 min at 4 °C and 9300 g and subsequently transferred to new reaction tubes and stored at -80 °C until analysis.

Individual plasma samples of adult zebrafish were thawed on ice, vortexed and diluted with assay-buffer (dilution factor 5000) immediately before VTG protein analysis. For quantification of VTG a commercially available enzyme-linked immunosorbent assay (ELISA) for zebrafish was used (Biosense Laboratories AG, Bergen, Norway). Purified zebrafish VTG from blood plasma was used as standard for an external calibration. The assay and VTG quantification was performed according to manufacturer instructions. One individual blood plasma sample per replicate tank (*n*=5 samples per dose group) was analyzed for vitellogenin protein. VTG protein titer and *vtg1* mRNA were investigated in the same individuals.

2.3.6. Histology. After anesthesia, one fish per replicate tank (*n*=5 per dose group) was opened at the abdominal-site and fixed in Bouin's solution for about 24 h. One additional fish per replicate tank of the positive control (EE2) was fixed in Formalin (10 %) for about 48 h to compare quality of fixation reagents afterwards. After fixation, fish were kept in 70 %

ethanol for about four weeks. The head and tail were removed and the remaining body was cut into four pieces of about 4-5 mm thickness using microtome-blades (Solingen). The samples were dehydrated applying the following protocol: 70 % ethanol (45 min), 80 % ethanol (45 min), 96 % ethanol (30 min), 96 % ethanol (45 min), 100 % ethanol (30 min), 100 % ethanol (60 min), 100 % ethanol (60 min), xylol (30 min), xylol (45 min), xylol (60 min), paraffin at 62 °C (45 min), paraffin at 62 °C (60 min) and paraffin at 62 °C (60 min). After dehydration samples were embedded in paraffin perpendicular meaning the four pieces per fish in one paraffin block. 3 µm thin sections were cut using a water-flow microtome (Microm HM 355 S). The sections were stained by standard Hematoxylin-Eosin (H&E) staining protocol.

The evaluation of cross sections was done using a Zeiss microscope (Zeiss, Germany) (40x magnification) and the images were evaluated using ImageJ software (free-download software). Sections were taken from three different levels along the gonad-axis. Testes staging was based on the relative frequencies of spermatogonia, spermatocytes and spermatids present (Johnson et al., 2009) by measuring their areas occupied in every fish and gonad. The data were evaluated by calculating the mean of every testicular stage and for every single fish, including all levels of both gonads. Finally, the overall mean was calculated for every stage and dose group and was taken for graphical representation.

2.3.7. Data analysis and statistics. All graphic data were graphically illustrated with GraphPad Prism 5 (GraphPadSoftware, San Diego, USA). The significance of differences in either transcript level for qRT-PCR, blood level of VTG, condition factor, and testicular stages in the histology caused by BP-3 and EE2, respectively, was analyzed by one way analysis of variance (ANOVA) followed by a Tukey post-hoc test (95% confidence interval). Graphical results are shown as mean ± standard deviation of mean. Differences were considered significant at $p \leq 0.05$.

2.4. Results

2.4.1. General toxicity.

Adult males. At the end of experiment, body length and weight of individual fish were measured in order to determine the condition factor (CF= weight [g]/length [mm] x 100) (see Table S2 in SI). Fish showed no abnormal behavior and only accidently mortality during exposure. They were similar in size (3.84 ± 0.03 cm) and weight (494.6 ± 14.9 mg wet weight) and did not significantly differ among replicates and treatments, therefore the condition factor (CF) did not vary (1.29 ± 0.03). Details of biological parameters are given in SI (Table S2).

Eleuthero-embryos. During the exposure of embryos, mortality and behavior were recorded daily and coagulated eggs or death eleuthero-embryos were removed immediately. As previously observed (Zucchi et al., 2012; Fraysse et al., 2006), the highest mortality occurs within the first 24 h. There was no difference in mortality between control and exposed eleuthero-embryos, and thus no relation to BP-3 exposure. Subsequently, the mortality was negligible. Eleuthero-embryos are very sensitive for water quality, however no relation of mortality among treatments and controls was observed. Eleuthero-embryos displayed normal swimming behavior by the end of the 120 h exposure. The overall survival of each treatment (*n*=4 replicates per treatment) and the sample pool size range (*n*=4 pools, 1 pool for every replicate and treatment) used for RNA extraction and subsequent qRT-PCR analysis is given in SI (Table S3).

2.4.2. Analytical chemistry.

2.4.2.1. Quality assurance.

Water and fish analysis. For all sample sequences measured, the standard deviation of system suitability (SST) was below 10 % and the linear regression coefficients (R^2) > 0.99 for all detected analytes. Arising from the chromatographic peak area of the calibration standards, the limit of quantification (LOQ) was set equal to the limit of detection (LOD) at 0.15 ng (water) and 0.03 ng (fish) for LC-MS/MS detection, respectively (signal to noise ratio

(S/N) > 20 (derived from Agilent software ChemStation)) and at 1.0 ng (water) for LC-DAD detection (S/N > 4 (derived from visual inspection)). The recovery rates for all detected analytes, BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB were acceptable (64 - 119 %) for water analysis of the adult and eleuthero-embryo study, respectively. The recovery rate of d_{10}-BP was reproducible beyond all exposed and unexposed spiked fish samples, ranging from 58 to 86 %. The variation of recovery rates for the other analytes in the unexposed spiked fish samples (BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB) was more abundant (37 - 129 %). A detailed summary of quality assurance for the water and fish analysis (linearity (R^2), SST, LOD/LOQ, recoveries of spike samples) is given in the SI (Table S4 and S5).

2.4.2.2. Analytical results.

Water. During the exposure of adult fish, water samples were taken three times randomly from different replicates tanks and for every treatment after 0, 24 and 48 h of exposure (*n*=3 for every time point). In control water, none of the analytes (BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB) could be detected, or were below the limit of quantification (LOQ = 0.15 ng). In general, only BP-3 and BP-1 were found in water samples, but no BP-2, 4,4'-DHB and 4-HB. BP-3 was degraded rapidly within 48 h, and the degradation product BP-1 increased from 24 to 48 h. Measured median concentrations of BP-3 ranged between 4.8 (0 h) - 1.7 (48 h) µg/L at nominal dose 10 µg/L, 124 (0 h) - 80 (48 h) µg/L at nominal dose 200 µg/L and 312 (0 h) - 294 (48 h) µg/L at nominal dose 600 µg/L (see Table S6 in SI). The actual median BP-3 concentrations over time (0-48 h) were 2.4, 84 and 312 µg/L, corresponding to 24 %, 42 % and 52 % of nominal levels, respectively (Table 2). The metabolite BP-1 could not be detected in any of the exposure water samples at time point 0 h. At later time points, the measured median concentrations of the metabolite BP-1 ranged between 0.3 (24 h) - 0.5 (48 h) µg/L at nominal 10 µg/L BP-3, 6.0 (24 h) - 10.4 (48 h) µg/L at nominal 200 µg/L BP-3, and 17.4 (24 h) - 23.3 (48 h) µg/L at nominal 600 µg/L BP-3 (see Table S6 in SI). The actual BP-1 concentrations over time in the three BP-3 treatment groups were 0.4, 8.2 and 20.4 µg/L, respectively (Table 2).

In contrast are the results of the eleuthero-embryo study, whereas only BP-3 and no BP-1 was detected in all water samples, except in controls, which were also free of any contamination. Similar as in the adult study, BP-3 was degraded rapidly within 24 h in the treated groups. The measured median concentrations of BP-3 ranged between 8.1 (0 h) - 8.3

(24 h) µg/L at nominal 10 µg/L, 117.4 (0 h) - 109.9 (24 h) µg/L at nominal 200 µg/L and 449.9 (0 h) - 425.6 (24 h) µg/L at nominal 600 µg/L (see Table S7 in SI). The actual median BP-3 concentrations over time (0-24 h) in the eleuthero-embryo experiment were 8.2, 114 and 438 µg/L, corresponding to 82 %, 57 % and 73 % of nominal exposure concentrations, respectively (Table S7 in SI). The EE2 concentrations (positive control) in exposure water were not measured analytically and considered to be stable within 48 h.

Table 2: Summary of chemical data for adult exposure study. Measured concentrations of BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB in exposure water and adult fish samples are shown. Measured actual water concentrations (µg/L) are shown as median concentrations (0-48 h) (*n*=3 water samples per time point and dose group). Concentrations in fish are given as mean ± standard deviation in ng/g wet body weight (bw) (*n*=10 fish samples per dose group). The actual concentrations in exposure water and fish are used to calculate the bioconcentration factor (BCF), expressed as mean ± standard deviation.

Dose groups	**measured concentrations**	**BP-3**	**BP-1**	**BP-2**	**4,4-DHB**	**4-HB**
Control	conc. in fish (ng/g bw)	n.d.	n.d.	n.d.	n.d.	n.d.
	conc. in water (µg/L)	-	-	-	-	-
	BCF					
Solvent control DMSO	conc. in fish (ng/g bw)	n.d.	n.d.	n.d.	n.d.	n.d.
	conc. in water (µg/L)	-	-	-	-	-
	BCF					
Low 10 µg/L BP-3	conc. in fish (ng/g bw)	44.5 ± 25.7	43.2 ± 26.9	n.d.	n.d.	n.d.
	conc. in water (µg/L)	2.4	0.4	-	-	-
	BCF	**19 ± 11**	**108 ± 67**			
Mid 200 µg/L BP-3	conc. in fish (ng/g bw)	7918 ± 2194	217 ± 228	n.d.	< LOD	n.d.
	conc. in water (µg/L)	84	8.2	-	-	-
	BCF	**94 ± 26**	**27 ± 28**			
High 600 µg/L BP-3	conc. in fish (ng/g bw)	20686 ± 8551	300 ± 166	n.d.	< LOD	n.d.
	conc. in water (µg/L)	312	20.4	-	-	-
	BCF	**66 ± 27**	**15 ± 9**			

Bioconcentration factor (BCF) calculated as BCF = actual conc. in fish [ng/g bw] / actual conc. in water [µg/L]. (n.d.: not detected; < LOD: below limit of detection (0.03 ng)).

Fish. Two fish (whole body) per replicate tank and treatment were analyzed for BP-3 and potential metabolites (BP-1, BP-2, 4,4'-DHB and 4-HB) by LC-MS/MS (*n*=10 fish per

treatment). None of the analytes could be detected in control fish. Appropriate to the water analysis of the adult study, only BP-3 and BP-1 were found in fish samples, whereas BP-2, 4,4'-DHB and 4-HB could not be detected. BP-3 and BP-1 were accumulated in adult zebrafish after 14 days of exposure in a dose-related manner. Measured mean concentrations of BP-3 and BP-1 were 44.5 ± 25.7, 7918 ± 2194, 20686 ± 8551 ng/g bw (wet weight), and 43.2 ± 26.9, 217 ± 228, 300 ± 166 ng/g bw (wet weight), respectively (Table 2). Bioconcentration factors (BCF=conc. in fish [ng/g bw] / conc. in water [µg/L]) were calculated for BP-3 and BP-1 based on actual concentrations in fish and water. BCFs increased with exposure concentrations with highest BCF of 94 ± 26 for BP-3 at 84 µg/L (Table 2). In contrast, the BCF for BP-1 decreased with increasing exposure concentrations (Table 2). A detailed representation of individual data for the water analysis of every time point (0, 24, 48 h) of the adult fish and eleuthero-embryo study, as well as for the fish analysis, including recovery rates of spiked samples for every workup series is given in the SI (Tables S6, S7, S8).

2.4.3. Targeted gene expression analysis by qRT-PCR and VTG protein analysis.

To test the hypothesis that BP-3 interferes with genes involved in sex hormonal signaling and steroidogenesis a suite of target genes was selected and investigated in different tissues of males, and additionally in eleuthero-embryos. We scrutinized the investigated genes for potential transcriptional difference between water and solvent control (0.01% DMSO in BP-3 and EE2 dose groups) samples by means of qRT-PCR. No significant differences occurred (data not shown), thus gene modulation arising from the use of the solvent can be excluded, and consequently, gene expression normalization and comparison of treatments were conducted versus solvent control samples.

Adult males. The expression of *esr1, ar*, *cyp19b* and *cyp1a1* (Fig. 1) were evaluated in the brain, *vtg1* in the liver (Fig. 2) and *hsd17b3, cyp19a* (Fig. 3), *hsd3b*, *cyp11b2* and *hsd11b2* (Fig. S1 in SI) in the testes. In the brain, BP-3 and EE2 led to a similar tendency for down-regulation of *esr1* and *ar*, being significant only at 84 µg/L BP-3 (Fig. 1). Expressional changes of steroidogenic enzymes, such as the aromatase *cyp19a* and *cyp19b* in testes and brain, respectively, were evaluated due to their critical role in catalyzing the final rate-limiting step in conversion of androgens into estrogens. BP-3 led to a significant down-regulation of the

cyp19b transcript at 84 µg/L (2.9-fold inhibition), similar to the positive control EE2 (2.5-fold inhibition) in the brain (Fig. 1). In the testes, transcripts of *cyp19a* showed a significant transient induction at 2.4 µg/L BP-3 (1.2-fold). Additionally, transcripts of *cyp1a1* were induced by BP-3 in the adult brain, being significant only at 312 µg/L BP-3; the same up-regulation trend (albeit not significant) occurred also in the positive control (EE2) (Fig. 1).

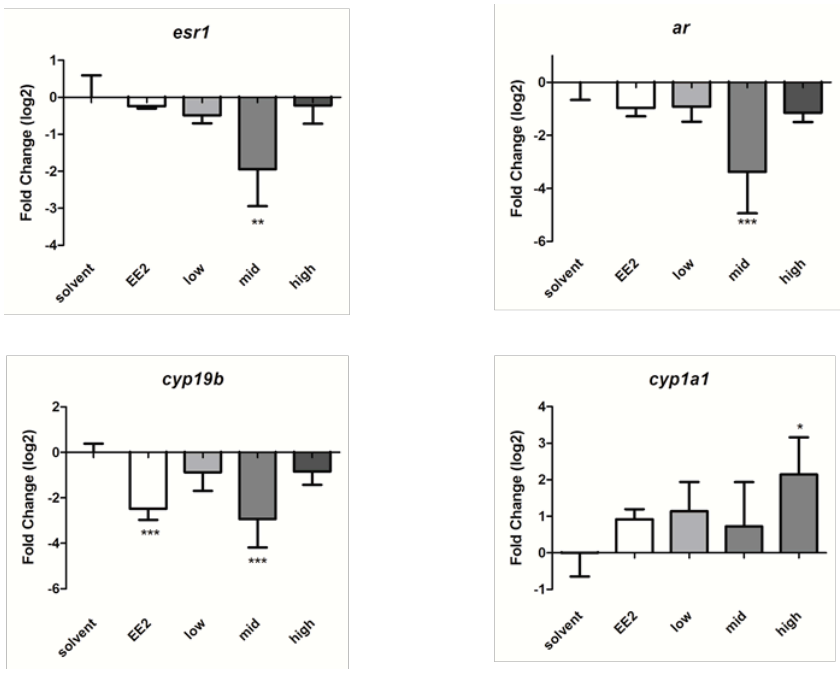

Figure 1: Relative gene expression in brain of adult zebrafish males of *esr1*, *ar*, *cyp19b* and *cyp1a1* after exposure to 2.4 (low), 8.4 (mid), 312 (high) µg/L BP-3 and the positive control (5 ng/L EE2). Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. Results are given as mean ± standard deviation (*n*=4-5 replicates per treatment). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

In the liver *vtg1* mRNA was not altered, while the positive control EE2 significantly induced vitellogenin at transcriptional and translational level (Fig. 2). The lack of *vtg1* induction by BP-3 was further confirmed by VTG protein analysis in the blood of the same individual male fish (Fig. 2).

In addition we also performed a microarray study (data and detailed methodological information are shown in the SI) using the same individual liver samples of solvent control, 2.4 and 312 µg/L BP-3 as used for qRT-PCR analysis. The microarray data revealed that no particular physiological or biological pathways were significantly affected by BP-3 in the liver of zebrafish. Thus, no additional information on potential molecular effects or the mode of action of BP-3 could be deduced from the microarray data.

In the testes BP-3 exposure led to an overall down-regulation of *hsd3b, hsd17b3, hsd11b2* and *cyp11b2* transcripts (Fig. 3 and Fig. S1 in SI), being significant at 84 µg/L BP-3 (0.8- fold inhibition) only for the *hsd17b3* transcript (Fig. 3). Similarly, *hsd17b3 was* down-regulated by EE2 (1.5-fold inhibition) (Fig. 3).

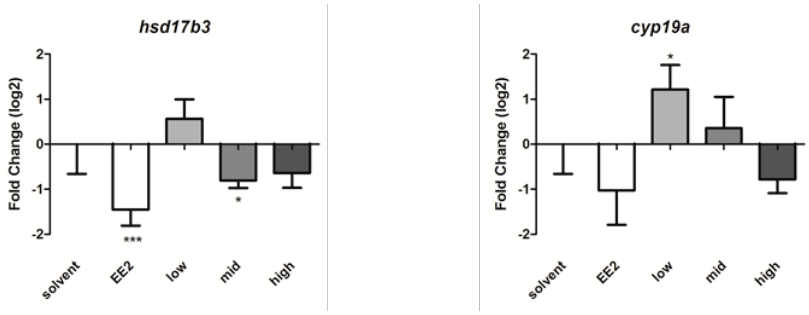

Figure 3: Relative gene expression in testes of adult zebrafish males of *hsd17b3* and *cyp19a* after exposure to 2.4 (low), 8.4 (mid), 312 (high) µg/L BP-3 and the positive control (5 ng/L EE2). Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. Results are given as mean ± standard deviation (*n*=4-5 replicates per treatment). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (*** $p < 0.0001$).

Eleuthero-embryos. The same target genes were analyzed in eleuthero-embryos. The *esr1* transcript shows a down-regulation, being significant at 438 µg/L BP-3 (0.6-fold inhibition (Fig. 4). Transcripts of *vtg1* did not vary upon BP-3 exposure as in adult fish (Fig. S2 in SI). There was a tendency for a down-regulation of the *ar* at 114 and 438 µg/L BP-3, albeit not significant (Fig. S2 in SI). Similarly as in the adult brain, the *cyp19b* transcript tended to be down-regulated by BP-3, albeit not significantly (Fig. S2 in SI). BP-3 led to significant down-regulation of *hsd17b3* at 114 and 438 µg/L BP-3, similar as observed in the adult testes

(Fig. 4). The transcript of *hsd3b* was also down-regulated (up to 1.5-fold inhibition), albeit not significantly (Fig. S2 in SI).

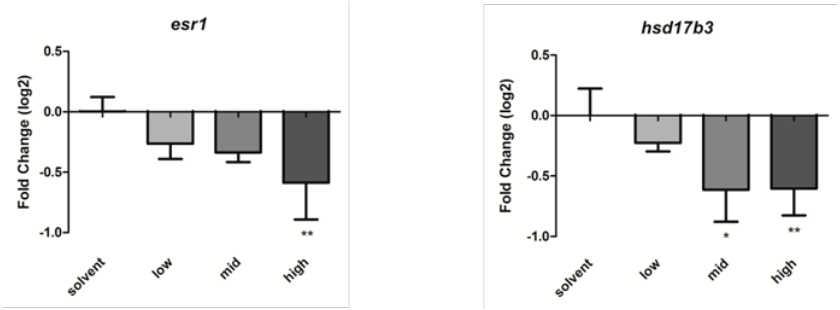

Figure 4: Relative gene expression in eleuthero-embryos of *esr1* and *hsd17b3* after exposure to 8.2 (low), 114 (mid) and 438 (high) µg/L BP-3. Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. Results are given as mean ± standard deviation (*n*=3-4 replicates per treatment). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$).

2.4.4. Histology.

Testes of all investigated adult zebrafish males were regular in structure showing a full spectrum of sperm differentiation stages (spermatogonia, spermatocytes, spermatids) in controls and treated fish. No significant differences occurred across treatments in terms of frequencies of various developmental stages. Testes of males exposed to 2.4, 8.4 and 312 µg/L BP-3, and 5 ng/L EE2, respectively, were characterized by a high frequency of mature sperms (spermatids) and a low frequency of spermatogonia, which apparently did undergo further differentiation to spermatocytes (Fig. 5). Therefore, no effect on testicular development and no intersex phenomenon occurred after exposure to BP-3. Typical histological sections of testes are shown in Fig. 6 and frequencies of individual stages are shown in Fig. S3 in the SI. Bouin's solution is preferred over Formalin 10% as fixation reagent for adult testes due to observed hardness of the tissue (data not shown).

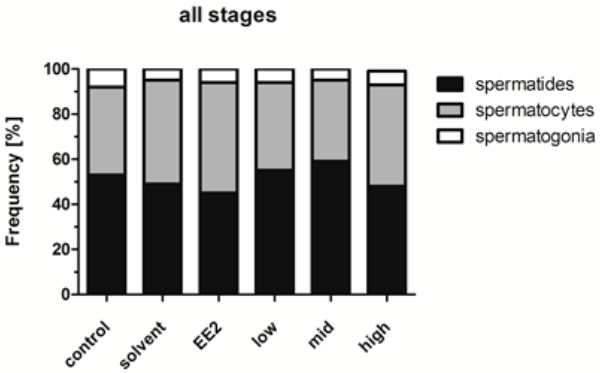

Figure 5: Relative frequencies of different testicular developmental stages (spermatogonia, spermatocytes, spermatids) in adult male zebrafish after exposure to 2.4 (low), 8.4 (mid) and 312 (high) µg/L BP-3, and positive control (5 ng/L EE2). Results are given as mean values (*n*=5 males per treatment).

2.5. Discussion

2.5.1. Analytical chemistry.

The quality assurance applied to verify the analyte concentrations detected show that recovery rates for all detected analytes (BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP) were in an acceptable range for water and fish analysis, whereas in fish, recovery rates were more variable, but reproducible for all individual analytes. The developed analytical method is a compromise for simultaneous detection of BP-3, four possible metabolites (BP-1, BP-2, 4,4'-DHB, 4-HB) and d_{10}-BP as internal extraction control standard, considering the varying chemical extraction properties of the different analytes (see Table S1 in SI).

BP-3 was partly transformed to BP-1, mainly via metabolism and subsequent excretion by adult fish. Thus, biotransformation is similar in adult fish as in humans (Kunisue et al., 2012). However, zebrafish eleuthero-embryos lack the capability to metabolize BP-3 to BP-1, probably because BP-3 metabolizing enzymes are not yet fully active at this early life-stage. Occurrence of BP-1 is not related to photodegradation under the photoperiod of 16:8 h light/dark, as a similar light/dark regime was used in the embryo exposure, where no BP-1 was formed. The BP-3 concentrations were rather stable within 48 h exposure for the highest dose (312 - 294 µg/L) compared to the lower doses (124 - 80 µg/L and 4.8 - 1.7 µg/L), which implies that degradation of BP-3 is more evident for lower concentrations.

Furthermore, this degradation of BP-3 was not so obvious in the embryo study, probably due to the more frequent water exchange in this study. Previous studies (Liu et al., 2012; Rodil et al., 2009b) described microbial biodegradation of BP-3 to BP-1 as a common and fast phenomenon under oxic conditions. *O*-demethylation was proposed as main pathway for BP-3 biodegradation (over photolysis and hydrolysis). Furthermore, BP-3 and BP-1 accumulated in adult zebrafish after 14 days of exposure in a dose-dependent manner. BP-1 body burdens are rather based on metabolism than direct uptake from exposure water. BCFs of BP-3 increased with exposure concentrations being highest at 84 µg/L BP-3. In contrast, the BCF for BP-1 decreased with increasing exposure concentrations. BP-3 and BP-1 have a higher tendency for bioconcentration in fish as compared to BP-2 (Weisbrod et al., 2007). Previous studies demonstrated BP-1 as the major metabolite of BP-3 also in humans (Kunisue et al., 2012).

2.5.2. Targeted gene expression analysis by qRT-PCR.

In the present study we analyzed the effects of BP-3 on the expression of genes involved in estrogenic, androgenic and steroidogenic pathways, motivated by previous studies which showed hormonal activity potential in other species (Coronado et al., 2008) and the structurally related UV filter benzophenone-4, respectively (Zucchi et al., 2011b). We show that exposure to BP-3 leads to interference with the expression of transcripts involved in hormonal and steroidogenic pathways in both adults and eleuthero-embryos.

Adult males. In the brain 84 µg/L BP-3 led to down-regulation of *esr1*, *cyp19b* and *ar* transcripts. The significant down-regulation of *ar* (3.4-fold) at 84 µg/L BP-3 is in accordance to our previous *in vitro* data pointing to an antiandrogenic activity both *in vitro* (Kunz and Fent, 2006b) and *in vivo*. In fish expressional changes of *ar* may translate to adverse effects on reproduction in both sexes (Gorelick et al., 2008). Down-regulation of the *ar* transcript by EE2 was expected (Filby et al., 2007), while *cyp19b* mRNA was unexpected (Martyniuk et al., 2006). Alterations in brain aromatase (*cyp19b*) expression during exposure to estrogenic compounds could also result in impaired sexual behavior in male fish (Hinfray et al., 2006; Fenske and Segner, 2004).

Transcripts of *cyp19b*, expressed in the brain of adult fish showed an induction, and *cyp19a*, mainly expressed in the testes, show a decrease after BP-3 exposure. A similar effect occurred with benzophenone-4 (BP-4) with induction of *cyp19b* in the brain, and down-regulation of *cyp19a* in the testes of male zebrafish (Zucchi et al., 2011b). Thus, the differential reactions of the aromatases in different tissues (up- and down-regulation, respectively) are shared by BP-3 and BP-4. In the testes expression of *hsd3b, hsd17b3, hsd11b2* and *cyp11b2* were analyzed to determine potential effects of BP-3 on the sex-steroid hormone synthesis (Busby et al., 2010). Significant alterations occurred only for the *hsd17b3* transcript at 84 µg/L BP-3, similar to EE2, as previously shown in fathead minnow (Filby et al., 2007).

Forthcoming studies are needed to determine whether these transcriptional changes affect plasma sex steroid levels in adult fish. Determination of estradiol, androstenedione, 11-keto-testosterone, 11β-OH-testosterone would further corroborate our hypothesis of an antiestrogenic and antiandrogenic activity of BP-3.

Additionally, transcripts of *cyp1a1* were induced by BP-3 in the adult brain, being significant at 312 µg/L BP-3, and a similar trend (albeit not significant) occurred in the positive control (EE2). The induction of *cyp1a1* by BP-3 may be a consequence of biotransformation responses in zebrafish due to *O*-demethylation, and may in turn lead to modulation of steroidogenesis (Arukwe et al., 2008).

Eleuthero-embryos. We followed a similar target gene expression approach in eleuthero-embryos to evaluate whether the effects found in adult are similar in early developmental stage. The aromatase *cyp19b* was analyzed in eleuthero-embryos due to its sensitive responsiveness to (xeno-) estrogens (Mouriec et al., 2009; Hinfray et al., 2006). The transcripts for *esr1*, *ar*, *cyp19b* show a down-regulation (albeit not significant, except for *esr1* at 438 µg/L BP-3). This suggests an overall antiestrogenic and antiandrogenic activity, similarly as in the brain of adults. This is supported by the observed down-regulation of *hsd17b3* and *hsd3b*. The data indicate that BP-3 interacts with steroid hormone synthesis also in eleuthero-embryos. Further studies are necessary to establish whether the transcriptional changes are translated into adverse developmental effects including gonadogenesis. Nevertheless, responses in zebrafish eleuthero-embryos and adult stage can differ, as recently reported for benzophenone-4 (Zucchi et al., 2011b). However, the data

obtained in the present study show similar transcriptional alterations in adults and eleuthero-embryos, which emphasizes that BP-3 affects expression of genes involved in hormonal pathways similarly in two different developmental stages.

2.5.3. Plasma vitellogenin.

In agreement with data obtained on the transcriptional level, VTG protein was not altered after exposure to 2.4 - 312 µg/L BP-3. This finding is in accordance with our previous study in fathead minnows (Kunz et al., 2006b), but differs from previous studies in Japanese medaka and rainbow trout, where a significant induction of VTG was observed at 620 and 749 µg/L BP-3 respectively (Coronado et al., 2008). The difference might be explained by the much lower concentrations used in our present study, but also by species-differences that may be based on different metabolism and consequently, different amounts of estrogenic metabolite BP-1.

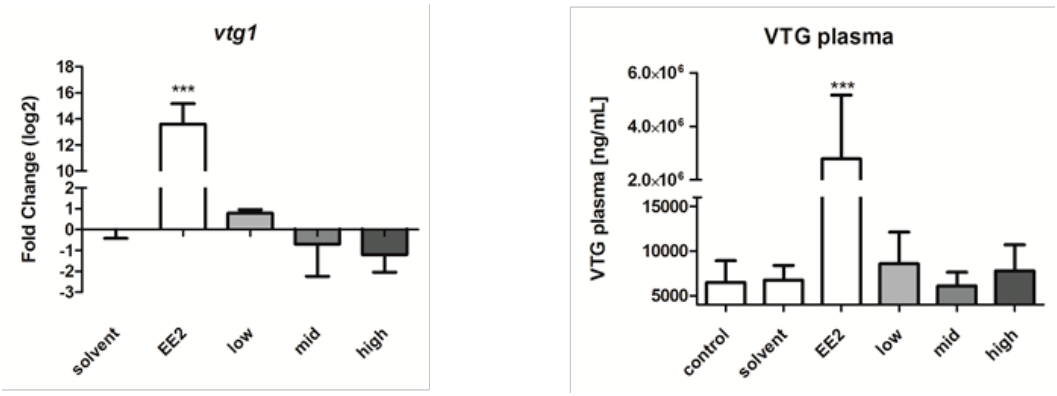

Figure 2: Left: Relative gene expression of *vtg1* in individual liver samples of adult zebrafish males after exposure to 2.4 (low), 8.4 (mid), 312 (high) µg/L BP-3 and the positive control (5 ng/L EE2). Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. **Right:** Vitellogenin (VTG) blood plasma concentrations (ng/mL) of same individual adult zebrafish after exposure to solvent (0.01% DMSO), 5 ng/L EE2 (positive control) and 2.4 (low), 8.4 (mid) and 312 (high) µg/L BP-3, determined by ELISA. All results are given as mean ± standard deviation (n=4-5 replicates per treatment). Asterisks indicate significantly different expression/concentration to solvent control (*** $p < 0.0001$).

These results may imply that the estrogenic BP-1, found in the present study as a metabolite of BP-3, did not exhibit an estrogenic activity at such concentrations (up to 266 ng/g bw). It is rather unlikely that the presence of BP-1 in combination with antiestrogenic BP-3 would elicit an estrogenic effect, as previously described for additive action of UV filters *in vivo* (Kunz and Fent, 2006a).

2.5.4. Histology.

The morphology of the testes in adult zebrafish males was not altered after BP-3 treatment. This observation seems in agreement with the lack of estrogenic activity, as revealed by the lack of VTG induction in the liver and plasma, respectively. Moreover, BP-3 differs from effects of 3-benzylidene camphor (Kunz et al., 2006a), BP-2 (Weisbrod et al., 2007) and EHMC (Christen et al., 2011), where different ratios of sperm developmental stages were observed. Changes in gonad morphology depend on the dose and exposure time, and obviously, they were too low or too short for inducing a detectable effect in the present study. It cannot be ruled out that more sensitive end-points (e.g. increased cell death, testicular fibrosis, eosinophilia) may have occurred. The lack of effects by low concentrations of EE2 is in agreement to previous data, where no morphological changes occurred in zebrafish gonads after exposure to 2 ng/L EE2 for 8 months despite significant vitellogenin induction (Soares et al., 2009). However, additional sensitive testicular end-points, not analyzed in the present study, should reveal whether testicular adverse effects occur after BP-3 treatment.

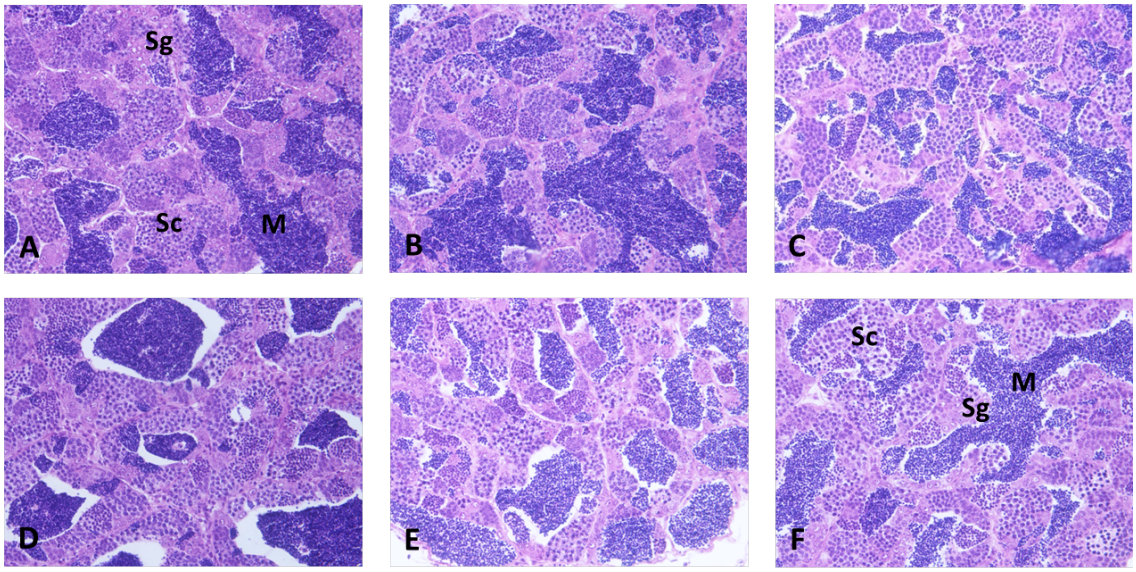

Figure 6: Typical histological sections of testes of adult zebrafish males exposed for 14 days to water control (A), solvent (0.01% DMSO) (B), positive control 5 ng/L EE2 (C), 2.4 (D), 8.4 (E) and 312 (F) µg/L BP-3. The evaluated sperm developmental stages, spermatogonia (Sg), spermatocytes (Sc) and mature sperms (spermatids, M) are marked.

2.6. Conclusions

The present study shows that low concentrations of BP-3 lead to alteration of genes involved in steroidogenesis and hormonal pathways in zebrafish at different developmental stages. Alterations at 2.4 - 312 µg/L BP-3 are similar in adult males and eleuthero-embryos. This bears the potential for adverse effects on the endocrine system. BP-3 is indicated to exhibit mainly antiestrogenic and antiandrogenic activities, confirming our previous *in vitro* data (Kunz and Fent, 2006b). Chemical analysis showed that BP-3 is metabolized to BP-1 in adult zebrafish, but not in eleuthero-embryos that lack transformation capacity in early stages. Thus, zebrafish has a similar transformation capacity of BP-3 as humans. The similar transcriptional effects in adults and eleuthero-embryos indicate that they arise from BP-3 itself, and the metabolite BP-1 noted in adult males may contribute. Effects occurred mainly at 84 µg/L BP-3, which is about one order of magnitude higher than highest environmental levels. Histological effects in the testes of adult fish were not noted, probably due to the short exposure time. Forthcoming studies in zebrafish are necessary to clarify whether transcriptional alterations translate to effects on plasma sex steroid levels, or affecting fertility and reproduction. The data of our present study suggest that the heavily used UV

filter BP-3 is probably of lower concern for endocrine disruptive effects on zebrafish as other UV filters, in particular at environmental relevant concentrations.

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Supplementary Data

In Appendix A1 a complete list of differentially expressed genes from microarray analysis in adult zebrafish liver is given. Detailed representations for the chemical analysis, including detection parameter, quality assurance and detailed results for the exposure water and fish analysis of the adult and eleuthero-embryo study are given. Graphical representations of qRT-PCR data of adult testes and eleuthero-embryo are shown, as well as individual frequencies of histological investigated testicular stages.

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Chapter 3

The UV-absorber benzophenone-4 alters transcripts of genes involved in hormonal pathways in zebrafish (*Danio rerio*) eleuthero-embryos and adult males

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3.1 Abstract

Benzophenone-4 (BP-4) is frequently used as UV-absorber in cosmetics and materials protection. Despite its frequent detection in the aquatic environment potential effects on aquatic life are unknown. In this study, we evaluate the effects of BP-4 in eleuthero-embryos and in the liver, testis and brain of adult male fish on the transcriptional level by focusing on target genes involved in hormonal pathways to provide a more complete toxicological profile of this important UV-absorber. Eleuthero-embryos and males of zebrafish were exposed up to 3 days after hatching and for 14 days, respectively, to BP-4 concentrations between 30 and 3000 µg/L. In eleuthero-embryos transcripts of *vtg1*, *vtg3*, *esr1*, *esr2b*, *hsd17ß3*, *cyp19b cyp19a*, *hhex* and *pax8* were induced at 3000 µg/L BP-4, which points to a low estrogenic activity and interference with early thyroid development, respectively. In adult males BP-4 displayed multiple effects on gene expression in different tissues. In the liver *vtg1, vtg3, esr1 and esr2b* were down-regulated, while in the brain, *vtg1*, *vtg3* and *cyp19b* transcripts were up-regulated. In conclusion, the transcription profile revealed that BP-4 interferes with the expression of genes involved in hormonal pathways and steroidogenesis. The effects of BP-4 differ in life stages and adult tissues and point to an estrogenic activity in eleuthero-embryos and adult brain, and an antiestrogenic activity in the liver. The results indicate that BP-4 interferes with the sex hormone system of fish, which is important for the risk assessment of this UV-absorber.

Keywords: UV-filter, UV-absorber, Benzophenone-4, Effects on eleuthero embryos, mRNA expression, transcription profile

3.2. Introduction

Ultraviolet (UV) filters are mainly organic chemicals that absorb UVA (315–400 nm) or UVB (280–315 nm) radiations. These chemicals, called UV-filters or UV-absorbers, are added to consumer sunscreen products in concentrations up to 10 % (Schreurs et al., 2002). Currently, 28 UV-filters may be used in sunscreens in Europe (Zenker et al., 2008), and further 43 chemicals are listed as UV-filters in ingredients used in cosmetics (Wahie et al., 2007). They are included in detectable amounts in creams, lipsticks, lotions, fragrances, skin lotions, hair sprays and shampoos. In addition to their large use in personal care products, these chemicals find also application in the UV-protection of numerous materials and products (Balmer et al., 2005; Fent et al., 2010). Consequently, there are several sources by which these compounds enter the aquatic environment. These include direct input as a result of recreational activities such as bathing, swimming with wash off from skin into surface water and indirect input as a result of showering, wash and rub off, via wastewater treatment plants (Balmer et al., 2005; Buser et al., 2006; Fent et al. 2010). Apart from their beneficial properties (preventing sun burn and subsequent dermal lesions which eventually can lead to skin cancer) some UV-filters interfere with the endocrine system of mammals *in vitro* (Schlumpf et al., 2001; Gomez et al., 2005), as well as *in vivo* in fish (Kunz et al., 2006a, 2006b; Weisbrod et al. 2007; Coronado et al., 2008), leading to adverse effects on reproduction and development. In fish, some UV-absorbers were demonstrated to be estrogenic (Schreurs et al., 2002; Inui et al., 2003; Kunz et al., 2006a; Coronado et al. 2008), and to impair fertility and reproduction (Kunz et al. 2006a; Weisbrod et al. 2007). In addition, some UV-absorbers are lipophilic and accumulate in human milk (Schlumpf et al., 2008) and in aquatic biota (Fent et al., 2010). Despite these few studies, only little is known on the ecotoxicological consequences of these ingredients in personal care products for aquatic life.

Aquatic organisms are continuously exposed to UV-absorbers in wastewater contaminated environments. Benzophenone-4 (BP-4) (CAS No 4065-45-6) was found to be the most prevalent UV-absorber among other detected compounds including benzophenone-3 (BP-3), 3- (4-methyl) benzylidene camphor (4-MBC) and 2-ethyl-hexyl-4-trimethoxy-cinnamate (EHMC) in a river in Switzerland (Zenker et al, 2008; Fent et al., 2010). BP-4 is used in a wide range of cosmetics (shampoos, soaps, lipsticks, etc.) and personal care products (Rodil et al. 2009), and it also appears to be used in coatings, in photographic films and lithographic

plates. As a result, BP-4 was identified as one of the most widespread UV-absorber in the aquatic environment (Fent et al. 2010), occurring up to 849 ng/L in rivers and up to 1480 ng/L in wastewater in Spain (Rodil et al., 2008).

To date, potential adverse effects of BP-4 in humans, mammals, and fish remain elusive. No estrogenic activity of BP-4 *in vitro* was reported by Miller et al. (2001) while, our *in vitro* studies showed multiple hormonal activities including, very low estrogenic and pronounced antiestrogenic and antiandrogenic activities (Kunz and Fent, 2006). The *in vitro* estrogenicity of BP-4 was higher in a recombinant yeast system bearing a rainbow trout estrogen receptor α compared to bearing the human ERα (Kunz and Fent, 2006a). Previously, BP-4 was not found to significantly induce vitellogenin in fathead minnows (Kunz et al., 2006a), and therefore, the estrogenic activity of BP-4 remains to be further investigated. In the light of the potential endocrine-disrupting activity of BP-4, in the present study we evaluate the effects on the expression of genes involved in hormonal pathways in two different developmental stages of zebrafish (*Danio rerio*). The aim was to elucidate mechanisms of action of BP-4 and its potential effects on estrogen and androgen signalling, on steroidogenesis, as well as on the early development of the thyroid hormone system after exposure of eleuthero-embryos and adult male fish.

In doing so we followed a targeted gene concept by focusing on target transcripts involved in these hormonal systems. We selected estrogen related genes (vitellogenin 1, vitellogenin 3), sex-steroid receptors, (estrogen receptor alpha, estrogen receptor beta 1, androgen receptor) and steroidogenic genes (hydroxysteroid 17-ß dehydrogenase-3, P450aromA, P450aromB) in both eleuthero-embryos and adults, while mRNA expression of paired box 8 (*pax8*) hematopoietically-expressed homeobox protein (*hhex*), expressed in the developing thyroid (Wendl et al., 2002; Elsalini et al., 2003; Shi et al., 2008) were assessed in developing eleuthero-embryos. We also aimed at elucidation of differential effects in different organs, therefore we investigated selected target genes in liver, brain and testis of adult males. By analysing differential gene expression in different development stages and different organs, we expected to provide a more complete toxicological profile of this important UV-filter.

3.3. Materials and Methods

3.3.1. Chemicals. Benzophenone-4 (BP-4, 5-Benzoyl-4-hydroxy-2-methoxybenzene sulfonic acid, CAS no. 4065-45-6, purity ≥ 97.0%) and benzophenone-2 (BP-2, 2,2',4,4'-Tetrahydroxy-benzophenone, CAS no. 131-55-5, purity 97%) were purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland). BP-2 was used as extraction control standard. The solvents used, acetonitrile, ethanol and methanol were in HPLC grade and obtained from J.T. Baker (Stehelin AG, Basel, Switzerland). The formic acid (98%) used for acidification of HPLC eluents was purchased from Fluka (Sigma-Aldrich, Buchs, Switzerland). All water used in sample preparation or HPLC was of HPLC grade and in-house prepared (Nanopure Diamond, Barnstead, Switzerland). The SPE columns, Strata-X (60 mg/3mL), used for extraction of the analytes from the water samples were purchased from Phenomenex (Brechbühler AG, Schlieren, Switzerland).

3.3.2. Analytical chemistry. During the experiment, aliquots of exposure water were taken three times randomly from the tanks to determine the actual exposure concentration of BP-4. In the experiments with eleuthero-embryos, BP-4 concentrations were not determined due to limited amounts of exposure water. In the adult fish experiment, water samples of each treatment group were taken at the beginning (0 h), after 24 h and prior to water renewal (48 h). This was done on days 1 to 3, 7 to 9 and 11 to 13, respectively, from different replicate tanks. The water samples were stored in the dark at -20 °C until analysis by HPLC-DAD. Extraction and analysis of water samples was performed as follows. Stock standard solutions of BP-4 and BP-2 were prepared separately in ethanol containing 101 mg/L each. From these stock solutions, eight working standard mixtures were prepared in methanol containing each compound in a concentration range of 1.01 to 50.5 mg/L. Working extraction control standard solutions of 7.5 g/L BP-2 and BP-4 were similarly prepared in ethanol. All standard and working solutions were stored in the dark at 4 °C prior to use. All glassware used was rinsed with ethanol and nanopure water before using to avoid contamination. Sample aliquots of exposure water were prepared by taking 250 mL of exposure water from control, lowest and medium BP-4 dose groups and 25 mL of exposure water from the highest exposure group (3 mg/L). 75 µg of the extraction control standard BP-2 was added to each sample aliquot in order to determine the recovery rate. Strata-X,

polymeric reversed phase sorbent columns were used for the extraction of BP-4 and BP-2. Before sample load, the SPE columns were pre-conditioned with methanol, HPLC grade water and reconstituted water. After washing the columns with HPLC grade water and 5% methanol, they were dried under vacuum. The analytes were eluted with methanol and directly collected in 2 mL measuring flasks. The final volume was adjusted using methanol and the samples were stored at -20 °C until analysis. Moreover, spike samples containing both, BP-4 and BP-2 in reconstituted water with nominal concentrations of 30 and 3000 µg/L of BP-4 respectively, and 300 and 3000 µg/L of BP-2 respectively, were prepared in duplicate for each workup series and served as additional quality control of the extraction method.

The analyte BP-4 and the extraction control standard BP-2 were determined by HPLC-DAD (Agilent 1200) at 290 nm. The chromatographic separation of the two UV-filters was achieved by using a Zorbax SB-C18 column (150 mm x 3.0 mm, 3.5 µm particle size) at a column temperature of 40 °C. The gradient mixture consisting of HPLC grade water + 0.1 % formic acid and acetonitrile + 0.1 % formic acid was used at a flow rate of 0.5 mL/min. The elution gradient started with a mixture of 90 % water and 10 % of acetonitrile, the latter increased to 90 % within 10 min, before the system was set back to initial conditions within 2.5 min. The sample injection volume was set to 5 µL and was injected in duplicate for each sample using an autosampler. Under these conditions BP-4 eluted after around 6.3 minutes and BP-2 after around 7.5 minutes from the column.

3.3.3. Maintenance of adult zebrafish. Adult zebrafish *(Danio rerio*) (>121 days) were obtained from a local dealer (Swisstropicalfish, Niederönz, Switzerland), transferred to culture tanks (300 L) and acclimatized 1 month in our laboratory prior to commencing the experiments. Fish of both sexes were held in reconstituted tap water with a total hardness of 125 mg/L as $CaCO_3$ and a conductivity of 270 µS/cm. The water temperature was held constant at 27±1 °C with the photoperiod set at 16:8 h light/dark. Fish were fed twice daily with TetraMin flakes (Tetra GmbH, Melle, Germany) and once a day with a combination of brine shrimp (*Artemia salina*) and white mosquito larvae.

3.3.4. BP-4 exposure experiment.

Embryo exposure. In this study, embryos refer to zebrafish prior to hatching (0–3 *dpf*), while larvae refer to post-hatch animals (over 3 *dpf*) (Kimmel et al., 1995). Zebrafish embryos were obtained from Harlan Laboratories (Itingen, Switzerland). Immediately after fertilization embryos were transferred to our laboratory, examined under a stereomicroscope and subsequently maintained at 27 ± 1 °C in reconstituted water. All embryos used in the experiments were about the same age of postfertilization (*i.e.*, fertilization occurred within 15 min for each embryo). At approximately 2 – 4 hours post-fertilization (*hpf*), around 60 blastula-stage embryos (Kimmel et al., 1995) were collected and placed for the experiment into 150 mL covered glass beakers containing 100 mL of reconstituted water and the appropriate concentration of BP-4 (soluble in water). A reconstituted water control group was also included in the experimental setup. Each treatment of 30 µg/L and 3000 µg/L of BP-4, and the control (reconstituted water), respectively, consisted of six replicates. The embryos were continuously incubated at 27 ± 1 °C for approximately 5 days throughout gastrulation, organogenesis, and early larval development using a static-water renewal procedure by replacing every 48 h new beakers containing the appropriate BP-4 concentrations. Eleuthero-embryos were not fed during exposure.

The quality of the exposure water was monitored by measuring the oxygen concentration (> 70%), pH value (6.7 - 7.2) and temperature (27 ± 1 °C). At the end of the exposure (120 *hpf*) eleuthero-embryos were anaesthetized in a clove oil solution (Fluka AG, Buchs, Switzerland). A total of 15 eleuthero-embryos per replicate were pooled and stored in RNA*later* for qRT-PCR. Total RNA was extracted from pooled samples using the RN*easy* Mini Kit (Qiagen, Basel, Switzerland).

Exposure of adult males. Adult male zebrafish were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated water (8 fish/tank). The experimental setup consisted of 4 groups, 30 µg BP-4/L, 300 µg BP-4/L and 3000 µg BP-4/L, and the water control. Each dose-group and the control consisted of five replicates. The doses were selected on the basis that the lowest concentration (30 µg/L) refers to the worst-case environmental concentration (contaminated raw wastewater) and the others were 10- and 100-times higher. The quality of the exposure water was continuously monitored by measuring the oxygen concentration (> 70%), pH value (6.7 - 7.2) and temperature

(27 ± 1 °C). During the experiment, appearance, mortality and abnormal behavior of fish were recorded daily.

Fish were exposed for 14 days to BP-4 in a static-renewal procedure; every 48 h fish were transferred to new tanks containing the appropriate BP-4 concentrations. During the exposure period fish were fed daily as previously described. At the end of the experiment, fish were euthanized in a clove oil solution (Fluka AG, Buchs, Switzerland), the total body length and body weight were measured and liver, brain and testis were immediately excised. Tissue pools out of five fish per replicate were collected in RNA*later* and stored at −80 °C for subsequent total RNA extraction.

RNA isolation. Total RNA was extracted from zebrafish eleuthero-embryos, and from pooled adult male liver, brain, testis using the RN*easy* Mini Kit (Qiagen, Basel, Switzerland). The samples were further treated with RNase free DNase set (Qiagen, Basel, Switzerland) used to purify the RNA preparations from DNA contamination and to subsequently remove DNase and divalent cations from the samples. RNA concentrations and quality was analyzed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington DE, USA); the purity of every RNA sample was between 1.8 and 2.0 (260 nm/280 nm ratio).

RT-qPCR analysis. Total RNA from a pool of 15 eleuthero-embryos, and a pool of 5 zebrafish liver, brain and testis was isolated as described above (n = 6 replicates for eleuthero-embryos, n = 5 replicates for adults). 1 µg of total RNA template was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma-Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C, and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min.

The cDNA was used to perform SYBR-PCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). Gene-specific primers for vitellogenin 1 (*vtg1*), vitellogenin 3 (*vtg3*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), hydroxysteroid 17-ß dehydrogenase-3 (*hsd17ß3*), androgen receptor (*ar*), P450aromB (*cyp19b*), P450aromA (*cyp19a*), hematopoietically-expressed homeobox protein (*hhex*), paired box 8 (*pax8*), steroidogenic acute regulatory (*StAR*), 3b-hydroxysteroid

dehydrogenase (*3ß-hsd*) and insulin growth factor binding protein 1 (*igfbp1*) were obtained from published zebrafish primers sequences (Table 1). The *RpL13α* was selected in this study as housekeeping gene for normalization, because their expression profile did not vary either under experimental conditions or in different analyzed tissue. In agreement to their high level of expression (Ct value) the use of *18S rRNA* as a housekeeping gene it was necessary in order to normalize *cyp19b*, and *igfbp1* transcripts.

Table 1: Primer sequences for quantitative real-time PCR analysis and sources: *vtg1* (vitellogenin 1), *vtg3* (vitellogenin 3), ERalpha (*esr1*), ERbeta1 (*esr2b*), *ar* (androgen receptor), hydroxysteroid 17-ß dehydrogenase-3 (*hsd17ß3*), P450aromB (*cyp19b*), P450aromA (*cyp19a*), hematopoietically-expressed homeobox protein (*hhex*), paired box 8 (*pax8*), 3b-hydroxysteroid dehydrogenase (*3ß-hsd*), steroidogenic acute regulatory (*StAR*), and insulin growth factor binding protein 1 (*igfbp1*), *RpL13α* and *18S rRNA* (18S ribosomal RNA).

Target gene	GenBank number	Sense primer (5'-3')	Antisense primer (5'-3')	Product size (bp)
vtg1 [a]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94
vtg3 [b]	AF254638	TTAGAACCAGCAAAGGATGC	CATCTCTTTTCTCCTTAAATAC	208
esr1 [c]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163
esr2b [d]	NM_174862.3	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT	80
ar [e]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237
hsd17ß3 [f]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121
cyp19b [g]	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94
cyp19a [g]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92
hhex [h]	NM_130934	GGTAAGCCTCTGCTGTGGTC	TCTTCTCCAGCTCGATGGTT	106
pax8 [h]	AF072549	GAAGATCGCGGAGTACAAGC	CTGCACTTTAGTGCGGATGA	142
3ß-hsd [f]	AY279108	GCAACTCTGGTTTTCCACACTG	CAGCAGGAGCCGTGTAGCTT	102
StAR [g]	NM_131663	ACCTGTTTTCTGGCTGGGATG	GGGTCCATTCTCAGCCCTTAC	81
Igfbp1 [a]	NM_173283	GTCATCCTGGAATGGGAAGA	TGTGTGACGGATCAGTGGTT	93
RpL13α [i]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100
18S rRNA [b]	BX296557	CCACTCCCGAGATCCAACTA	CAAATTACCCATTCCCGACA	215

Data sources: [a] Hoffmann et al. 2006; [b] Meng et al. 2010; [c] Martyniuk et al. 2007; [d] Chandrasekar et al. 2010; [e] Hossain et al. 2008; [f] Hoffmann et al. 2008; [g] Arukwe et al. 2008; [h] Shi et al. 2008; [i] Oggier et al. 2010.

The real-time PCR program included an enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 57–60 °C, depending on transcript target as shown in Table 1 (30 s) and 72 °C (30 s), followed by a melting curve analysis post run.

For calculating expression levels, normalization was performed against the appropriate housekeeping gene (*Rpl13α* or *18S rRNA*). The ΔCt values were calibrated against the control ΔCt values for all the target genes. The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

The delta Ct value was derived by subtracting the threshold cycle (Ct) value for the housekeeping genes (*RpL13α* or *18S rRNA*), which served as an internal control, from the Ct value of the target gene, respectively. All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). The mRNA expression level of the different genes was expressed as fold change (log2) according to the formula: $2^{\Delta Ct(\text{untreated sample})- \Delta Ct(\text{treated sample})}$.

3.3.5. Data analysis and statistics. Data from qRT-PCR, were graphically illustrated with GraphPad® Prism 5 (GraphPadSoftware, San Diego, CA, USA). The significance of differences in transcript levels caused by BP-4 was analyzed by one way analysis of variance (ANOVA) and Newman-Keuls post-hoc test. Results are given as mean ± standard error of mean. Differences were considered significant at $p \leq 0.05$.

3.4. Results

3.4.1. BP-4 exposure concentrations.

Water concentrations of BP-4 were determined by HPLC-DAD after 0, 24, and 48 h of exposure for each treatment group. The recovery rates of internal standard added to each sample aliquot (served as extraction control) ranged from 88 % to 162 % (Table 2). The recovery rates of spike samples, which served as additional quality control for each workup series, ranged from 89 % to 120 % for the analyte (BP-4) and from 87 % to 121 % for the internal standard (BP-2). The actual concentrations of BP-4 were similar for all sampled time points (Table 2), thus BP-4 was stable for at least two days at a photoperiod of 16:8 h light/dark. The results demonstrate that actual BP-4 concentrations (expressed as median concentrations) were close to nominal values (Table 2). The actual BP-4 concentrations were

corrected by the appropriate recovery rates of internal standard added to the sample aliquots.

The BP-4 concentration in the exposure water in the eleuthero-embryo experiment could not be measured due to limited amounts of sampled exposure water. However, the stability of the analyte BP-4 represented by the chemical analysis of the exposure water of the adult male experiment is suggested to be similar for the eleuthero-embryo experiment using equal conditions.

Table 2: Nominal concentration and measured concentrations of BP-4 in exposure waters after 14 days of exposure.

Exposure concentrations **Nominal**	**Measured**					
[µg/L]	**0 h [a] [µg/L]**	**24 h [a] [µg/L]**	**48 h [a] [µg/L]**	**Median [b] [µg/L]**	**Recovery range of IS [%]**	**Corrected Analyte Conc. [c] [µg/L]**
0	0	0	0	0	93 - 116	**0**
30	31.2	27.4	35.8	31.2	88 - 130	**28.1**
300	242.2	318.7	322.2	318.7	92 - 121	**321.1**
3000	3802.5	3364.9	4083.2	3802.5	118 - 162	**2781.8**

[a] Median exposure concentrations.
[b] Median of actual exposure concentration after 0, 24 and 48 h.
[c] Corrected analyte concentrations were obtained by taking into account the appropriate recovery rates of internal standard added to each sample aliquot. The corrected analyte concentrations are expressed as median concentrations over the time period of 48 h.

3.4.2. Eleuthero-embryos and fish survival and gross observations.

During the exposure of embryos, only incidental mortality occurred and no relation among treatments and controls was evident. Eleuthero-embryos behaved normally and displayed normal swimming behaviour by the end of the 5 d-exposure. No adult male fish died during the 14-day experiment, and there were no indications of abnormal behaviour during the

exposure. Fish were similar in size (4.02 ± 0.25 cm total length, 0.73 ± 0.20 g wet mass) and did not significantly differ among tanks.

3.4.3. Expression of target genes in zebrafish eleuthero-embryos.

In eleuthero-embryos, the mRNA expressions of *vtg1* (Fig. 1A), *vtg3* (Fig. 1B), *esr1* (Fig. 1C), *esr2b* (Fig. 1D), *hsd17ß3* (Fig. 2A), *cyp19b* (Fig. 2B), *cyp19a* (Fig. 2C), *hhex* (Fig. 3A) and *pax8* (Fig. 3B) were significantly up-regulated at 3000 µg/L BP-4. Among these genes, the *esr2b* (Fig. 1D) transcript was significantly induced at 30 µg/L BP-4. The transcripts of the *ar* remained unaffected by BP-4 (Fig. S1, supplementary data). The magnitude of mRNA induction for all the investigated transcripts was below 1.27 fold change (log2).

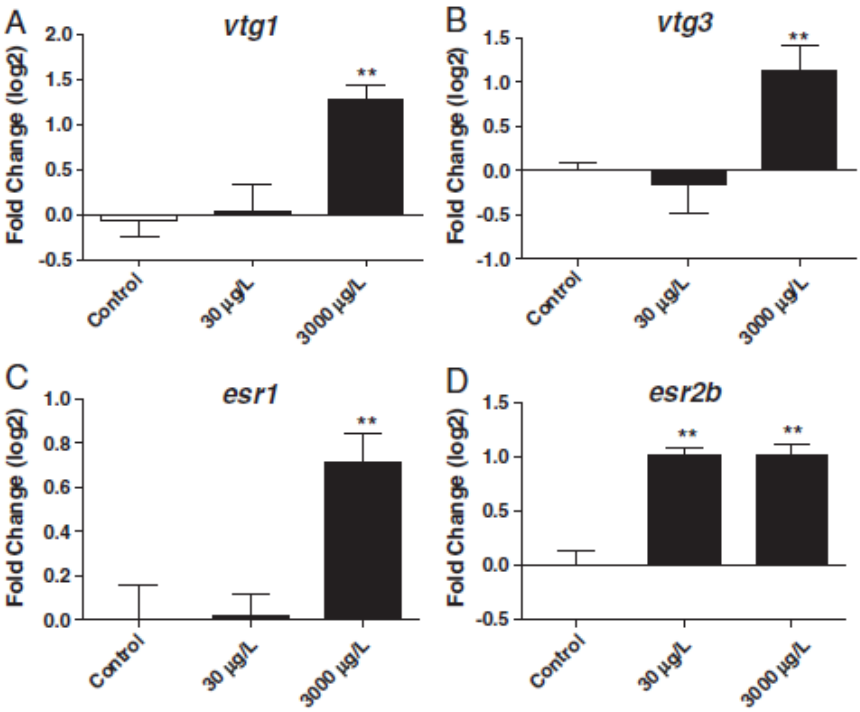

Figure1: Relative gene expression of *vtg1* (A) *vtg3* (B), *esr1* (C) and *esr2b* (D) mRNA levels in zebrafish eleuthero embryos after exposure to 30 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *vtg1*, *vtg3*, *esr1*, and *esr2b* mRNA abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=6 replicates for eleuthero embryos). Asterisks indicate significantly higher expression than control (*p< 0.05), and (**p<0.01).

3.4.4. Expression of target genes in adult male zebrafish.

The expression of *vtg1*, *vtg3*, *hsd17ß3*, *esr1* and *ar* were evaluated in all the tissue (testis, liver and brain), while relative transcript abundance of *esr2b* was determined in liver and brain only. In addition to those genes, in the liver (L), brain (B) and testis (T), transcriptional levels of *cyp19b* (B), *cyp19a* (T), *StAR* (T) and *3ß–hsd* (T) and *igfbp1* (L) were evaluated separately by qRT-PCR.

Estrogen receptor regulated genes. Our data showed a differential effect of BP-4 on *vtg1*, *vtg3*, *hsd17ß3*, *esr1,* and *ar* mRNA in testis, liver and brain. Specifically, the *vtg1* mRNA was down-regulated in the testis and liver. In the liver (Fig. 4A), a clear dose-response was observed with a significant down-regulation at 300 µg/L BP-4 (1.54-fold inhibition) and 3000 µg/L BP-4 (2-fold inhibition). Similarly, the *vtg3* mRNA (Fig. 4B) transcript was down-regulated (2-fold inhibition) at 3000 µg/L BP-4. In the testis *vtg1* expression was significantly down-regulated at 3000 µg/L BP-4 (0.85-fold inhibition) (Fig. 4A), while alterations in the *vtg3* transcript was not significant (Fig. 4B). In contrast, a dose-related and parallel increase in *vtg1* and *vtg3* transcripts occurred in the brain (Figs. 4 A and 4B).

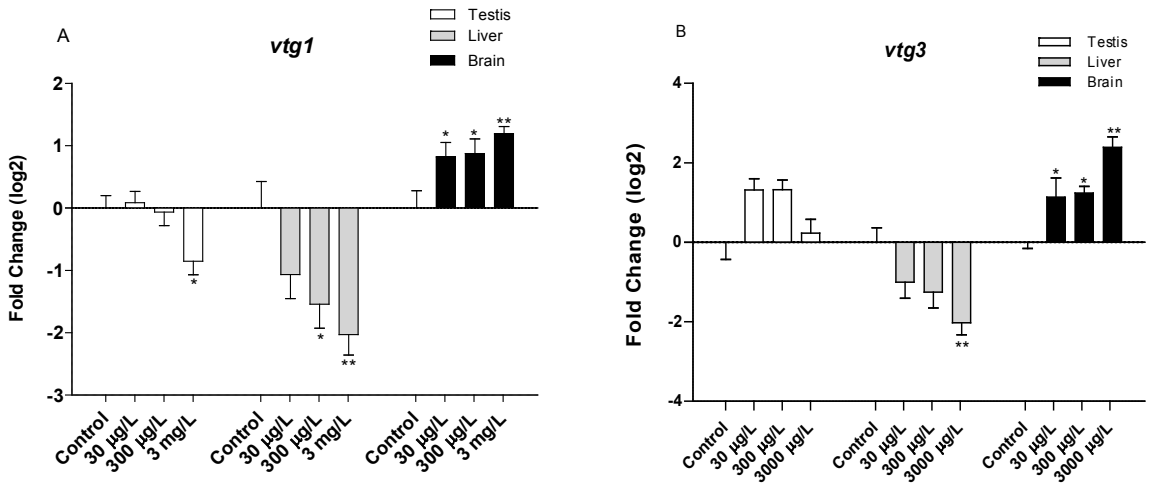

Figure 4: Relative gene expression of *vtg1* (A) and *vtg3* (B) in testis, liver and brain of adult male zebrafish after exposure to 30, 300 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *vtg1* and *vtg3* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=5 replicates for adults). Asterisks indicate significantly higher expression than control (**p*< 0.05), and (***p*<0.01).

Steroid hormone receptors. In the liver the expression of *esr1* and *esr2b* (Fig. 5A and B) was significantly down-regulated at 300 µg/L and 3000 µg/L BP-4, while in the brain only the *esr1* transcript was down-regulated (0.83-fold inhibition) at 3000 µg/L BP-4 (Fig. 5A). The *ar* transcript was significantly down-regulated in the liver at 300 µg/L and 3000 µg/L BP-4 (0.89-fold and 1.38-fold inhibition, respectively), but not in the testis and brain (Fig. 5A).

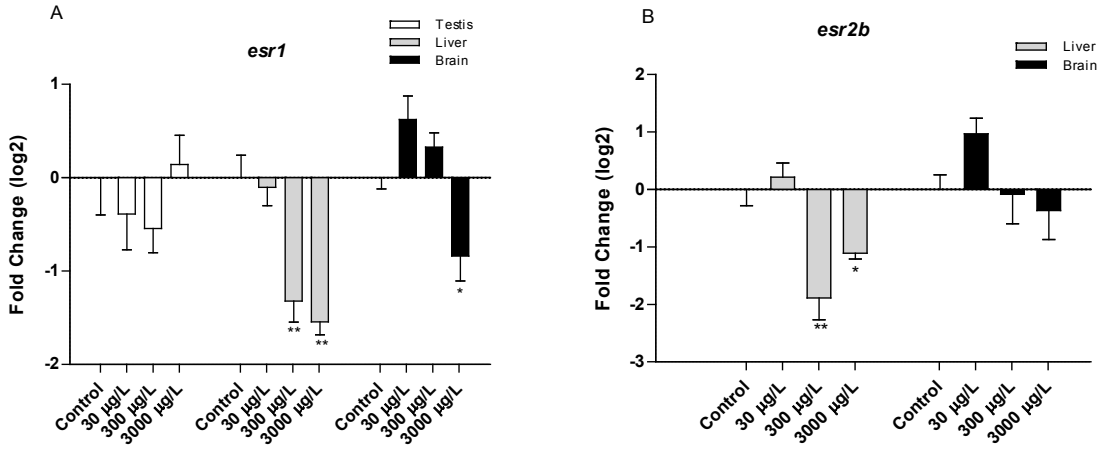

Figure 5: Relative gene expression of *esr1* (A) in testis, liver and brain and relative gene expression of *esr2b* (B) in liver and brain of adult male zebrafish after exposure to 30, 300 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *esr1* and *esr2b* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=5 replicates for adults).). Asterisks indicate significantly higher expression than control (**p*< 0.05), and (***p*<0.01).

Expression of genes involved in sex steroid synthesis. An overall down-regulation of the *hsd17ß3* transcript occurred in all of the investigated tissues. In the testis of males exposed to 300 µg/L and 3 mg/L BP-4, *hsd17ß3* mRNA was slightly down-regulated (0.38-fold and 0.42 inhibition, respectively) (Fig. 6B), while in the liver a significant alteration of *hsd17ß3* expression (2-fold inhibition) occurred at 3000 µg/L BP-4. In the brain a significant down-regulation of *hsd17ß3* transcripts was observed at 300 µg/L and 3000 µg/L (1.4-fold and 1.65 inhibition, respectively) (Fig. 6B).

In the brain the *cyp19b* transcription encoding the aromatase B was strongly induced in fish exposed to 3000 µg/L BP-4 (4-fold increase) (Fig. 7A). Meanwhile, the transcription of *cyp19a* encoding for aromatase A was significantly down-regulated at all of the BP-4 doses in

the testis. The down-regulation of *cyp19a* occurred already at 30 µg/L (3.5-fold inhibition) (Fig. 7B).

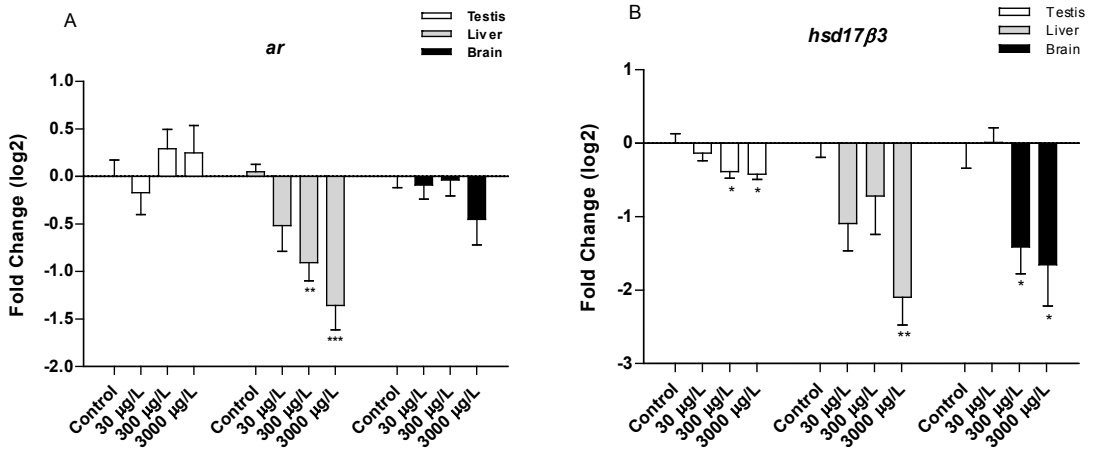

Figure 6: Relative gene expression of *ar* (A) and *hsd17ß3* (B) in testis, liver and brain of adult male zebrafish after exposure to 30, 300 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *ar* and *hsd17ß3* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=5 replicates for adults). Asterisks indicate significantly higher expression than control (**p*< 0.05), and (***p*<0.01).

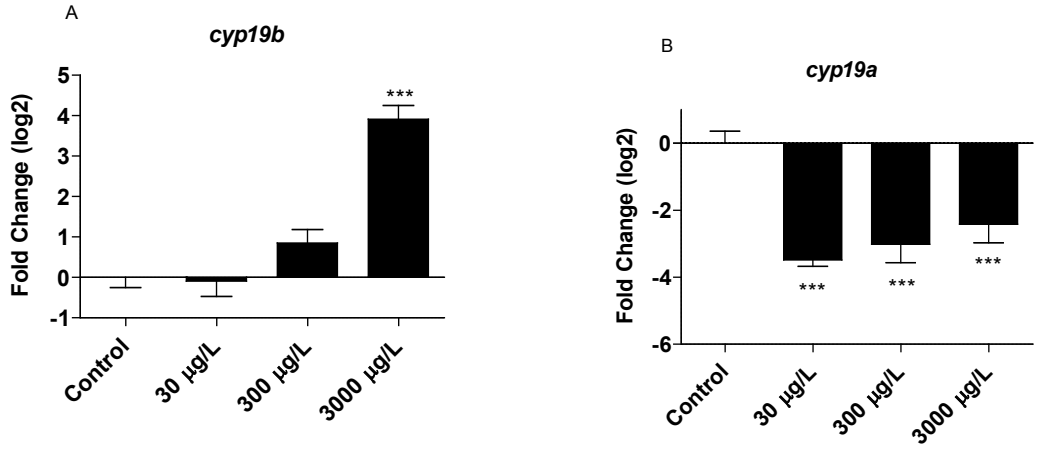

Figure 7: Relative gene expression of *cyp19b* (A) and *cyp19a* (B), respectively, in brain and testis of adult male zebrafish after exposure to 30, 300 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *cyp19b* and *cyp19a* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=5 replicates for adults). Asterisks indicate significantly higher expression than control (**p*< 0.05), (***p*<0.01) and (****p*<0.001).

An up-regulation in *StAR* mRNA expression (1-fold increase) was detected in the testis of fish exposed to 300 µg/L BP-4 (Fig. 8A). The *3ß–hsd* transcription in the testis was significantly increased by 300 µg/L and 3000 µg/L BP-4 (1.12-fold and 0.7-fold increase, respectively) (Fig. 8B). In the liver the transcription of *igfbp1* was significantly induced at 30 µg/L, 300 µg/L and 3000 µg/L in a concentration-dependent manner (1-fold, 1.5-fold and 2.5-fold increase, respectively) (Fig. 8C).

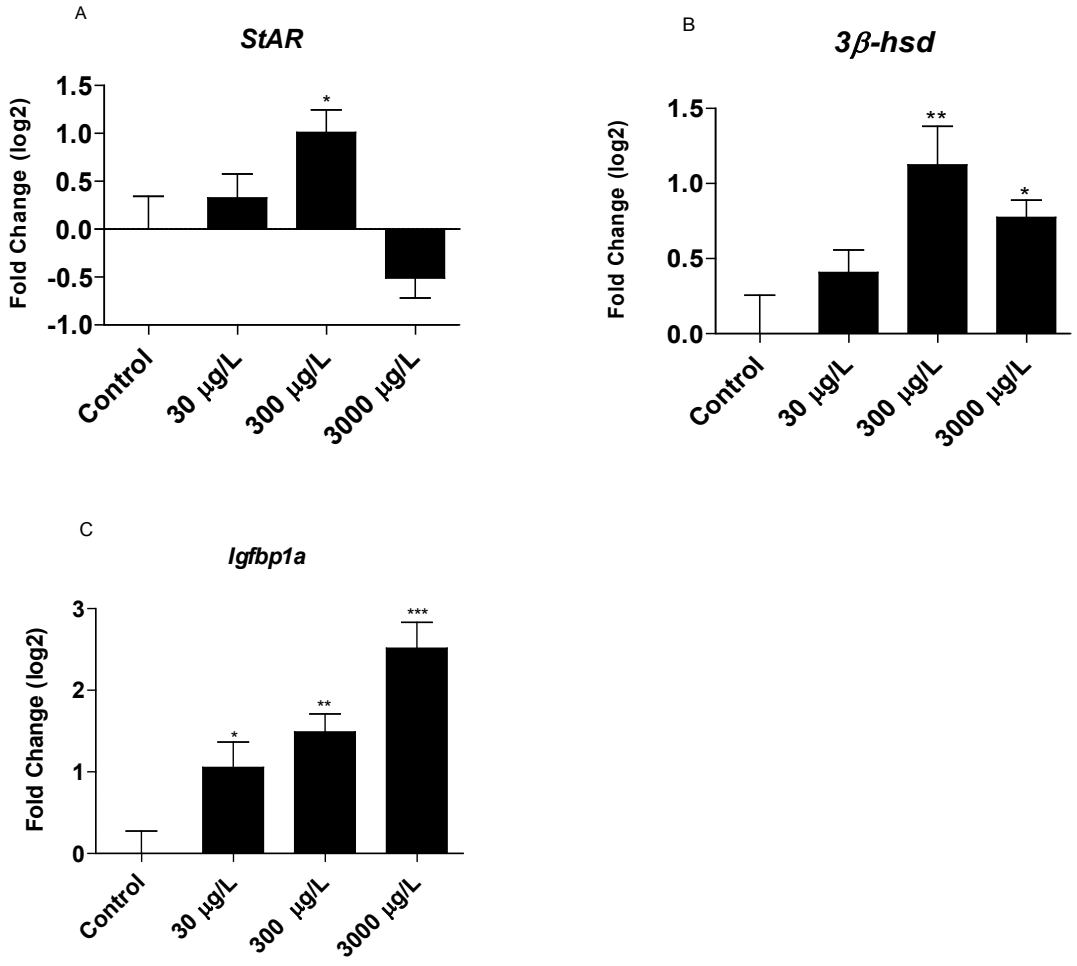

Figure 8: Relative gene expression of *StAR* (A) and *3ß–hsd* (B) in testis and relative gene expression of *Igfbp1* (C) in liver of adult male zebrafish after exposure to 30, 300 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *StAR*, *3ß–hsd* and *Igfbp1* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=5 replicates for adults). Asterisks indicate significantly higher expression than control (**p*< 0.05), (***p*<0.01) and (****p*<0.001).

3.5. Discussion

We analysed the effects of BP-4 on the expression of genes involved in estrogenic, androgenic and steroidogenic pathways in two different zebrafish developmental stages, and genes involved on thyroid gland development in eleuthero-embryos. By applying this targeted gene expression approach, we aimed at elucidation of the unknown modes of action of BP-4. We showed for the first time that BP-4 interferes with the expression of genes involved in the sex hormone system in both life stages and in genes involved in the embryonic development of the thyroid hormone system. We analysed actual BP-4 concentrations and found that they were close to nominal and remained constant during exposure. Hence, BP-4 was stable in exposure waters.

3.5.1. Eleuthero-embryos.

In zebrafish eleuthero-embryos we evaluated whether BP-4 affects gene expression during development, and whether effects are similar in the early developmental period as in the adult stage. At 3000 µg/L in eleuthero-embryos the transcripts of *esr1*, *esr2b, vtg1*, *vtg3*, *hsd17ß3*, *cyp19b* and *cyp19a* were significantly up-regulated. The vitellogenin synthesis is under hormonal control, and its expression is tissue-, stage- and sex-dependent (Gündel et al., 2007). Vitellogenin genes are already expressed in eleuthero-embryos, and estrogenic compounds lead to induction of *vtg1* and *vtg3* (Jin et al., 2009; Henry et al., 2009). Consequently, the induction of *vtg1* and *vtg3* transcripts by BP-4 (3000 µg/L) reflects an estrogenic response. At the lower BP-4 concentration, only *esr2b* was significantly induced, which indicates that BP-4 has only a low estrogenic potency on expression of *vtg1*, *vtg3*, *esr1* and *esr2b*.

BP-4 led to a significant induction of aromatase *cyp19b* and *cyp19a* transcripts at 3000 µg/L, which points to an effect on steroidogenesis. The aromatases convert testosterone to estradiol. Most fish have two *cyp19* genes, the *cyp19a* gene encoding aromatase A is mainly expressed in gonads, whereas *cyp19b* encoding for aromatase B is mainly expressed in the brain of adult zebrafish (Mouriec et al. 2009a; Mouriec et al. 2009b). These genes are expressed during zebrafish embryogenesis, in particular *cyp19b* (Kishida and

Callard, 2001, Mouriec et al. 2009a). The aromatases are fully functional in eleuthero-embryos, and *cyp19b* is strongly increased by 17ß-estradiol (E2) and relies on estrogen receptors (Mouriec et al. 200b). In consequence, BP-4 is indicated to have an estrogenic action on the expression of aromatases.

The alteration of expression of *hsd17ß3* by BP-4 was not as pronounced compared to the aromatases, although a significant induction was noted at the high concentration. The changes in the expression of genes involved in steroidogenesis may potentially result in a different production of enzymes and, consequently to changes in the steroid synthesis.

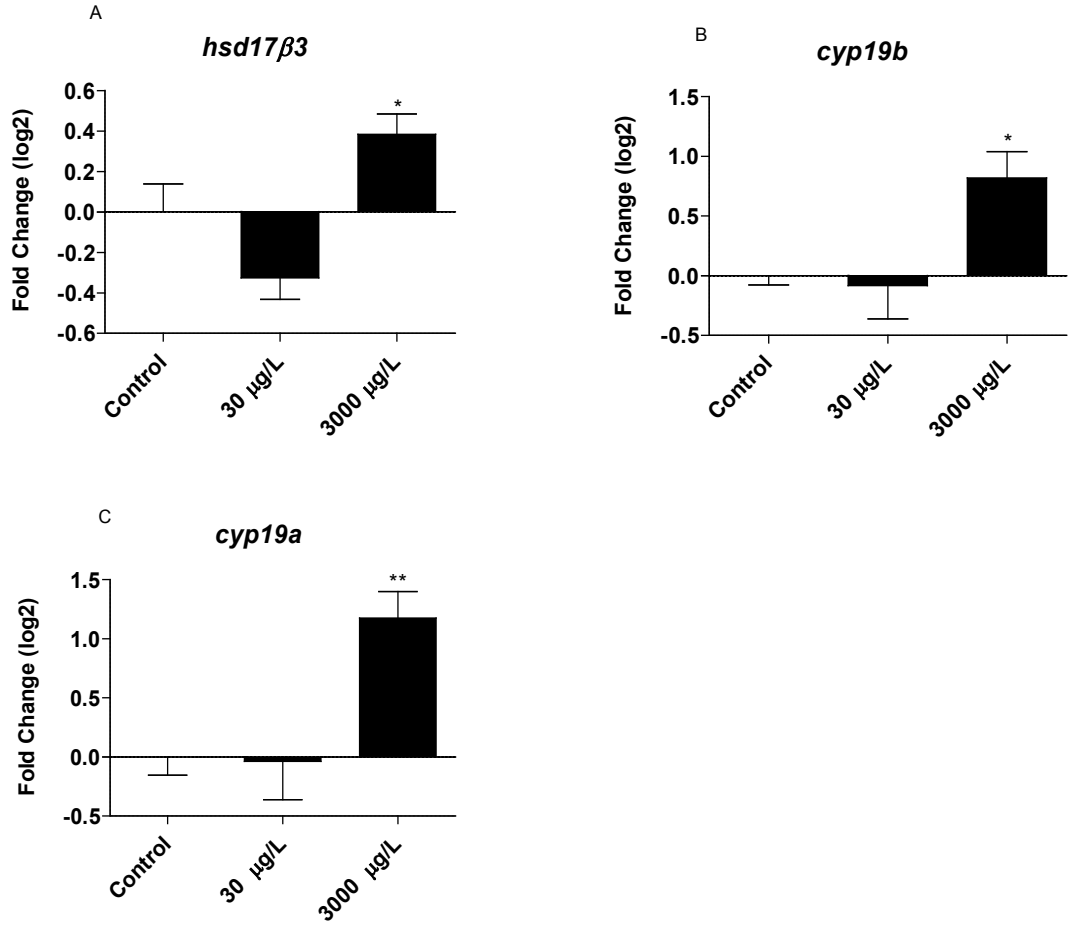

Figure 2: Relative gene expression of *hsd17ß3* (A), *cyp19b* (B) and *cyp19a* (C) mRNA levels in zebrafish eleuthero embryos after exposure to 30 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *hsd17ß3*, *cyp19b* and *cyp19a* mRNA abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=6 replicates for eleuthero embryos). Asterisks indicate significantly higher expression than control (**p*< 0.05), and (***p*<0.01).

Several genes are involved in the thyroid development during embryogenesis. The genes *hhex* and *pax8* are required for the differentiation and formation of thyroid follicles and can be regarded as marker genes in the developing thyroid of zebrafish (Elsalini et al., 2003), as transcripts are expressed in the developing thyroid (Wendl et al., 2002). The over-expression of *hhex* results in an enlarged thyroid primordium (Elsalini et al., 2003). In our study, the expression of *hhex* and *pax8* was significantly up-regulated by BP-4, albeit at low extent (Fig. 3 A, B). To our knowledge, little is known on the effects of environmental compounds on genes involved in thyroid development. The expression of *hhex* was significantly up-regulated by perfluorooctanesulfates, whereas *pax8* expression occurred at lower concentrations (Shi et al., 2008). In our study, 3000 µg/L BP-4 increased the expression of *hhex* and *pax8*. This may point to an induction of thyroid gland development at early developmental stages in zebrafish. However, the relationship between the over-expression of these genes and thyroid development, or on thyroid hormone levels remains unknown. Whether BP-4 has a potential effect on the thyroid system needs to be investigated more specifically in forthcoming experiments.

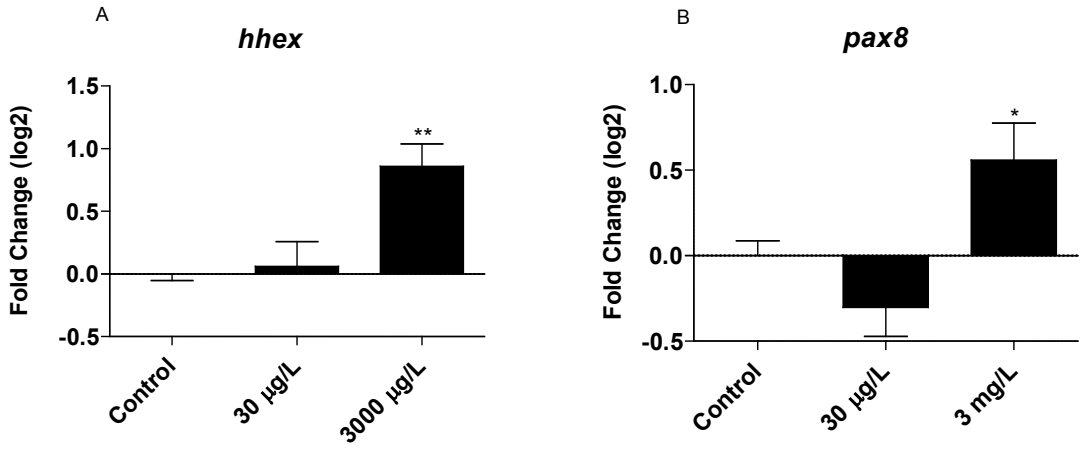

Figure 3: Relative gene expression of *hhex* (A) and *pax8* (B) mRNA levels in zebrafish eleuthero embryos after exposure to 30 and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *hhex* and *pax8* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (n=6 replicates for eleuthero embryos). Asterisks indicate significantly higher expression than control (*p< 0.05), and (**p<0.01).

3.5.2. Adults.

By analyzing the same transcription profiles in adult males, we aimed to determine whether the effects found in eleuthero-embryos are identical in the adult stage. Moreover, we aimed to analyse the differential expression of the targeted genes in different tissues to obtain better insights into the mode of action of BP-4 and related molecular effects. The induction of *vtg1* and *vtg3* indicates exposure to estrogenic compound, (Miracle et al., 2006). BP-4 led to a significant and dose-dependent down-regulation of *vtg1* and *vtg3* transcripts in the liver. This points to an antiestrogenic activity of BP-4 in the liver. On the other hand, *vtg1* and *vtg3* transcripts were significantly up-regulated in the brain in a dose-dependent manner. In the testes no clear change in expression was noted. The differential effect of BP-4 on expression of *vtg1* and *vtg3* in different tissues highlights the importance for analyzing transcription profiles in multiple tissues to elucidate molecular effects and mode of actions of environmental chemicals. Is known that vitellogenin genes are primarily expressed in the liver (Islinger et al., 2003; Tong et al., 2004; Wang et al., 2005); but recent findings in fish indicated that E2 induces *vtg1* not only in liver, but also in heart and brain (Yin et al. 2009; Ma et al., 2009).

In the liver transcripts of *esr1* and *esr2b* were significantly down-regulated at 300 and 3000 µg/L BP-4, which correlates with the down-regulation of *vtg1* and *vtg3* transcripts (Fig. 5 A, B). This points to an antiestrogenic activity of BP-4 in the liver, which is in accordance with our previous *in vitro* data (Kunz and Fent, 2006a). In recombinant yeast carrying either the rainbow trout (rtERα), or the human (hERα) estrogen receptor, we found a low estrogenic, and a strong anti-estrogenic activity (Kunz et al., 2006b). Similarly to our present *in vivo* study, two model anti-estrogens, letrozole and tamoxifen, led to down-regulation of *vtg* and *esr1* transcripts (Sun et al., 2010). In the testis and brain, no clear profile of expression changes of *esr1* and *esr2b* transcripts were observed. Again, these results indicate the differential expression profile induced by BP-4 in different tissues.

In the liver, BP-4 led to down-regulation of *ar* transcripts (Fig. 6A), while this was lacking in eleuthero-embryos (Fig. S1). This effect is paralleled by the dose-related down-regulation of *vtg1, vtg3* and *esr1* in the liver of males. This is in accordance to previous *in vitro* data showing that BP-4 strongly inhibited the *ar* related transactivation in recombinant yeast carrying the human *ar*, pointing to an anti-androgenic activity (Kunz and Fent, 2006a).

As in eleuthero-embryos BP-4 led to changes in the expression of genes involved in steroidogenesis. The major enzyme involved in testosterone formation is the 17*ß*-hydroxysteroid dehydrogenase type 3 (*hsd17ß3*), which catalyzes the reduction of androstenedione to testosterone. The *hsd17ß3* gene is expressed during zebrafish embryogenesis, and in different tissues of adult fish (Mindnich et al., 2004). BP-4 led to down-regulation of *hsd17ß3* in all tissues, being more pronounced in liver and brain than in testis (Fig. 6B). Previously, it was shown that 17α-ethinylestradiol (EE2) led to inhibition of *hsd17ß3* expression in the testis of fathead minnows (Filby et al. 2007). The down-regulation of *hsd17ß3* by BP-4 in testis, liver and brain reflects an estrogenic activity via reduced formation of *hsd17ß3,* and in turn, a potential reduction of testosterone synthesis.

Estrogens may result in up-regulation of the *cyp19* gene, in particular *cyp19b*, in both male and female fish (Callard et al., 2001), but also androgens regulate *cyp19* gene expression in both directions depending on the tissue (Govoroun et al., 2001, Mouriec et al. 2009b). For instance, in zebrafish gonads, 17α-methyltestosterone decreased *cyp19a* expression (Fenske and Segner, 2004), but also exposure of adult female zebrafish to 10 nM of E2 for 7 days suppressed *cyp19a* expression in the ovary (Hinfray et al., 2006). Our data demonstrate that in the testis *cyp19a* was down-regulated by BP-4 at all concentrations (Fig. 7B). On the other hand, the expression of *cyp19b* was significantly up-regulated (up to 4-fold, log2) in the brain (Fig. 7A). Forthcoming studies are needed to show whether this affect plasma sex steroid levels and consequently, fertility and reproduction in adult fish.

In the testis we observed the highest variability in terms of Ct value and the lowest responsiveness among the selected genes. In order to better understand the effects of BP-4 in zebrafish testis, we further characterized the mRNA expression of two key steroidogenic enzymes. *3ß-hsd* is involved in the conversion of pregnenolone to progesterone, and *StAR* in mediating cholesterol transfer from the outer to the inner mitochondrial membrane (Stocco, 2000; Arukwe et al., 2008). Both transcripts were mainly up-regulated at 300 µg/L BP-4. Deng et al. (2010) reported an up-regulation of *3ß-hsd* mRNA in zebrafish testis after 3 µg/L tribromophenol (2,4,6-TBP) treatment associated with increased levels of testosterone. E2 and EE2 exposure inhibited *StAR* transcript in testis (Filby et al. 2006; Garcia-Reyero et al., 2009), but the action of estrogens in regulating steroidogenic gene expression is currently not fully understood.

In the liver, BP-4 led to a dose-dependent up-regulation of *igfbp1*. This transcript was selected because of its alteration by estrogens (Riley et al., 2004; Hoffman et al., 2006; Baker et al., 2009). In mammals, the IGFBP-1 gene possesses glucocorticoid-regulated elements and steroids might increase IGFBP-1 levels (Lee et al., 1997). Paassilta et al. (2000) demonstrated that oral administration of estradiol increases circulating levels of IGFBP-1, and it is also up-regulated during catabolic states, such as insulin-dependent diabetes mellitus and after stress conditions in mammals and fish (Lee et al., 1997; Kelley et al., 2001). We cannot univocally ascribe the *igfbp1* up-regulation as an estrogenic response, since in mammals IGFBP expression appears to be regulated by a variety of physiologically relevant factors (Kelley et al., 2002; Riley et al., 2004); but in accordance with the increasing number of studies that have shown *igfbp1* alteration after exposure with relevant estrogens, it seems to be a promising target gene to further investigated in future studies.

3.6. Conclusions

This work shows for the first time that BP-4 led to alteration of gene expression in eleuthero-embryos and adult males of zebrafish. In eleuthero-embryos BP-4 induced an estrogenic activity as reflected by up-regulation of *vtg1, vtg3, esr1* and *esr2b*. Furthermore, genes involved in thyroid development were altered. In adult male zebrafish BP-4 displayed multiple hormonal effects in different tissues. In the liver, down-regulation of *vtg1, vtg3, esr1* and *esr2b* points to an overall anti-estrogenic activity of BP-4, which is in accordance to our *in vitro* study (Kunz and Fent, 2006a). The effect of BP-4 on the transcription profile differs in different organs. Contrary to the liver, *vtg1* and *vtg3* transcripts were up-regulated in the brain. In the testis, *vtg1*, *hsd17ß3* and *cyp19a* were down-regulated.

In addition expressions of genes involved in steroidogenesis were altered. The expression of *cyp19a* and *cyp19b* were up-regulated in eleuthero-embryos as well as in the brain of adults. On the other hand, *hsd17ß3* was down-regulated in the testis, liver and brain of adult males. The effects of BP-4 on transcription of genes important for steroidogenesis in brain and testis may alter steroid hormone levels, which may affect sex determination during embryogenesis and reproductive output in adults.

At an early developmental stage, the alteration of the expression profile occurred at high concentrations and at low magnitude, which points to an overall low activity of BP-4. Effect concentrations are well above environmental concentrations (Rodil et al. 2008; Fent et al., 2010), and the lowest effect concentration in our study was 30 µg/L. However, due to the prevalence of this UV-absorber in the aquatic environment, this UV-absorber may have environmental consequences due to additive effects of UV-filters (Kunz and Fent, 2006b).

Our targeted gene approach focusing on multiple tissues enabled us to obtain a more complete picture about the toxicological profile of BP-4 in fish, but at the same time, it makes data interpretation more complex, especially if the compound of interest displays multiple hormonal activities (Kunz and Fent, 2006b). However, our *in vivo* data confirms the *in vitro* data (Kunz and Fent, 2006a), showing that BP-4 interferes with the sex hormone system. Our study points out that effect in eleuthero-embryos can, but not always, be paralleled in the adult fish. Furthermore, the study clearly shows the importance of focusing on several genes in more than one tissue to elucidate molecular mechanisms and effects of a chemical compound. To obtain a more complete toxicological profile of BP-4, further studies are needed to relate the transcription profile to physiological effects, and to potential effects on the fertility and reproduction.

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Supplementary Data

In Appendix A2 additional gene expression data in eleuthero-embryos are provided.

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Chapter 4

Accumulation and effects of the UV-filter octocrylene in adult and embryonic zebrafish (*Danio rerio*)

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4.1 Abstract

Wide application of the UV-filter octocrylene (OC) in cosmetics leads to contamination of the aquatic environment, but effects of OC remain unclear. Here we determine bioaccumulation and molecular effects of OC. Adult male zebrafish were exposed to 22, 209 and 383 µg/L and embryos to 69, 293 and 925 µg/L OC. OC accumulated in fish up to 17µg/g. Calculated BCF varied between 41 and 136. Microarray analysis in brain and liver following exposure to 383 µg/L OC revealed alteration of 628 and 136 transcripts, respectively. Most prominent GO processes included developmental processes, organ development, haematopoiesis, formation of blood vessels, blood circulation, fat cell differentiation and metabolism. Validation by RT-qPCR in brain and liver of adult fish and embryos included a series of genes. Blood levels of 11-ketotestosterone were not altered. The transcriptomics data suggest that OC mainly affects transcription of genes related to developmental processes in the brain and liver and metabolic processes in the liver.

Keywords: UV-filter, octocrylene, transcriptomics, microarray, gene expression, zebrafish, personal care products, bioaccumulation

4.2. Introduction

UV-filters are ingredients in many personal care products such as sunscreens, shampoos, soaps, creams, lotions, perfumes, lipsticks and other cosmetics (Hauri et al., 2003). In addition, they are also present in food contact materials including plastics and cartons (Muncke, 2010). UV-filters are mainly (aromatic) organic molecules, which absorb harmful UV-irradiation by their conjugated pi-systems (chromophores), and thus protect human skin and commercial products from degradation. Many UV-filters are high production volume chemicals and enter the aquatic environment either directly, during bathing activities, or indirectly via incomplete removal in wastewater treatment plants. UV-filters are mostly lipophilic, found in all aquatic compartments of fresh and sea water and in biota (Fent et al., 2010; Balmer et al., 2005) and are sometimes increased in summer months (Magi et al., 2013; Buser et al., 2006).

Octocrylene (OC) is one of the most widely and increasingly used UV-filters (Avenel-Audran et al., 2010). Thus, the major input source in the environment occurs via application in cosmetics. OC has been detected up to 4400 ng/L in rivers and lakes (Rodil et al., 2009a) and 167 ng/L in tap water (Díaz-Cruz et al., 2012). An average of 38 µg/L OC was found in (household) gray water (Hernández-Leal et al., 2010). Concentrations in the ng/L range (12-390 ng/L) up to 12 µg/L OC have been detected in wastewater (Magi et al., 2013; Balmer et al., 2005). Due to the high lipophilicity of OC ($\log K_{OW}$ = 6.9) and its low biodegradability (Rodil et al., 2009b; Hernández-Leal et al., 2010), OC has a considerable tendency for bioaccumulation. Up to 2400 ng/g lipid weight (lw) and 30 ng/g dry weight (dw) of OC were found in river fish in Switzerland (Buser et al., 2006) and Spain (Gago-Ferrero et al., 2013), respectively. In marine mussels at shores, up to 7100 ng/g dw was found (Bachelot et al., 2012). Higher sediment concentrations were reported in lakes (up to 642 µg/kg dw) compared to rivers (25 µg/kg dw) (Kaiser et al., 2012; Rodil et al., 2008). Recently, 89-782 ng/g lw OC were measured in the liver of marine mammals along the Brazilian coast (Gago-Ferrero et al., 2013).

Previously, we have shown that some UV-filters possess hormonal activities *in vitro* and in fish (Blüthgen et al., 2012; Zucchi et al., 2011a,b; Christen et al., 2011; Kunz and Fent, 2006). Benzophenone-1 and benzophenone-2 show estrogenic activity and lead to reproductive effects in fish (Kunz and Fent, 2006; Weisbrod et al., 2007). For most UV-filters biological

activities and modes of action are poorly understood. Benzophenone-3 (Blüthgen et al., 2012) and EHMC (Zucchi et al., 2011a) alter the transcription profile in fish, including genes involved in the sex hormone system and steroidogenesis.

To date, the toxicological profile and modes of action of OC are poorly known. OC possesses anti-estrogenic and (anti)-androgenic activity *in vitro* (Kunz and Fent, 2006). However, it is not known whether OC has endocrine activity in fish *in vivo* as well. Moreover, chronic effects in fish have not been investigated, nor its molecular effects. Therefore, the aim of the present study was to elucidate the modes of action of OC in zebrafish (*Danio rerio*) after chronic exposure as revealed by transcriptomics. We evaluated the transcription profiles in brain and liver of adult zebrafish males and eleuthero-embryos, respectively, by microarrays and RT-qPCR at different exposure times. We also tested the hypothesis, whether the *in vitro* hormonal activity was reflected *in vivo* by means of RT-qPCR and 11-ketotestosterone level in blood of males. The transcriptomics approach was chosen in order to get first insights into molecular mechanisms of OC in fish and to estimate potential (eco) toxicological impacts of OC.

4.3. Materials and Methods

4.3.1. Chemicals. Octocrylene (OC, 2-Ethylhexyl 2-cyano-3,3-diphenylacrylate, CAS no: 6197-30-4, purity > 97 %), dimethylsulfoxide (DMSO), pentane and formic acid were purchased from Sigma-Aldrich (Buchs, Switzerland). The solvents used, ethyl acetate, acetonitrile, methanol, ethanol and diethyl ether (anhydrous), were of HPLC grade and obtained from J.T. Baker (Stehelin AG, Basel, Switzerland). Water used for sample preparation and HPLC was of HPLC grade and in-house prepared (Nanopure Diamond, Barnstead, Switzerland). SPE columns Strata-X (200 mg/6 mL) were purchased from Phenomenex (Brechbühler AG, Schlieren, Switzerland) and Oasis HLB (6cc 200 mg) from Waters (Waters AG, Baden-Dättwil, Switzerland), respectively. Syringe filters Titan 2 HPLC Filter (17 mm, 0.45 µm, PVDF membrane) were purchased from ThermoScientific (US). KoiMed Sleep (Ethylenglycolmonophenylether) was purchased from KOI&BONSAI Zimmermann (Bühlertann, Switzerland). Heparin ammonium salt (100 KU) was obtained from Sigma-Aldrich (Buchs, Switzerland), and BD Micro-Fine+Innen sterile insulin syringes

(0.5mL, 0.33 mm (29G) x 12.7 mm) from Becton Dickinson (Allschwil, Switzerland). The Kits used for RNA extraction (RN*easy* Mini Kit 74104 and RN*ase*-Free DN*ase* Set 79254) and enzymes for cDNA synthesis (M-MLV Reverse Transcriptase (M1705)) were purchased from Qiagen (Basel, Switzerland) and Promega (Dübendorf, Switzerland), respectively.

4.3.2. Zebrafish. Juvenile zebrafish were obtained from Harlan Laboratories (Itingen, Switzerland) and cultivated to adulthood in culture tanks (300 L). Fish of both sexes were held in reconstituted deionized water (salt concentrations: $CaCl_2x2H_2O$ 147.0 mg/L, KCl 2.9 mg/L, $MgSO_4x7H_2O$ 61.6 mg/L, $NaHCO_3$ 32.4 mg/L) with a conductivity of 350-360 µS/cm, and applying a static-water renewal weekly. Water temperature was 26 ± 1 °C and the photoperiod 16:8 h light/dark. Fish were fed twice daily with a combination of frozen brine shrimps (*Artemia salina*), mosquito larvae and *Daphnia magna*. Water parameters, such as nitrate, nitrite and pH were controlled regularly using Test strips (Easy Test, JBL) and oxygen was ensured ≥ 80 %.

4.3.3. Exposure (study design). The study design was chosen based on the OECD guideline No. 204 (Fish, prolonged toxicity test). Adult male zebrafish (about 2 years old, mean body length 3.9 cm, mean weight 441 mg) were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated reconstituted water (15 fish / tank). The fish loading rate in the different dose groups was between 0.64-0.68 g/L in the first 8 days of exposure and 0.47-0.50 g/L between 8 and 16 days of exposure. The experimental design consisted of five groups with five replicates each, including water control, solvent control (0.005 % DMSO), 100, 1000 and 2000 µg/L OC (nominal concentration, dissolved in DMSO). The solvent concentration was 0.005 % DMSO in all tanks, except in water controls. Exposure concentrations have been chosen to address both environmental levels and effect concentrations. Depending on what is regarded as environmental concentration, our lowest nominal concentration is about 4-times (household grey water, Hernández-Leal et al., 2010), 8-times (wastewater, Balmer et al., 2005) or 23-times higher (river water, Rodil et al., 2009a). However, real concentrations were much lower. In addition, the higher but non-

toxic concentrations were chosen to address metabolism of OC by determination of metabolites.

Thus, the lowest OC concentration can be regarded as representative of a ten-times higher than worst-case environmental concentration (Magi et al., 2013; Balmer et al., 2005), and higher concentrations were used to elucidate metabolism and molecular mechanism of OC toxicity. Oxygen (≥ 80 %), pH value (6.4-6.8), nitrate and nitrite concentration (0 mg/L) were continuously monitored to ensure high water quality, temperature was 26 ± 1 °C, and light cycle was 16:8 h light/dark. Appearance, mortality and abnormal behavior of fish were recorded daily, and fish were fed twice daily.

After an acclimatization period of 4 days adult male fish were exposed to OC for 8 and 16 days, respectively. At both exposure times, water and solvent controls were included. A static-water renewal procedure was applied with complete water exchange every 48 h, meaning that fish were transferred to new tanks containing reconstituted water and the appropriate chemical and solvent concentrations, respectively. Exposure concentrations were assessed prior and after water renewal.

No significant OC-related mortality occurred. Fish were anesthetized in KoiMed Sleep (1.5 mL/1 L water) before sampling. Total body length and weight of each individual fish were measured. After 8 days, four fish of every replicate tank were dissected immediately, brain and testes were pooled per replicate, collected in RNA*later* and stored at -80 °C for subsequent RNA extraction. After 16 days, five fish per replicate tank were dissected and brain, liver and testes were pooled per replicate, collected in RNA*later* and stored at -80 °C for subsequent RNA extraction. Weights of individual testes were determined before pooling. In addition, blood samples were taken prior to dissection using heparinized needles after 16 days and pooled per replicate (n=5). After centrifugation, blood plasma was stored at -80 °C for sexual hormone analysis. The remaining fish were frozen in liquid nitrogen after sex determination and stored at -80 °C for subsequent OC body residue analysis.

Embryos were obtained by in-house breeding using unexposed males and females from culture tanks. About 1 h after spawning fertilized embryos were examined for vitality under a stereomicroscope (Zeiss, DV4) and randomly distributed into covered glass beakers containing 500 mL of reconstituted water and the appropriate concentration of OC and

solvent control (0.005 % DMSO). The experimental design consisted of five groups (water and solvent control, 100, 1000 and 2000 µg/L OC nominal) with four replicates of each (200 fertilized eggs/replicate). Water was completely exchanged every 24 h (static-water renewal) by transferring embryos to new beakers containing appropriate OC concentrations. Embryos were continuously exposed for 144 h post fertilization (*hpf*) at 27 ± 1 °C in well-aerated reconstituted water under a photoperiod 16:8 h light/dark, and not fed during exposure. Appearance, mortality, development and abnormal behavior were visually inspected and recorded daily and dead embryos were removed. After 48, 96 and 144 *hpf* samples were taken, pooled and stored in RNA*later* at -80 °C for subsequent RNA extraction. The sample pool size consisted of 14-50 eleuthero-embryos per replicate, depending on the survival rate and developmental stage.

4.3.4. Analytical chemistry.

Water. Exposure water samples were taken after 3, 9 and 13 days of exposure during the adult zebrafish study and each sampling day at the beginning (0 h), after 24 h and prior to water renewal (48 h) (*n*=9 samples per treatment). Water samples during the eleuthero-embryo study were taken after 1, 3 and 6 days of exposure and each sampling day at 0 and 24 h (*n*=6 samples per treatment). The water samples were stored at -20 °C in glass bottles for 5-6 months (adult study) and 3 months (embryo study), respectively, and thawed at room temperature overnight in the dark a day before the analysis. Special care was taken during sampling and sample preparation to avoid any (cross-) contamination of samples due to the ubiquitous presence of UV-filters. All materials were rinsed several times with ethanol, glassware was heated at 500 °C (for 3 h) prior to use and the whole extraction procedure was performed in the dark to avoid possible degradation.

Stock standard solutions of OC were prepared in ethanol containing 1200 µg/mL OC. From stock solution, eight to thirteen working standard solutions were prepared in ethanol in a concentration range of 1 to 120 µg/mL OC. Working spike solutions for the quality control samples were similarly prepared in ethanol containing 500 and 250 µg/L OC. All standard and working solutions were stored in the dark at 4 °C.

Sample aliquots of 25-250 mL, depending on the nominal concentration, were subjected to solid-phase-extraction (SPE) using polymeric reversed-phase sorbent columns, Strata-X (200 mg/6 mL) (Phenomenex, Brechbühler AG, Schlieren, Switzerland). Water samples of the embryo study were extracted using Oasis HLB 6cc 200 mg (Waters AG, Baden-Dättwil, Switzerland) that had a very similar property. The columns were equilibrated subsequently with 2 x 4 mL of ethyl acetate, HPLC-water and reconstituted water. After sample load the columns were washed with 2 x 4 mL HPLC-water and 5% methanol and subsequently dried under vacuum for about 30 min. After cleanup OC was eluted and collected using 2 x 5 mL ethyl acetate + 2 % formic acid. Eluates were dried under a gentle stream of nitrogen at 30 °C and re-suspended in 250 µL ethanol and stored at 4°C until analysis. Samples of higher exposure groups were diluted into calibration range with ethanol.

Fish. After 16 days, fish were sampled and stored at -80 °C until analysis. One fish per replicate tank (*n*=5 samples per treatment) was analyzed applying similar precautions and pre-treatments of materials used as described for water analysis. The same standard solutions prepared for the water analysis were used for quantification of the fish samples. Working spike solutions for the quality control samples were prepared in ethanol containing 10 and 50 µg/mL OC, respectively.

The individual fish samples were homogenized with 4 mL HPLC water using an Ultra-Turrax mixer (IKA Ultra-Turrax T8 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany). The liquid-liquid extraction was performed stepwise by adding first 4 mL diethyl ether and 1 mL of 10 % NaCl solution. After shaking, 5 mL pentane was added and the mixture was shaken vigorously for about 1 min. After centrifugation at 2'300 g for 15 min at 4 °C the organic phase was collected. The extraction was repeated by using fresh diethyl ether (4 mL) and pentane (5 mL). After centrifugation, the organic extracts were combined and filtrated through a pentane-purged 0.45 µm PVDF syringe filter (Titan 2 HPLC Filter, Thermoscientific, US). The extracts were evaporated to dryness using a gentle stream of nitrogen at 30 °C and analytes were re-suspended in 100 µL ethanol. In a last purification step, the residues were centrifuged at 16'300 g for 10 min at room temperature and the clear supernatant was subjected to LC-DAD analysis. The fish samples of higher exposure groups were diluted within the calibration range with ethanol.

Liquid Chromatographic - Diode Array Detection (HPLC-DAD). OC was detected using an Agilent 1100 HPLC system, HPLC-DAD (Agilent Technologies) at 290 nm. The software used for quantification was the Agilent ChemStation. The chromatographic separation was achieved by using a Zorbax SB-C18 column (150 mm x 3.0 mm, 3.5 µm particle size) and a C-18 guard column (4.6 mm x 12.5 mm, 5 µm particle size), both from Agilent Technologies (Basel, Switzerland) at column temperature of 30 °C. The gradient mixture consisted of HPLC-grade water and acetonitrile and was used at a flow rate of 1 mL/min. The elution gradient started with a mixture of 95 % water and 5 % of acetonitrile, the latter increased to 95 % within 3 min and was kept for 5 min, before the system was set back to initial conditions within 2 min. The total run time of 10 min was followed by a re-equilibration of 2 min prior the next injection. The single injection volume was set to 5-10 µL for each sample using an autosampler and each sample was injected in duplicate. A series of external calibration standards was injected on every day of the analysis and used for the quantification of OC.

Quality Control. The extraction method of water was validated prior sample extraction using reconstituted water spiked with known amounts of OC. The following setup was validated for water: 1 x extraction blank (reconstituted water only), 2 x 100 µg/L, 2 x 1000 µg/L and 2 x 2000 µg/L OC. The extraction of fish was validated prior sample extraction using unexposed male zebrafish. The following setup was validated for fish: 1 x solvent-blank (no fish matrix), 1 x matrix-blank (unexposed zebrafish), 2 x 1000 ng and 2 x 5000 ng OC.

During preparation of exposure water samples, two spike samples were run in parallel for each workup series (n=2 per treatment). The water spike samples consisted of reconstituted water (25 - 250 mL, depending on the OC concentration), which was spiked with 100, 1000 and 2000 µg/L OC, according to nominal exposure concentrations. During preparation of fish samples, two spike samples were extracted in parallel for each work-up series (n=2 per treatment). The fish spike samples consisted of individual unexposed zebrafish males, which were spiked with 1000 and 5000 ng OC, depending on the treatment of the exposed fish samples. For both matrices, water and fish, the spike samples served as quality control of the extraction method and were quantified similarly to the exposed samples.

In water and fish, OC was quantified by external standard calibration. A linear regression model was applied for a concentration range of 1 - 120 µg/mL OC. Instrumental repeatability

was assessed by six times injections of a standard solution (10 µg/mL) and is expressed as standard deviation of system suitability (SST). The detection (LOD) and quantification (LOQ) limits of the analytical method were assessed for a signal to noise ratio (S/N) > 7 and derived from visual inspection. This was the best compromise between identification and quantification. Gomez et al. (2012) used a S/N of 3 (LOD) and 10 (LOQ), respectively.

Chemical properties test. Chemical properties of OC in water without the influence of organic matter, such as fish, larvae, food and excretions, were assessed in a small test using similar conditions (exposure container, 16:8 h light/dark rhythm, oxygen) to those in the exposure studies. Nominal concentrations of 100 and 2000 µg/L OC were tested in stainless steel tanks (adult study) and glasses (embryo study), consisting of three replicates each. Water samples were taken at 0, 24 and 48 h and stored at -20°C for 10 months until analysis, which was performed as described above.

4.3.5. Molecular und biochemical analyses.

RNA extraction. Total RNA was extracted from pooled adult zebrafish tissues (n=4-5 fish per replicate) and pooled eleuthero-embryos (n=14-50 embryos per replicate) using the RN*easy* Mini Kit (Qiagen, Basel, Switzerland). To purify RNA and to remove DNA and divalent cations, samples were further treated with RN*ase*-Free DN*ase* (Qiagen, Basel, Switzerland). RNA concentration and quality was analyzed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington DE, USA) by measuring the absorbance at 260 and 280 nm. Only RNA samples of purity between 1.8 and 2.0 for both ratios 260/280 nm and 260/230 nm were subjected to transcriptomics analyses (microarray and RT-qPCR). In addition, the purity of RNA samples was assessed by electrophoresis (rRNA intensity ratio for 28S/18S about 2:1) (Taylor et al., 2010). RNA samples were stored at -80 °C for subsequent microarray and RT-qPCR analysis.

Microarray analysis. Total RNA of pooled brain and liver samples from two treatments (solvent control, 383 µg/L OC after 16 days) each were selected for microarray analysis, performed at the Functional Genomics Center Zürich (FGCZ). This concentration was selected to obtain significant expressional alterations related to the potential mode of action of OC. The integrity of extracted total RNA samples was verified using an Agilent 2100

Bioanalyzer (Agilent Technologies, Basel, Switzerland). RNA quality criteria required for hybridization of microarrays were met by using only samples fulfilling 260/280 nm ratios > 1.8, 28S/18S ratios > 1.4 and RNA integrity numbers (RIN) > 8. A total of 16 arrays (Agilent 1-Color, 4 x 44 K zebrafish microarray) were used, including four biological replicates (arrays) for every tissue and treatment. Total RNA was reverse-transcribed into double-stranded cDNA, Cy3 labeled and hybridized according to manufacturer's manual at the FGCZ. The raw microarray data obtained were analyzed using GeneSpring GX 12.0 software (Agilent Technologies).

RT-qPCR analysis. The isolated total RNA of pooled adult brain, liver and testes samples as well as from pooled eleuthero-embryos was subjected to RT-qPCR analysis. In total, the number of samples were *n*=5 replicates for adult tissue samples (each after 8 (brain and testes) and 16 days (including liver) of exposure), and *n*=4 replicates for eleuthero-embryos (each after 48, 96 and 144 *hpf*). Total RNA (1 µg) was reverse-transcribed using the cDNA Synthesis Kit from Promega (Dübendorf, Switzerland). The total volume of 14 µL (RNA + RN*ase*-free water) was incubated with 1 µL of random hexamers (Roche, Switzerland) at 70 °C for 5 min to melt secondary structures within template. A volume of 10 µL of a master-mix solution, containing 5 µL M-MLV 5x- reaction buffer, 1.25 µL dNTPs (Sigma-Aldrich, Switzerland), 0.4 µL M-MLV reverse transcriptase and 3.35 µL RN*ase*-free water, were added to each sample. The complete reaction mixture was incubated at 37 °C for 60 min, following 5 min at 95 °C to stop the reaction. The cDNA was stored at -20 °C.

Finally, 50 ng of cDNA per reaction were used for RT-qPCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). The following two-step real-time PCR profile was used: enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 57.5-60 °C (60 s) depending on the target transcript as shown in Table 1, followed by a melting curve analysis post run (65-95 °C).

For validation of microarray data, transcripts were selected based on biological pathways and significance and magnitude of alterations. Thus, selected transcripts for RT-qPCR validation were involved in several important biological/physiological pathways, and thus hypothesized to play a major role in the effects of OC. Gene-specific primers from published zebrafish primer sequences were used as given in Table 1.

Table 1: Primer sequences for quantitative RT-PCR analysis, sequence sources and annealing temperature. The following transcripts were analyzed in adult brain: thyroid hormone receptor beta/alpha (*thrb/a*), melatonin receptor 1B (*mtnr1bb*, *zMel1b-1*), retinol binding protein 2a (*rbp2a, CRBPII*), P450 enzymes (*cyp1c1*, *cyp1c2*), platelet derived growth factor receptor alpha (*pdgfra*), estrogen receptor alpha (*esr1*), androgen receptor (*ar*) and P450aromataseB (*cyp19b*); in adult liver: CCAAT/enhancer binding protein alpha (*cebpa*), retinol binding protein 4 (*rbp4*), pregnane X receptor (*pxr, nr1i2*), lipoprotein lipase (*lpl*), aryl hydrocarbon receptor nuclear translocator-like 1a (*arntl1a, bmal1*) and vitellogenin 1 (*vtg1*); in adult testes: 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), 11β-hydroxylase (*cyp11b2*) and 11β-hydroxysteroid dehydrogenase type 2 (*hsd11b2*). A selection of all target genes was investigated in eleuthero-embryos. The ribosomal protein L13a (*RpL13a*) was selected as housekeeping gene for normalization due to stable expression among treatments, time points and tissues.

Target gene	GenBank number	Sense primer (5' - 3')	Antisense primer (5' - 3')	Product size (bp)	Annealing temp. (°C)
thrb [a,b]	NM_131340	TGGGAGATGATACGGGTTGT	ATAGGTGCCGATCCAATGTC	110	57.5
thra [a,b]	NM_131396	CTATGAACAGCACATCCGACAAGAG	CACACCACACACGGCTCATC	85	57.5
mtnr1bb [c]	NM_001201565	TCGGTGTTCAGGAATCGTAAACTG	GAAGGCCAGACTGACCACAAA	63	60
rbp2a [d]	NM_153004	GGAGATGCTCAGCAATGACA	CTGTTGTCCAGGCCTTTTGT	216	60
cyp1c1 [e]	NM_001020610	AGTGGCACAGTCTACTTTGAGAG	TCGTCCATCAGCACTCAG	130	60
cyp1c2 [e]	NM_001114849	GTGGTGGAGCACAGACTAAG	TTCAGTATGAGCCTCAGTCAAAC	117	60
pdgfra [f]	NM_131459	ACCGAATGACCAAACCTGAG	CAGGTCTCCTGAGTCCAAGC	506	60
cebpa [g]	NM_131885	AACGGAGCGAGCTTGACTT	AAATCATGCCCATTAGCTGC	250	60
rbp4 [d]	NM_130920	GCAGAGAGCTGGATGAGGAC	GCTCCTTGATGCAATGGTTT	194	60
pxr [h]	NM_001098617	ATGCGGCGACAAATCTACTGGC	TGTGAAGTGTGGCAGAGAGGTG	577	60
lpl [i]	NM_131127	ACCCTCGAAGAACCTGAAGA	CGTCCATCCATGAATAACTATG	117	57.5
arntl1a [c]	NM_131577	CAGAGCTTCGCCACAAACC	CTGTGATCAATGCATGTCCTTTCA	78	60
esr1 [j]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163	60
ar [k]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237	60
cyp19b [l]	AF_183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94	58.5
vtg1 [m]	AY_034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94	57
hsd17b3 [n]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121	57
cyp11b2 [o,p]	NM_001080204	GCTCATGCACATTCTGAGGA	TGTGCTGAAGGTGATTCTCG	115	60
hsd11b2 [o,p]	NM_212720	TGCTGCTGGCTGTACTTCAC	TGCATCCAACTTCTTTGCTG	123	60
RpL13a [q]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100	57.5 - 60

Data sources: [a] (Chen et al., 2012b), [b] (Yan et al., 2012), [c] (Shang et al., 2007), [d] (Chen et al., 2012a), [e] (Jönsson et al., 2009), [f] (Lien et al., 2006), [g] (Shieh et al., 2010), [h] (Bresolin et al., 2005), [i] (Levi et al., 2012), [j] (Martyniuk et al., 2007), [k] (Hossain et al., 2008), [l] (Arukwe et al., 2008), [m] (Hoffmann et al., 2006), [n] (Hoffmann et al., 2008), [o] (Fuzzen et al., 2011), [p] (Alsop et al., 2008), [q] (Oggier et al., 2010).

The ribosomal protein L13a (*RpL13a*) was selected as housekeeping gene for normalization due to prior investigation of stable expression among treatments, time points and tissues. Primers were obtained from Microsynth (Basel, Switzerland) and PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled eleuthero-embryo

cDNA (R^2 > 0.98 for all primers). A no template control reaction was included in every run for each primer pair to test for potential contamination and to assess the presence of primer-dimers (Taylor et al., 2010).

All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). Normalization and calculation of expression levels was performed as previously (Blüthgen et al., 2012). The mRNA expression levels of different target genes are expressed as fold change (log2) according to the formula: $2^{-(\Delta Ct(\text{treated sample})-\Delta Ct(\text{untreated sample}))}$ and referred to solvent control.

4.3.6. Radioimmunoassay of 11-KT. After 16 days, the blood plasma samples of five individual male fish per replicate was pooled and used for analysis of 11-ketotestosterone (11-KT) (*n*=5 samples per treatment). Prior to radioimmunoassay (RIA), pooled plasma volume (20-40 µL) was made up to 100 µL with PBS-buffer and steroids were extracted using 500 µL ethyl acetate. After centrifugation (2500 g, 5 min, 4°C), the organic phase, which contains the steroids, was transferred into a new tubes and stored at -20°C until analysis. The RIA was performed following the protocol described by Scott et al. (1980). 11-KT antibody (1: 50'000 dilution) and ^{3}H-11-KT label (6000 DPM) was used from previous studies (Margiotta-Casaluci and Sumpter, 2011). 150 µL sample aliquots were evaporated using a vacuum centrifuge and re-suspended in 100 µL steroid-assay-buffer. The assay was carried out in duplicate, including total counts, non-specific binding and maximum binding, and the same calibration curve (0.005 – 10 ng/mL 11-KT) was used for quantification of all samples.

4.3.7. Data analysis and statistics. Raw microarray data were imported in GeneSpring GX 12.0 software (Agilent Technologies) in txt format. All data were quantile normalized and the baseline was set to the median of all samples. The following workflow, suggested by GeneSpring, was applied separately for brain and liver samples. The experiments were grouped non-numerically. Quality control included correlation plots, quality control metrics, correlation coefficients and principal component analysis (PCA, 3D-scatter plot). Only samples matching quality criteria (correlation coefficient ≥ 0.98) were used. The non-averaged entity list was filtered by detected flags (filter set to 50 %). This entity list was

used for statistical analysis by applying *T*-test unpaired (asymptotic *p*-value) and Benjamini-Hochberg FDR as multiple testing corrections. The resultant entity list (setting cut-off at $p \leq 0.1$ (brain) and ≤ 0.3 (liver), respectively) was filtered on volcano plot by applying unpaired *T*-test (asymptotic *p*-value) and Benjamini-Hochberg FDR to get the final list of differentially expressed genes between solvent control and 383 µg/L OC. Genes were considered differentially expressed when *p*-values ≤ 0.05 and the fold change cut-off (FC) varied between 1 and 2. To identify and visualize the involvement of the differentially expressed genes in specific pathways, the entity lists of liver and brain, including all significantly different expressed genes (up- and down-regulated), were imported separately (by tissue) in the MetaCore™ software (GeneGo, San Diego, CA). Finally, priority lists (sort by *p*-value) of gene ontology (GO) processes, pathway maps and process networks were obtained by enrichment analysis (EA) (threshold set to 1.0, p=0.05). It is notable that EA showed similar results for gene entity lists setting the FC at 1 and 2 for both liver and brain, whereas more transcripts involved in the pathways were included in liver for FC ≥ 1, and hence was used for data interpretation.

Data were graphically illustrated by GraphPad Prism 5 (GraphPadSoftware, San Diego, USA). The significance of differences in transcript levels for RT-qPCR was analyzed by one way analysis of variance (ANOVA) followed by a Tukey post-hoc test (95% confidence interval). Results are shown as mean ± standard deviation. Differences were considered significant when $p \leq 0.05$.

4.4. Results

4.4.1. Gross observations. OC showed no overt toxicity to zebrafish, meaning that no statistically significant effects on survival, phenotype and behavior were observed in adult males. OC did not significantly affect survival and hatching rate in embryos and no significant changes occurred in condition factors and gonadosomatic index (GSI) in adults (Tables S1, S2). However, at 209 and 383 µg/L OC the GSI showed a trend for increase, but this was statistically not significant. Furthermore, eleuthero-embryos exposed to OC had a lighter pigmentation compared to controls.

4.4.2. Analytical chemistry. All recovery rates obtained from spike samples, which served as quality control for the water and fish extraction methods, are given in SI (Table S5, S6), as well as the results of previously performed validations (Tables S3, S4). The results of an exposure-condition simulated study to test the properties of OC in water (without organic matter) are shown in Table S9. The overall mean water concentrations (0 - 48 h) in the adult experiment were 22, 209 and 383 µg/L OC, corresponding to 22, 21 and 19 % of nominal concentration as shown in Table 2. No OC was detected in control samples (limit of detection (LOD) set to 1 µg/mL). The average concentrations in the embryo exposure water were 69, 293 and 925 µg/L OC, respectively, corresponding to 69, 29 and 46 % of the nominal dose (Table S8). OC concentrations in water samples showed relatively high standard deviations for the triplicate analysis per treatment and time point in both *in vivo* studies (Tables S7, S8).

Table 2: Measured averaged octocrylene (OC) concentrations in exposure water and adult fish after 16 days exposure and calculated bioconcentration factor (BCF).

Treatment	Measured concentrations in water (0-48h) (mean) [a] [µg/L]	Measured concentrations in fish (mean ± sd) [b] [ng/g bw]	BCF [c]
Control	n.d.	n.d.	-
DMSO (0.005 %)	n.d.	n.d.	-
100 µg/L OC	22	2956 ± 1475	136
1000 µg/L OC	209	16751 ± 7168	80
2000 µg/L OC	383	15810 ± 2472	41

[a]: Measured actual water concentration (µg/L) as mean (0-48 h) (*n*=3 samples per time point and treatment); [b]: Concentrations in fish as mean ± S.D. in ng/g wet body weight (bw) (*n*=5 samples per treatment); [c]: Bioconcentration factor calculated as BCF = conc. in fish [ng/g bw] / mean conc. in water [µg/L]. n.d.: not detected.

After 16 days of exposure mean OC concentrations in fish were 2956 ± 1475, 16'751 ± 7168 and 15'810 ± 2472 ng/g wet body weight (bw), respectively (Table 2). No OC was detected in control fish samples. Calculated bioconcentration factors (BCF = conc. in fish [ng/g bw] / conc. in water [µg/L]) ranged between 41 and 136 (Table 2).

4.4.3. Microarray analysis and selection of target genes.

Brain. After 16 days of exposure of adult males to 383 µg/L OC, 628 transcripts were differentially expressed (fold-change (FC) ≥ 2, $p \leq 0.05$) in brain relative to the solvent control (Table S10). Following enrichment analysis (EA), various GO processes and biological pathways were found to be affected. The GO terms, listed by significance and number of altered and involved transcripts within a certain GO process, are given in Table 3 and in more detail in Table S12. They include developmental processes (80 transcripts), anatomical structure development (73 transcripts), system development (68 transcripts), multicellular organismal processes and development (169 transcripts), response to steroid hormone stimulus (22 transcripts), anatomical structure morphogenesis (44 transcripts), positive regulation of cellular processes (58 transcripts), organ development (51 transcripts) and cellular component organization (63 transcripts).

The enrichment by pathway maps is shown in detail in Table S13 and predominant pathways based on the type and known function of genes is given in Table 4. It includes, among others, hypoxia-induced EMT (epithelial-to-mesenchymal transition) in cancer and fibrosis, cytoskeleton remodeling role of PDGFs in cell migration, signal transduction JNK pathway, translation regulation of EIF4F activity, transport macropinocytosis regulation by growth factors, reproduction GnRH signaling and immune response antigen presentation by MHC class I. Based on enrichment analysis by process networks we hypothesize that OC may interfere with cell adhesion platelet-endothelium-leucocyte interactions, in development of EMT regulation, protein folding, development of cartilage, calcium transport and reproduction at gonadotropin regulation and GnRH signaling pathways (Table 4 and details in Table S14).

Based on EA we hypothesize that the following transcripts are the most relevant due to their known function and involvement in many different pathways and GO-terms: thyroid hormone receptor beta (*thrb*), melatonin receptor 1B (*mtnr1bb, zMel1b-1*), cellular retinol binding protein 2a (*rbp2a, CRBPII*), P450 enzymes (*cyp1c1, cyp1c2*), and platelet-derived growth factor receptor alpha (*pdgfra*). Among these, *rbp2a* (FC = -2.8), *cyp1c1* (FC = -2.0), *cyp1c2* (FC = -2.5) and *pdgfra* (FC = -2.7) were down-regulated, whereas *thrb* (FC = 2.0) and *mtnr1bb* (FC = 2.5) were up-regulated. In addition to *thra*, these genes were selected for validation by RT-qPCR.

Table 3: Ten most significantly altered GO processes by 383 µg/L OC in brain and liver of adult male zebrafish as obtained by MetaCore pathway analysis on 628 transcripts in brain (FC ≥ 2, p ≤ 0.05) and 136 transcripts in liver (FC ≥ 1, p ≤ 0.05) are listed by the their significance. The number of transcripts affected in each process is included and of them the targets selected for RT-qPCR analysis.

	GO processes affected by 383 µg/L OC	*p* -value [a]	No. of genes [b]	Genes selected for RT-qPCR analysis
BRAIN	Developmental processes	2.8E-10	80	*rbp2a* , *pdgfra* , *thrb*
	Anatomical structure development	2.8E-10	73	*rbp2a* , *pdgfra* , *thrb*
	System development	5.8E-10	68	*rbp2a* , *pdgfra* , *thrb*
	Multicellular organismal processes	1.3E-09	95	*rbp2a* , *pdgfra* , *thrb* , *mtnr1bb*
	Multicellular organismal development	1.6E-09	74	*rbp2a* , *pdgfra* , *thrb*
	Response to steroid hormone stimulus	1.4E-08	22	*pdgfra* , *thrb*
	Anatomical structure morphogenesis	1.4E-08	44	*pdgfra* , *thrb*
	Positive Regulation of cellular processes	7.6E-08	58	*pdgfra* , *thrb*
	Organ development	1.1E-07	51	*rbp2a* , *pdgfra* , *thrb*
	Cellular component organization	3.9E-07	63	*pdgfra*
LIVER	Xenobiotic metabolic processes	1.8E-06	6	*pxr*
	Cellular response to xenobiotic stimulus	1.9E-06	6	*pxr*
	Urea cycle	2.1E-06	3	*cebpa*
	Response to xenobiotic stimulus	2.2E-06	6	*pxr*
	Amide biosynthetic processes	3.5E-06	3	*cebpa*
	Urea metabolic processes	4.3E-06	3	*cebpa*
	Nitrogen cycle metabolic processes	5.3E-06	3	*cebpa*
	Lung development	1.0E-05	6	*cebpa* , *rbp4*
	Respiratory tube development	1.1E-05	6	*cebpa* , *rbp4*
	Response to vitamin B2	1.4E-05	2	*cebpa*

[a] GO processes are listed by significance; [b] Number of altered genes involved within GO process by 383 µg/L OC.
rbp2a: retinol binding protein 2a, *pdgfra:* platelet derived growth factor receptor alpha, *thrb:* thyroid hormone receptor beta, *mtnr1bb:* melatonin receptor 1B, *pxr:* pregnane X receptor, *cebpa:* CCAAT/enhancer binding protein alpha, *rbp4:* retinol binding protein 4.

Liver. Additionally, the global transcription profile was determined in the liver that may be a potential target organ of OC in addition to brain (however, it should be noted that there is no previous information on target organs of OC). Exposure to 383 µg/L OC for 16 days resulted in 136 (fold-change (FC) ≥ 1, $p \leq 0.05$) and 45 differentially expressed transcripts (FC ≥ 2, $p \leq 0.05$), respectively (Table S11). Enrichment analysis showed that manifold pathways and GO processes were affected as shown in Table 3 and in more detail in Table S12, including the names of all involved transcripts. The most prominent GO processes

include xenobiotic metabolic processes (6 transcripts), (cellular) response to xenobiotic stimulus (12 transcripts), urea cycle and metabolic processes (6 transcripts), amide biosynthetic processes (3 transcripts), nitrogen cycle metabolic processes (3 transcripts), lung and respiratory tube development (12 transcripts) and response to vitamin B2 (2 transcripts).

Table 4: Most relevant pathways and process networks affected by 383 µg/L OC in brain and liver of adult male zebrafish as obtained by MetaCore pathway analysis on 628 transcripts in brain (FC ≥ 2, p ≤ 0.05), and 136 transcripts in liver (FC ≥ 1, p ≤ 0.05). The number of transcripts affected in each biological pathway is included, and of them the targets selected for RT-qPCR analysis.

	Pathways and Process networks affected by 383 µg/L OC	No. of genes [a]	Genes selected for RT-qPCR analysis
BRAIN	Cytoskeleton remodeling, cell migration	3	*pdgfra*
	Epithelial-to-mesenchymal transition (EMT) in cancer cells	3	*pdgfra*
	Transport, macropinocytosis regulation by growth factors	3	*pdgfra*
	Cell adhesion, platelet-endothelium-leucocyte interactions	9	*pdgfra*
	Development, regulation in EMT	9	*pdgfra*
LIVER	Fat cell differentiation	5	*cebpa* , *lpl*
	Development in granulopoiesis	1	*cebpa*
	Inflammation, IL-6 signalling	3	*cebpa*
	Multiple signal transductions	6	*cebpa*
	Transcription regulation of nuclear receptors	2	*pxr*

[a] Number of altered genes involved within pathway and process network by 383 µg/L OC.
pdgfra: platelet derived growth factor receptor alpha, *cebpa:* CCAAT/enhancer binding protein alpha, *lpl:* lipoprotein lipase, *pxr:* pregnane X receptor.

The enrichment analysis revealed various pathway maps (Table S13), whereas the most relevant pathways, based on known function of transcripts and their observed frequently involvement in different pathways, are highlighted in Table 4. They include, among others, the role of chemicals on fat cell differentiation, signal transduction activin A signaling regulation, regulation of metabolism triiodothyronine and thyroxine signaling, polyamine metabolism, arginine metabolism and developmental role of nicotinamide in G-CSF-induced

granulopoiesis. Selected process networks are included in Table 4 and details are given in Table S14. They include, among others, inflammatory mediator IL-6 signaling, signal transduction of the ESR1 nuclear receptor pathway, cytoskeleton intermediate filaments, DNA damage, signal transduction of the androgen nuclear receptor pathway, cell cycle mitosis and transcriptional regulation of nuclear receptors in general.

The larger entity list (FC ≥ 1.0) was used for the pathway analysis of liver samples. It shows that some transcripts (with known function) were predominant and are involved in various GO processes and pathways, including pregnane X receptor (*pxr, nr1i2*), aryl hydrocarbon receptor nuclear translocator-like 1a (*arntl1a, bmal1*), CCAAT/enhancer binding protein alpha (*cebpa*), plasma retinol binding protein 4 (*rbp4*) and the lipoprotein lipase (*lpl*). All of these transcripts are up-regulated by 383 µg/L OC, (*pxr* (FC = 1.8), *arntl1a* (FC = 2.1), *rbp4* (FC = 2.2) and *lpl* (FC = 1.7)), except *cebpa* which is down-regulated (FC = -2.4). These transcripts were selected for validation by RT-qPCR.

4.4.4. Validation by RT-qPCR. Altered genes identified by microarrays with known functions and predominant presence in various GO processes and pathways (Tables 3, 4) were selected for validation by RT-qPCR in brain and liver tissues using the same RNA samples. In addition to samples of controls, 22 and 209 µg/L OC, selected transcripts were analyzed in brain samples after 8 days of exposure, and in eleuthero-embryos after different exposure times. As there was no difference between water and solvent control, mRNA alterations by OC were detected by comparison with solvent controls (Figs. 1-3 and Figs. S1-S5). Transcript alterations revealed by RT-qPCR are expressed as log2.

Brain. Fig. 1A-B shows transcripts of *rbp2a* and *cyp1c2*. After 8 days, both transcripts were up-regulated, while at 16 days they show significant and dose-dependent down-regulation (1.5-fold and 0.7-fold, respectively), confirming microarray data. Fig. S1 summarizes transcription profiles of *thrb, thra, mtnr1bb* and *cyp1c1*. The expression level of *thrb* and *mtnr1bb* was not altered by any treatment. The same is true for *thra*, which was analyzed in addition, although microarrays did not show a significant alteration. A down-regulation (0.6-fold) among all treatments was observed for *cyp1c1*, confirming the more sensitive microarray data. Finally, the overall down-regulation of *pdgfra* (0.8-fold) at 8 and 16 days is in accordance with microarray data (Fig. 1C).

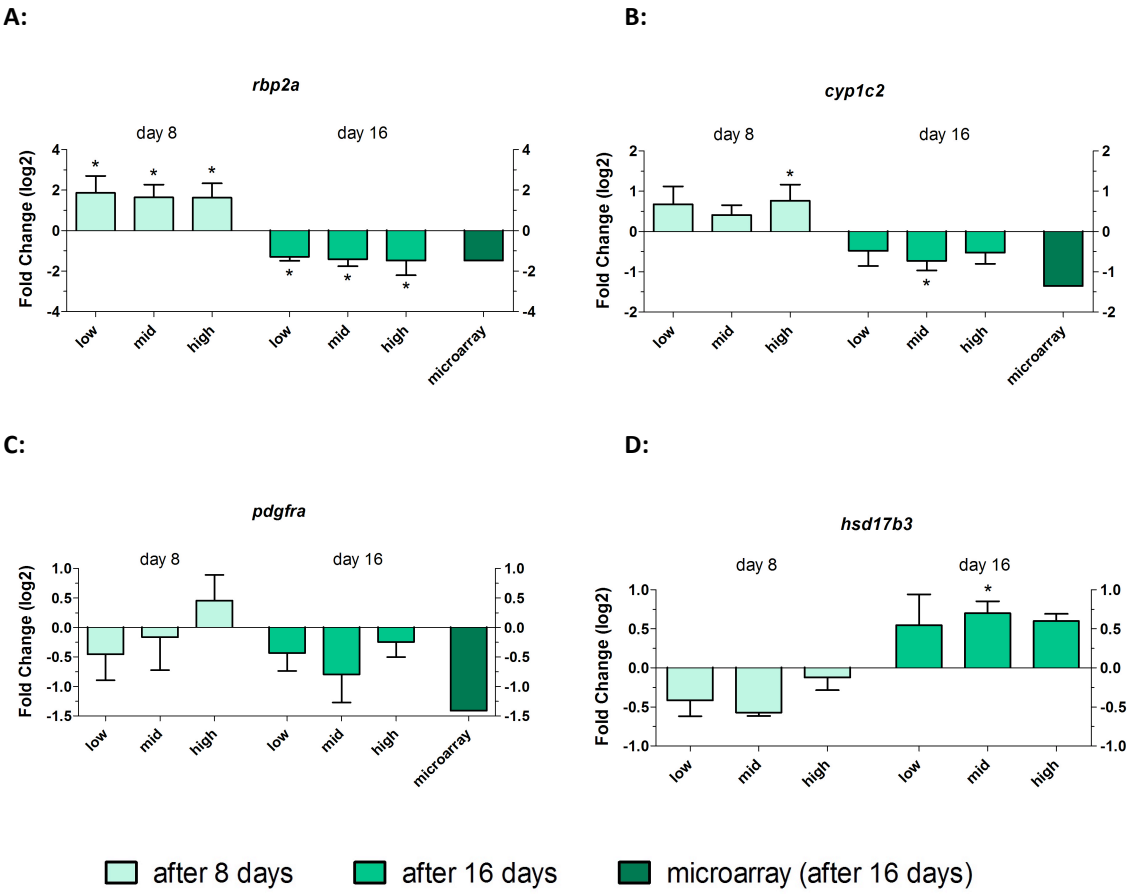

Fig. 1: Relative gene expression in different tissues of adult zebrafish males after exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Brain: Transcripts of retinol binding protein *rbp2a* (**A**), cytochrome P450 enzyme *cyp1c2* (**B**) and platelet-derived growth factor receptor alpha *pdgfra* (**C**) after 8 and 16 days, including microarray data after 16 days. Testes: Transcripts of 17β-hydroxysteroid dehydrogenase type 3 *hsd17b3* (**D**) after 8 and 16 days. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$).

Liver. Transcripts of *cebpa* show a significant and dose-dependent down-regulation (2.0-fold) after 16 days as in microarrays (Fig. 2A). An up-regulation was observed for *rbp4* (0.6-fold) (Fig. 2B). Fig. S3 shows no significant changes of *pxr* and *lpl* after 16 days exposure among all treatment, whereas microarrays show up-regulation. The transcripts of *arntl1a* show a transient up-regulation, significant at 383 µg/L OC (0.6-fold), confirming microarray data (Fig. 2C).

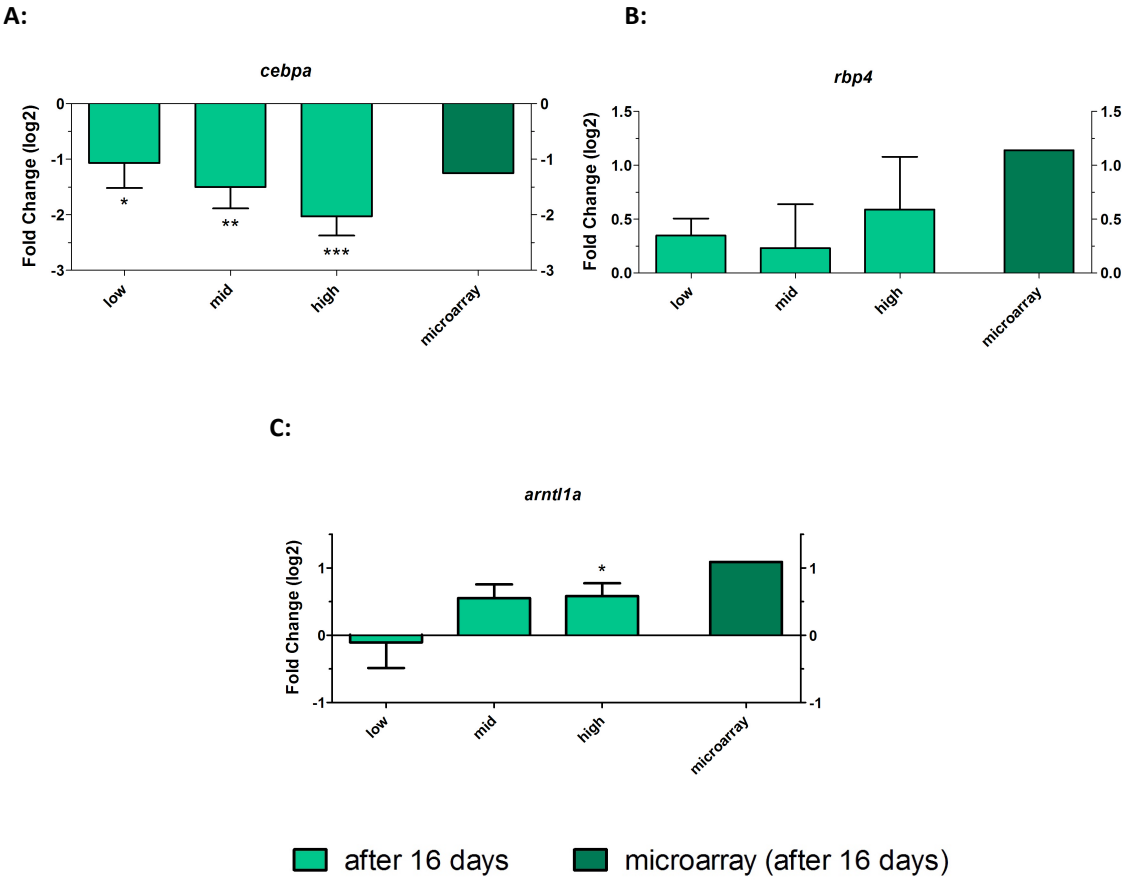

Fig. 2: Relative gene expression in liver of adult zebrafish males after exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Transcripts of CCAAT/enhancer binding protein alpha *cebpa* (**A**), retinol binding protein 4 *rbp4* (**B**) and aryl hydrocarbon receptor nuclear translocator-like 1a *arntl1a* (**C**) after 16 days, including microarray data. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

Eleuthero-embryos. A similar and significant up-regulation of *pdgfra* occurs at all times and among all treatments (1.9-fold after 48 *hpf* and 0.8-fold after 144 *hpf*) (Fig. 3A). In contrast, expression of *cebpa* (Fig. 3B) and *mtnr1bb* (Fig. S5) show an up-regulation after 48 *hpf* with subsequent down-regulation after 144 *hpf* among all treatments (0.4-fold for *cebpa* and 0.5-fold for *mtnr1bb*). The expression levels of *rbp2a, cyp1c2, lpl, thrb* and *thra* show no significant alteration at 144 *hpf* (data not shown). At 96 *hpf* (exposure to 293 µg/L OC) data are missing for all investigated genes because three of four RNA samples failed the quality criteria after extraction.

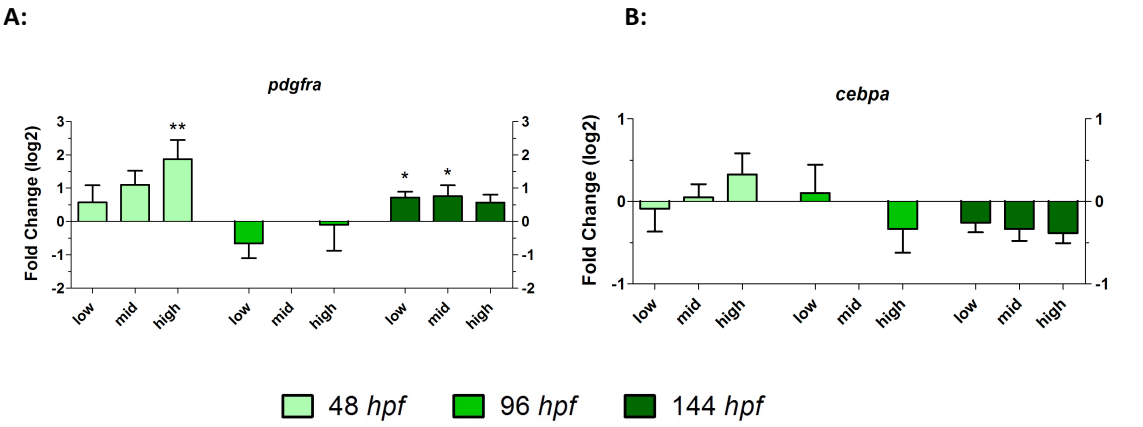

Fig. 3: Relative gene expression in eleuthero-embryos after exposure to 69 (low), 293 (mid) and 925 (high) µg/L OC. Transcripts of platelet-derived growth factor receptor alpha *pdgfra* (**A**) and CCAAT/enhancer binding protein alpha *cebpa* (**B**) after 48, 96 and 144 *hpf*. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=3-4 replicates). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$).

***Genes related to hormone system*.** Based on *in vitro* activity, additional emphasis is given to potential hormonal activity of OC by analyzing expression of *ar, esr1,* and *cyp19b* in the brain, *vtg1* in the liver, and *hsd17b3*, *cyp11b2* and *hsd11b2* in testes after 16 and 8 days (*ar* and *hsd17b3*). No significant alteration of *ar, esr1* and *cyp19b* were noted in the brain at 22 - 383 µg/L OC (Fig. S2). The same is true for *vtg1* in the liver (Fig. S3). However, in testes, *hsd17b3* expression differs after 8 and 16 days (Fig. 1D), and *hsd11b2* and *cyp11b2* show a tendency for up-regulation (Fig. S4). In eleuthero-embryos, transcripts of *esr1*, *ar*, *vtg1* and *hsd17b3* show no significant alterations among all treatments and time points (Fig. S5).

4.4.5. Steroid hormone levels. Due to the limited blood sample amount, concentrations of only the main androgen (11-KT) in fish were analyzed. 11-KT concentrations in blood plasma samples were calculated using the linear range of standard binding curve (22-90 % binding, LOD =39 pg/mL 11-KT). As shown in Fig. S6, OC had no effect on 11-KT concentrations in adult males.

4.5. Discussion

4.5.1. Analytical chemistry.

Analysis of all exposure water and the chemical properties test show comparable results; measured concentrations were 20-50 % of nominal concentrations. We hypothesize that this might be rather due to strong sorption properties of OC to both materials, stainless steel tanks (adult study) and glass (embryo study) than due to organic matter. This is based on similar results obtained in the chemical properties test (without fish or embryos). Relatively high standard deviations in triplicate analysis per time point and treatment suggest inhomogeneous distribution of OC. A similar behavior was noted in analogous experiments with the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) that has a log K_{ow} of 6.1, which is in the same range of the log K_{ow} of OC of 6.88 (Fent et al., 2008; Christen et al., 2011).

Table 2 shows that the BCF decreased at higher exposure levels. We do not know whether physiological parameters were altered at high exposure concentrations affecting OC uptake or metabolism. Without adverse outcomes associated with exposure, BCFs are expected to be constant regardless of the exposure concentration. Fish samples collected in rivers had OC residues of up to 2400 ng/g lipid weight in Switzerland (Buser et al., 2006) and 30 ng/g dw in Spain (Gago-Ferrero et al., 2013), respectively. Thus, whole body tissue analysis shows that the lipophilic OC accumulated in adult zebrafish to the same extent as in river fish and with similar concentration variations (Buser et al., 2006).

Calculated BCF varied between 41 and 136. This is in a similar and lower range, respectively, as other UV-filters including benzophenone-3 (average BCF 19 - 94) (Blüthgen et al., 2012) and benzophenone-2 (average BCF 0.3 - 6) (Weisbrod et al., 2007). Benzophenone-3 showed fast metabolism, and we assume that this is similar with OC, which would explain such a low BCF despite the high lipophilicity of OC (logK_{ow} 6.88). However, the BCF in fish is lower than in marine mussels, where up to more than 7 mg/kg (dry weight) OC was found at contaminated beaches (Bachelot et al., 2012). A similar bioaccumulation profile of OC was found in mussels for the highly lipophilic UV filter EHMC (log K_{ow} 5.80) (Gomez et al., 2012). However, concentrations of OC in mussels were higher than those of EHMC as the latter compound degraded rapidly by photolysis (Gomez et al., 2012). As in mussels, dietary uptake

can be a source of OC uptake in zebrafish in our present experiments as well. Zebrafish were fed and adsorbed OC may have been taken up in addition to uptake via water. Dietary uptake may even be the primary way of uptake.

4.5.2. Transcriptomics.

GO processes based on transcriptional alterations indicated that developmental processes, including anatomical structure, multicellular and organ development were affected after exposure to 383 µg/L OC. In addition responses to steroid hormone stimulus and cellular processes were affected. Based on enrichment analysis we hypothesize that OC may have also an influence on EMT (epithelial-to-mesenchymal transition), meaning formation of blood vessels, cytoskeleton remodeling and protein folding. In addition microarray data indicated that OC influenced to a lower extent reproduction (gonadotropin regulation and GnRH signaling) due to lower expressional changes.

The transcriptomics data revealed that some genes were frequently involved in many different GO processes, pathways and network processes, and hence were selected for analysis by RT-qPCR due to their known function in zebrafish. For example, *pdgfra, rbp2a, cyp1c1* and *cyp1c2* are involved in blood circulation, and hence we hypothesize that pathways including blood vessel formation, blood circulation and developmental processes are relevant for OC. Forthcoming experiments should evaluate the relationship to heart development by evaluation of heart rate to provide additional relevance to this hypothesis. The growth-factor receptor *pdgfra*, a fibroblast marker, plays an important role during heart regeneration (Fredriksson et al., 2004; Kim et al., 2010). The transcription level of this tyrosine kinase receptor is up-regulated in the regenerating zebrafish heart, whereas an OC-related inhibition of *pdgfra* may cause a lack of blood vessels and endothelial cells formation (Kim et al., 2010; Lien et al., 2006). Different regulation of *pdgfra* in adults (down) and embryos (up) may be related to regenerative processes in zebrafish heart (Lien et al., 2006). *Cyp1c1/2* is associated with a decrease in mesencephalic vein (MsV) blood flow in zebrafish embryos (Kubota et al., 2011), and the reduced blood flow can be rescued by *Cyp* inhibitors (Dong et al., 2002). However, biological consequences of differential gene regulation of *cyp1c2* and *rbp2a* after 8 (up) and 16 days (down) exposure remains unclear,

and have to be investigated in further experiments. The transcript alterations of thyroid hormone receptors *thrb* and *thra*, which are involved in developmental processes and steroid hormone stimulus responses, seem to be of minor importance due to their negligible mRNA alterations (FC log2 < 0.5). The same is true for the melatonin receptor 1B (*mtnr1bb*), which is involved in the circadian rhythm (Shang et al., 2007).

GO processes and biological pathways affected by 383 µg/L OC in the liver include xenobiotic metabolic and stimulus processes, overall nitrogen processes, such as urea cycle and amide biosynthesis as well as fatty acid metabolism and fat cell differentiation. Consequently, transcriptional alteration of *cebpa, rbp4, lpl, pxr* and *arntl1a* were assessed by RT-qPCR due to their predominance in these biological processes. The protein encoded by *cebpa* plays a crucial role in the regulation of carbohydrate and lipid metabolism in the liver and adipose, as well as in myelopoiesis (blood cell formation). It is also critical for the commitment of myeloid precursor cells to granulocytic differentiation (Berman et al., 2005). The similar dose-dependent down-regulation of *cebpa* in adult liver and embryos suggests that OC may have an impact on blood cell formation, and presumably may inhibit the maturation of granulocytes (Lyons et al., 2001), similarly in different life stages. This is in accordance with our findings in the brain.

Moreover, the transcriptomics data indicate that the retinal synthesis is influenced by OC. Retinoids (e.g. retinol, better known as vitamin A) are important in various physiological processes, including reproduction, cell growth and differentiation, angiogenesis, developmental processes, immunity and embryogenesis (Chen et al., 2012a) and also involve lipoprotein lipases (*lpl*) (Levi et al., 2012). The retinol binding protein 4 (*rbp4*) is a specific carrier for retinol and delivers retinol from liver deposits to peripheral tissues via the blood stream (Tingaud-Sequeira et al., 2006; Chen et al., 2012a). In relation to our transcriptional results, which suggest an overall inhibition of blood cell/vessel formation, based on down-regulation of transcripts involved in these processes, it might be reasonable that *rbp4* is induced in the liver as a feedback response. In addition, expressional changes of *arntl1a* and *pxr* indicate an interference of OC with xenobiotic metabolism (Bresolin et al., 2005; Tseng et al., 2005).

4.5.3. Endocrine effects.

Based on *in vitro* data (Kunz and Fent, 2006) and our experience with other UV-filters (Blüthgen et al., 2012; Zucchi et al., 2011a, 2011b; Christen et al., 2011; Kunz and Fent, 2006), we included the investigation of potential hormonal activities of OC in zebrafish by a combination of transcriptomics and steroid hormone levels. In general, transcription levels of investigated genes related to the endocrine system and steroidogenesis are low and within biological variability, in particular in eleuthero-embryos. One exception is *hsd17b3* investigated in adult testes, whose expression differs in time. These *in vivo* results differ from *in vitro* data that showed at much higher concentrations partially agonistic and full antagonistic androgenic activity to hAR, and full antagonism to hER (Kunz and Fent, 2006). However, the *in vitro* yeast based system is not a full representative of complex biological responses to chemical exposure in an organism and thus the effects may differ. Our data indicate that the influence of OC on the sex hormone system and steroidogenesis in zebrafish males and eleuthero-embryos may exist, although no influence on KT-11 levels were noted. It appears that the impacts on GnRH signaling pathway and gonadotropin regulation in brain (Tables S13, S14) coupled with the results on *hsd17b3* would indicate that endocrine impacts may occur. In conclusion, our results suggest that a reproductive effect on zebrafish males up to 383 µg/L OC may occur. Therefore, reproduction should be evaluated in forthcoming experiments. However, endocrine activity of OC is weaker compared to the UV-filter benzophenone-1 (Kunz and Fent, 2006), and benzophenone-2 (Weisbrod et al., 2007) that negatively affect reproduction. Thus, impacts on the endocrine system and potential reproductive effects of OC on fish cannot be ruled out as usually only drastic reproductive effects are reflected in sex steroid hormone levels (Blüthgen et al., 2013; Runnalls et al., 2013).

The present study focused on the global transcription profile and on selected transcripts in zebrafish after exposure to OC to provide first insights into potentially affected biological processes by this UV-filter. Although microarray analysis in brain and liver of adult zebrafish revealed potentially new and unexpected modes of action of OC, such as an influence on haematopoiesis, blood flow, blood vessel formation and organ development, it is essential to further investigate how these transcriptional alterations translate into physiological changes in zebrafish. Further experiments should include appropriate physiological endpoint

measurements to corroborate the influence of OC on haematopoiesis, blood vessel formation and circulatory and developmental processes as indicated by our transcriptomics data. Such additional experiments including reproduction should provide a link between our transcriptional data and physiological responses of OC for a comprehensive risk assessment.

4.6. Conclusions

Our applied transcriptomics approach revealed first and unexpected insights into molecular mechanism of the largely used UV-filter OC in zebrafish. The similar transcriptional responses in adult males and embryos provide some confidence that the expressional changes are driven by OC exposure, and that OC acts similarly at different life stages. Our transcriptomics data suggest that OC may have an impact on haematopoiesis, blood flow, blood vessel formation and organ development (e.g. heart) in zebrafish, and impacts on the endocrine system cannot be ruled out. The focus in our study was to elucidate potential mechanism of action of OC in zebrafish based on transcriptomics by using a multiple tissue and exposure time approach in adult fish and embryos. By now, neither mammalian nor ecotoxicology data are available to compare our results. It should be noted, however, that many transcriptional alterations had a threshold of FC < 2.0, which suggests that some of the changes have not a strong statistical basis. Our study should be expanded to show that the transcriptionally affected genes are also functionally affected. Further experiments are necessary to investigate how transcriptional alterations translate into physiology (e.g. haematopoiesis, blood flow, blood vessel formation, heart rate), and developmental processes in zebrafish. Although our transcription profiles on endocrine targets and 11-KT level in blood plasma indicate a rather low potential for reproductive effects by OC in zebrafish males, this should be corroborated by investigating the reproductive performance of both genders.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supporting Information Available

In Appendix A3 detailed results of chemical analysis (water, fish, chemical properties test and quality control), results of exposure experiments (adult and eleuthero-embryo), biological parameters and 11-KT level are given. Complete lists of significantly different transcripts of genes in microarrays, detailed lists of GO processes, pathway maps and process networks and additional RT-qPCR results are given in Appendix A3.

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Chapter 5

Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 1. Reproductive and early developmental effects

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5.1 Abstract

Effects of synthetic progestins have recently been reported in fish, but potential effects of the synthetic antiprogestin mifepristone (MIF), also called RU486, have not been studied. The present study provides first insights into reproductive effects of MIF in zebrafish in comparison to the progesterone receptor agonist, progesterone (P4). We carried out a reproductive study using breeding groups of adult zebrafish. After a 14 day pre-exposure, zebrafish were exposed for 21 days to 5, 39, 77 ng/L MIF, 25 ng/L P4 and water and solvent controls. In addition, embryos originating from exposed adult fish were continuously exposed to 3, 15, 26 ng/L MIF, and 254 ng/L P4, respectively, for 96 h post fertilization. We found a significant U-shaped increase in egg production after exposure to 5 and 77 ng/L MIF, but no effects at 25 ng/L P4. Levels of sex steroid hormones in blood plasma of adult males (11-ketotestosterone) and females (17β-estradiol) were not altered. In addition to an increase of mature vitellogenic oocytes in ovaries of females exposed to MIF and P4, we observed several histopathological changes in ovaries, including post-ovulatory follicles, atretic follicles and proteinaceous fluid. Male gonads showed no or less alterations and no histopathological effects. Fertility of eggs and hatching success of embryos (F1 generation) was not affected at 3-26 ng/L MIF and 254 ng/L P4, respectively. The data lead to the conclusion that trace quantities of MIF affect reproduction of zebrafish and ovaries of female zebrafish. Effects on transcriptional changes in adult and embryonic zebrafish of this study in comparison to *in vitro* effects are reported in the associated report (Blüthgen et al. 2013).

Keywords: antiprogestin, mifepristone, RU486, progesterone, reproductive effects

5.2. Introduction

Human pharmaceuticals have increasingly become of concern as some possibly cause adverse effects on aquatic organism (Fent et al., 2006). Due to phylogenetic conservation of the targets of these drugs (e.g. receptors and enzymes) responses in fish may be similar to those in humans (Christen et al., 2010; Fent et al., 2006; Gunnarsson et al., 2008). Among the most active compounds encountered in the aquatic environment are natural and synthetic steroid hormones (Sumpter and Johnson, 2005), which originate mainly from wastewater effluents. Synthetic steroid hormones including ethinylestradiol (EE2) and progestins, which are present in surface waters in the lower ng/L-range, are demonstrated to cause reproductive effects in fish (Länge et al., 2001; Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009).

Fish reproduction is mainly controlled by gonadotropins, which are released from the pituitary, and are analogous to the mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH). They are responsible for stimulating the synthesis of sex steroids (androgens, estrogens and progestins), which act in turn on target tissues to regulate spermatogenesis, oogenesis, reproduction, sexual phenotype and behavioral characteristics (Arukwe, 2001; Patiño and Thomas, 1990). Progestins play an important role in stimulation of oocyte maturation, ovulation in females, stimulation of spermiation and sperm motility in males, initiation of meiosis in both sexes and in some species also act as sex pheromones (Thomas, 2012; Tokarz et al., 2013).

Two progestins have been identified as major maturation inducing steroids (MIS) in fish, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and 17α,20β,21-trihydroxy-4-pregnen-3-one (THP) (Canario and Scott, 1990; Scott et al., 2010; Thomas et al., 2012). They induce oocyte maturation in teleosts by activation of nuclear and membrane-bound progesterone receptors, depending on season, spermiation, time of day and hormonal stimulation (Scott et al., 2010; Thomas et al., 2012). This is in contrast to the situation in humans, where the natural progesterone (P4) is an important regulator during oocyte maturation and is also necessary to maintain pregnancy via progesterone receptor binding. All progestins bind to the progesterone receptor (PR), but depending on their parent structure (progesterone, testosterone or spironolactone), also to other steroid hormone receptors (e.g. androgen,

estrogen, glucocorticoid and mineralcorticoid receptors). Thus, many progestins exhibit multiple hormonal activities.

Mifepristone (commercial name: Mifeprex™, RU486, Mifegyne™) is a synthetic antiprogestin and its consumption is increasing (in the UK, in 2006: 18.6 kg; 2007: 21 kg) (Runnalls et al., 2010). It is used as an effective non-invasive method of medical termination of up to 49 days of pregnancy. MIF acts by a competitive interaction with natural P4 at the PR, and inhibits the activity of P4, and thus terminating pregnancy (Spitz et al., 1996). MIF can prevent ovulation in mammals, but not in all cases (Spitz et al., 1996). MIF exhibits mixed agonistic and antagonistic activity to the PR *in vitro* and *in vivo*, and its mode of action as an antiprogestin is strongly dependent on the presence of other steroid hormones (e.g. P4 and 17β-estradiol (E2)), on the phase of the menstrual cycle and predominant isoform of the PR (PRα, PRβ) (Spitz et al., 1996; Spitz, 2003). In addition, MIF poses some antiestrogenic and weak antiandrogenic activity in addition to antiglucocorticoid activity (at high doses) (Spitz et al., 1996). Due to its interaction with various steroid receptors, MIF is clinically used also for other treatments, such as labor induction, menstrual regulation, endometriosis, contraception, Cushing Syndrome and steroid receptor containing tumors (e.g. in breast, ovary, prostate), although its main application is termination of pregnancy (Spitz, 2003).

Recent ecotoxicological research has focused mainly on reproductive effects of synthetic progestins in fish, and antiprogestins lack attention. Low ng/L concentrations of synthetic progestins, such as levonorgestrel (Runnalls et al., 2013; Zeilinger et al., 2009), drospirenone (Zeilinger et al., 2009), norethindrone (Paulos et al., 2010), gestodene (Runnalls et al., 2013), and at higher concentrations (µg/L) desogestrel (Runnalls et al., 2013), as well as P4 (DeQuattro et al., 2012) showed reduced or even ceased egg production in fish. In addition, some of them possess androgenic activities *in vivo* and *in vitro* (Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009).

MIF has recently been demonstrated to affect gene expression in zebrafish embryos, indicating antiprogestogenic and androgenic activities (Zucchi et al., 2012). MIF (log K_{OW}=5.4) was detected in wastewater effluents at concentrations of 195 ng/L (Liu et al., 2011), and at similar concentrations in hospital effluents (Liu et al., 2010). Surface water concentrations have not yet been reported thus far, but are expected to be in the lower ng/L-range. Environmental concentrations of P4 are reported to be in a similar range; 0.3-1.4 ng/L in

rivers in Spain (Kuster et al., 2008) and 14-27 ng/L in freshwater rivers in the U.S.A. (Pal et al., 2010).

In our present work we evaluate the so far unknown effects of MIF in adult zebrafish (*Danio rerio*) at low ng/L concentrations on reproduction, fertility and hatching success. We performed a reproductive assay using adult zebrafish males and females exposed for 21 days to 5, 39, 77 ng/L MIF, and 25 ng/L P4, respectively. Reproductive effects, sex steroid hormone levels of adult fish and histology of gonads of both sexes are reported here, whereas *in vitro* activities and effects on gene expression are reported in the associated paper (Blüthgen et al., 2013). We hypothesize that MIF affects egg production and other physiological endpoints based on its reported antiprogestogenic and antiglucocorticoid activities (Zucchi et al., 2012).

5.3. Materials and Methods

5.3.1. Chemicals. Mifepristone (MIF, RU486, 11β-(4-dimethyl-amino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one, CAS no: 84371-65-3, purity ≥ 98 %), progesterone (P4, 4-pregnene-3,20-dione, CAS no: 57-83-0, purity ≥ 99 %), medroxyprogesterone (used as internal standard (IS) for chemical analysis, 17α-hydroxy-6α-methylprogesterone, CAS no: 520-85-4) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Buchs, Switzerland). Solvents used, methanol, ethanol, acetone and acetonitrile, were of HPLC-grade or higher and obtained from J.T. Baker (Stehelin AG, Basel, Switzerland), Fluka (Buchs, Switzerland) and Carlo Erba Reagents (Milan, Italy). Acetic acid and ammonium hydroxide solution (25%) were from Fluka (Buchs, Switzerland). Water used for sample preparation and HPLC was of HPLC grade and in-house prepared (Nanopure Diamond, Barnstead, Switzerland, and MILLI-RO PLUS 90, MILLIPORE, Molshelm, France, respectively). Solid-phase extraction cartridges Strata-X (200 mg/6 mL) were purchased from Phenomenex (Brechbühler AG, Schlieren, Switzerland).

KoiMed Sleep (Ethylenglycolmonophenylether) was purchased from KOI&BONSAI Zimmermann (Bühlertann, Switzerland). Heparin ammonium salt (100 KU) was obtained from Sigma-Aldrich (Buchs, Switzerland), and BD Micro-Fine+Innen sterile insulin syringes

(0.5mL, 0.33 mm (29G) x 12.7 mm) from Becton Dickinson (Allschwil, Switzerland). Bouin's solution (HT10132) used for fixation was obtained from Sigma-Aldrich (Buchs, Switzerland).

5.3.2. Zebrafish. Adult zebrafish were obtained from Harlan Laboratories, Itingen, Switzerland, and maintained in culture tanks (300 L) for two months. Fish of both sexes were held in reconstituted deionized water (salt concentrations: $CaCl_2x2H_2O$ 147.0 mg/L, KCl 2.9 mg/L, $MgSO_4x7H_2O$ 61.6 mg/L, $NaHCO_3$ 32.4 mg/L) with a conductivity of 350-360 µS/cm, and applying a static-water renewal at least once per week. Water temperature was 26 ± 1 °C and the artificial photoperiod 16:8 h light/dark. Fish were fed twice daily with a combination of frozen brine shrimps (*Artemia*), mosquito larvae and *D. magna*. Water parameters, such as nitrate, nitrite and pH, were controlled regularly using Test strips (Easy Test, JBL) and always below the limit of detection and the oxygen concentration was ≥ 80 %.

5.3.3. Exposures. Exposure stock solutions of 0.04, 0.4 and 4 µg/mL MIF and 4 µg/mL P4 were prepared daily and weekly (for P4), respectively, in DMSO. These stock solutions were dispensed in reconstituted water to obtain final (nominal) exposure concentrations of 4, 40 and 400 ng/L MIF and 400 ng/L P4, respectively.

Zebrafish were exposed in a computer-controlled and taylor-made flow-through system (Péquitec MSR Systeme, Rothrist, Switzerland) which consisted of six custom-made dosing units, one for each treatment group. Each dosing unit supplied 5 replicate tanks, made of stainless-steel. For each treatment, four 10 L tanks were used for adult exposure and one 20 L tank for embryo exposure. All tanks were aerated (oxygen ≥ 80 %) and the temperature was kept constant at 26 ± 1 °C using the same water-bath for all treatments. The photoperiod during the experiment was set to 16:8 h light/dark. The MIF and P4 exposure stock solutions were dosed into the appropriate mixing chamber by a computer-controlled dispenser (100 µL) using Hamilton syringes and peristaltic pumps, and mixed with reconstituted water (1 L) using a magnetic stirrer prior to introduction to the exposure system. The final solvent concentration in all exposure tanks, except water control, was 0.01 %. The procedure for the dosing units of the controls was the same, whereas for the

solvent treatment, pure DMSO was dosed, and no solvent was used in the water control. Homogenous exposure solutions from the mixing chamber were equally divided into five reservoirs, belonging to the same treatment, and immediately released into exposure tanks through Teflon tubing and by gradient. The flow-through system was set to a renewal rate of 1 L (in the mixing chamber) every 28 min, meaning that 0.2 L fresh exposure water was supplied to each replicate tank every 28 min. Finally, this leads to one complete water-renewal per day in each tank.

Every tank was equipped with a spawning tray consisted of a stainless-steel tray (265 x 185 x 30 mm) covered with a stainless-steel grid (mesh size: 2.5 mm). Two stainless-steel cable ties were attached to the trays for manipulation from outside the tank and to reduce handling stress for the fish. Artificial weed (plastic dummies) was attached to the center of the grid, surrounded by a few glass stones to provide an appropriate spawning substrate for the fish. Conductivity (350-360 µS/cm), temperature (26 ± 1 °C), pH value (6.4-6.8), oxygen (≥ 80 %), nitrate and nitrite concentration (≤ 0 mg/L) were continuously monitored to ensure high water quality.

5.3.4. Experimental design. Adult zebrafish (about 10 months old) were stimulated for spawning using spawning trays prior to selection for the experiment and randomly placed into 10 L stainless steel tanks in well-aerated reconstituted water. Each spawning group consisted of four females and six males.

The experimental design consisted of six groups with four replicate tanks for each treatment, including water control, solvent control (0.01 % DMSO), nominal 4, 40, 400 ng/L MIF and 400 ng/L P4 (dissolved in DMSO). After an acclimatization of 4 days (including spawning trays), the experiment started with a pre-exposure period of 14 days, to establish the baseline rate of reproduction for each tank (spawning group), followed by one day of equilibration when chemical-dosing started and finally 21 days of exposure. During the whole exposure period, appearance, mortality and abnormal behavior of fish were recorded daily, and fish were fed twice daily. The study was conducted based on OECD Guideline 229/230 (OECD, 2009a,b).

Every morning the spawning trays were removed from all exposure tanks to check for eggs and replaced by clean spawning trays in the afternoon, both times before feeding the fish. The embryos were transferred to Petri-dishes and examined under a stereomicroscope (Zeiss, DV4) to determine fertilization success. Total number of eggs was recorded daily and from each clutch (per tank) a maximum of 50 fertilized embryos were randomly selected and transferred to an egg-cup, consisting of a glass tube (80 x 60 mm) and closed with a stainless-steel grid (mesh size: 0.36 mm) at the bottom-side. These egg-cups were placed in the 5th replicate tank (20 L) of each treatment group and surrounded by appropriate exposure water. For example, embryos arising from adult fish exposed to 4 ng/L MIF were further exposed to the same chemical concentration in a separate tank (maximum *n*=4 replicate egg-cups per day). After 4 days (96 h post fertilization (*hpf*)), the embryos were analyzed for viability and hatching. In addition, the embryos arising from adult fish after 19 days exposure were further exposed for 120 *hpf* and not fed during exposure, to collect samples of an early second generation stage (F1) for further gene expression analysis (Blüthgen et al., 2013a).

At the end of the experiment, parental fish (F0 generation) were anesthetized in KoiMed Sleep (1.5 mL/1 L water) before sampling. Total body length and weight of each individual fish were measured. Three fish of each gender and of every replicate tank were dissected immediately; brain, liver and gonads were pooled independently (*n*=4 samples per tissue per gender and per treatment), collected in RNA*later* and stored at -80 °C for subsequent RNA extraction. Weights of individual gonads were determined before pooling. In addition, blood samples of individual adult fish were taken prior to dissection using heparinized needles (*n*=12 samples per gender and treatment). After centrifugation, blood plasma was stored at -80 °C for sexual hormone analysis. One female and one male fish per replicate tank were fixed in Bouin's solution for histological analysis (*n*=4 samples per gender and treatment). The embryos exposed for 120 *hpf* were pooled per egg-cup and collected in RNA*later* and stored at -80 °C for subsequent RNA extraction (*n*=4 samples per treatment).

5.3.5. Analytical chemistry.

Sample collection. During exposure, all stock exposure-solutions of MIF and P4 were replaced daily and weekly, respectively, by freshly prepared stocks (dissolved in DMSO). Due to different tank volume (10 L for adults, 20 L for embryos) and expected variation in chemical concentration, water samples were taken from adult and embryo tanks separately for each treatment. Exposure water samples were taken after 4, 10 and 18 days of exposure from adult and embryo tanks (each time from different replicate tanks) and at each sampling day in the morning (T0, immediately after stock-solution exchange), in the afternoon (T1) and the day after (T2, about 24 h later) before re-placement of the stock-solution. At the same time, an aliquot of all stock exposure-solutions was taken at each sampling time point and stored at -20°C for further stability analysis. In addition, exposure water samples were taken two times during the pre-exposure period (after 4 and 12 days pre-exposure) (only one time-point each day) from adult tanks. The water samples were stored at -20°C in the dark in pre-heated glass bottles until analysis. Pre-exposure water samples after 12 days and exposure water samples after 4 and 18 days were analyzed. Later on, exposure water samples after 10 days were analyzed from the water control and 4 ng/L MIF treatments.

Sample preparation. The extraction method of MIF and P4 in water samples was adapted from Zucchi et al. (2012) using different solid-phase-extraction cartridges. The water samples were thawed at room temperature overnight and worked up in different series including samples of one exposure group per series (n=6 for adult tanks and n=4 for embryo tanks). All material was rinsed twice with acetone and ethanol and glassware was heated to 500 °C (for 3 h) prior to use.

Stock standard solutions of MIF, P4 and medroxyprogesterone (used as internal standard, IS) were prepared in methanol and further diluted in methanol to obtain working spike solutions of a mixture of 1.0, 0.1 and 0.01 µg/mL of MIF and P4, respectively, and 0.5 µg/mL of IS. All standard solutions were stored at 4 °C and further analyzed for stability.

Different sample aliquots (100 - 250 mL) of exposure water were prepared for solid-phase-extraction (SPE) depending on analytes concentration. In addition, one extraction-method blank sample, using reconstituted water only (200 mL), was prepared on every sample preparation day. A calibration curve was extracted, preparing 6 aliquots of reconstituted

water, which were spiked with a mixture of 0 (blank), 0.3, 1, 3, 10 and 30 ng of MIF and P4, respectively. The pH of all water samples was adjusted to 7 with 10% ammonium hydroxide and 2M hydrochloric acid and each sample aliquot (including blank and calibration samples) was spiked with 5 ng of IS. Polymeric reversed-phase sorbent columns, Strata-X (200 mg/6 mL) (Phenomenex, Brechbühler AG, Schlieren, Switzerland) were used for sample extraction. The cartridges were conditioned subsequently with 2 x 4 mL of methanol, HPLC-water (pH 7) and 2 x 2 mL reconstituted water (pH 7). Samples were then passed through the columns under vacuum at a flow-rate of 10 mL/min. Afterwards cartridges were vacuum dried for about 1 h and analytes were eluted with 2 x 4 mL methanol. The eluates were dried under a gentle stream of nitrogen at room temperature.

Liquid Chromatographic - Tandem Mass Spectrometry (HPLC-MS/MS). Dried samples were re-dissolved in different aliquots of Milli-Q water:methanol 70:30 (50-400 µL according to analytes concentration and volume of extraction), centrifuged for 2 min at 2500 rpm (Megafuge 1.0, Heraeus Instruments) and transferred into glass vials for instrumental analysis. The HPLC system consisted of a 1200 Series Binary Pump SL and Autosampler (Agilent Technologies, Santa Clara, CA). The MS system was an API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB–Sciex, Thornhill, Ontario, Canada). Additionally to the extracted calibration curve, a six-point external calibration curve was constructed by injecting standard solutions containing different amounts of target analytes (0-30 ng/100 µL of Milli-Q water:methanol 70:30) and a fixed amount of IS (5 ng/100 µL of Milli-Q water:methanol 70:30). The first point was spiked only with IS and was also used as an instrumental blank. Quantitative analysis was performed by calculating the ratios of the peak areas of MIF and P4, respectively, and the peak area of the IS for each sample. Further details on the chromatographic separation and MS parameter are given in the supporting data (SD).

Quality Control. The extraction method was validated using reconstituted water spiked with a mixture of known amounts of MIF and P4. The following setup was validated: 2 x extraction blank (reconstituted water only), 3 x 40 ng/L MIF+P4 and 3 x 400 ng/L MIF+P4. During preparation of exposure water samples, three randomly selected exposure water samples of nominal 4, 40 and 400 ng/L MIF were prepared in duplicate, using one of them as a quality control (QC) for the extraction method. These QC samples were spiked with a

mixture of 1 and 10 ng MIF+P4, respectively, and 5 ng of internal standard (IS) immediately before extraction and were treated as described above. The accuracy was calculated as follows: measured concentration/expected concentration x 100. The detection (LOD) and quantification (LOQ) limits of the analytical method were calculated from sample chromatograms as concentrations for which the signal to noise ratios were 3 and 5, respectively.

Chemical Stability Test. The following experimental design was used to determine the stability of MIF and P4 at -20 °C during storage in glass bottles, and thus to support the analytical results. Reconstituted water samples were spiked with a mixture of MIF and P4 at nominal concentrations of 4, 40 and 400 ng/L. A control containing deionized water was spiked with a mixture of 40 ng/L MIF and P4. All samples were set up in triplicate and were analyzed immediately, and after 3 months storage at -20 °C in the dark, following the same procedure as described above. Each set of analysis included a procedural blank to check for contamination.

5.3.6. Radioimmunoassay of sex steroids. After 21 days exposure, individual blood plasma samples (2-5 µL) were used for analysis of 11-ketotestosterone (11-KT) in males and 17β-estradiol (E2) in females. For both sex steroids *n*=2 individual samples per replicate and *n*=8 samples per treatment, respectively, were analyzed using radioimmunoassays (RIA).

Prior to RIA, individual plasma volume was made up to 100 µL with PBS-buffer and steroids were extracted using 500 µL ethyl acetate. After centrifugation (2500 g, 5 min, 4°C), the organic phase, which contains the steroids, was transferred into a new tube and stored at -20°C until analysis. The RIA was performed following the protocol described by Scott et al. (1980). Appropriate antibodies dilutions, 11-KT antibody (1: 50,000 dilution) and E2 antibody (1: 40,000 dilution), were prepared in steroid-assay-buffer (SAB) and working aliquots of labels, ^{3}H-11-KT and ^{3}H-E2, were prepared in SAB at radioactivities of approximately 6000 dpm per 100 µL. Labels and antibodies were used from previous studies (Margiotta-Casaluci et al., 2013). 200 µL sample extract aliquots were evaporated using a vacuum centrifuge and re-suspended in 100 µL SAB. The 11-KT and E2 RIA, respectively, were carried out in duplicate, including total counts, non-specific binding and maximum

binding, and the same calibration curve (0.005 – 10 ng/mL 11-KT and E2) was used for quantification of all samples. 11-KT and E2 concentrations in blood plasma samples of males and females, respectively, were calculated using the linear range of the standard curve (14-89 % binding for 11-KT and 15 - 78 % binding for E2). The limit of detection (LOD) was assessed from the linear binding range and determined at 39 pg/mL for 11-KT and 10 pg/mL for E2.

5.3.7. Histology. After anesthesia, one male and one female of each replicate tank (*n*=4 males and females, respectively, per treatment) were opened at the abdominal-site and fixed in Bouin's solution for about 24 h. After fixation, fish were kept in 70 % ethanol and all further procedures, including dehydration and embedding in paraffin, were performed as previously described (Blüthgen et al., 2012). Tissues (whole body) were sectioned to 3 µm thickness using the RM 2235 microtome (Leica Inc.; blades from Shandon MB35, Thermo). The sections were stained using a standard Hematoxylin-Eosin (H&E) staining protocol. The cross-sections were examined using an Olympus BX51 light microscope and photographs were taken using a digital camera (Olympus Q Imaging) and the Q-Capture Pro software (Version 51). Several cross-sections were taken from different tissue levels along the gonad-axis from each fish.

Males. Two cross-sections, including both testis, per fish were analyzed using the free-download software ImageJ (Version 1.46r, http://rsb.info.nih.gov/ij/download.html). Testes were staged based on the relative percentage of immature spermatocytes (spermatogonia and spermatocytes) and mature spermatocytes (spermatids and spermatozoa), based on the area they occupied at 100x magnification. Testes staging and histopathological alterations were evaluated according to histological (Dietrich and Krieger, 2009) and histopathological guidelines in fish (OECD, 2010; U.S. EPA, 2007). The data were evaluated by calculating the mean relative percentages of occupied areas compared to the total area of each individual testis, and for all fish within the same treatment.

Females. Two cross-sections per fish were analyzed at 20x magnification using the free-download software Image Tool (Version 2.0 Alpha 3, http://union-d.ru/projects/imagetools). Ovaries were staged based on numbers of perinucleolar oocytes

(PO), cortical alveolar (CO), early vitellogenic oocytes (EV) and mid-late vitellogenic oocytes (LV). The following criteria were applied for all oocytes: PO: no vacuoles, CO: vacuoles and chorion present, EV: early presence of yolk-filled vesicles, LV: minimum 50-70 % presence of yolk-filled vesicles. Ovary staging and histopathological alterations were evaluated according to guidelines (OECD, 2010; U.S. EPA, 2007; Dietrich and Krieger, 2009) and conducted using 100x magnifications. In females, additional sections of 100x magnification were analyzed in each fish for histopathology. The ovary data were evaluated by calculating the mean proportion of every individual oocyte stage from every fish and within one treatment.

5.3.8. Data analysis and statistics. Data were graphically illustrated and statistically analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, USA). Significant differences in hormone level (RIA) and different cell stages in the gonads were analyzed by one way analysis of variance (1-way ANOVA) followed by a Tukey post-hoc test (95% confidence interval) and are referred to the solvent (DMSO) control. Results are shown as mean ± standard deviation (S.D.) of mean, unless otherwise indicated. Significant differences in the reproductive performance (number of eggs/female/week, number of eggs/female/day, spawning frequency, fertility success, F1 hatching rate and embryo survival) were analyzed using two way analysis of variance (2-way ANOVA) followed by Bonferroni post-hoc test and comparing pre-exposure to exposure within the same treatment group. Significant differences were considered when $p \leq 0.05$.

5.4. Results

5.4.1. Analytical chemistry.

Concentrations of MIF and P4 were measured in pre-exposure and exposure waters and the method allowed to detect low amounts (LOQs were 0.23 ng/L for MIF and 0.14 ng/L for P4). Detailed results on the analytical chemistry including the validation, quality controls and stability during storage can be found in the supporting data (Table S2-S5). We found a concentration-dependent loss of MIF (up to 83 %) during storage at -20 °C within 3 months

(see Table S5), but no significant loss for P4. The measured concentrations of MIF (without taking the storage loss into account) and P4 in exposure water samples are shown in Table 1.

Table 1: Measured mifepristone (MIF) and progesterone (P4) concentrations in exposure water of adult tanks (10 L) and embryo tanks (20 L), respectively. The intra-day variability for three different time points (*n*=3) of each sampling day (after 4, 10 and 18 days exposure) are shown as well as the inter-day variability, considering 1-3 sampling days. All concentrations are represented as mean values ± S.D. in ng/L (without taking the stability loss into account).

Nominal Concentration		Measured Concentrations [ng/L]									
		PRE - EXPOSURE		EXPOSURE							
				after 4 days		after 10 days		after 18 days		Mean concentrations ± S.D.	
Water Control	Adult	N/A	MIF			N/A	MIF			**N/A**	**MIF***
		N/A	P4			N/A	P4			**N/A**	**P4***
	Embryo					N/A	MIF			**N/A**	**MIF***
						N/A	P4			**N/A**	**P4***
Solvent Control	Adult	N/A	MIF	N/A	MIF			N/A	MIF	**N/A**	**MIF**
		N/A	P4	0.2 ± 0.0	P4			0.4 ± 0.3	P4	**0.3 ± 0.2**	**P4**
	Embryo			N/A	MIF			N/A	MIF	**N/A**	**MIF**
				0.4 ± 0.2	P4			0.3 ± 0.1	P4	**0.4 ± 0.1**	**P4**
Low (4 ng/L MIF)	Adult	N/A	MIF	7.3 ± 1.1	MIF	1.2 ± 0.1	MIF	5.0 ± 0.7	MIF	**4.5 ± 3.1**	**MIF**
		N/A	P4	2.7 ± 3.3	P4	N/A	P4	1.7 ± 1.5	P4	**1.5 ± 1.4**	**P4**
	Embryo			4.1 ± 0.5	MIF	0.5 ± 0.0	MIF	3.8 ± 0.5	MIF	**2.8 ± 2.0**	**MIF**
				0.9 ± 0.4	P4	N/A	P4	0.8 ± 0.5	P4	**0.6 ± 0.5**	**P4**
Mid (40 ng/L MIF)	Adult	3.61	MIF	51.9 ± 27.2	MIF			27.0 ± 8.1	MIF	**39.4 ± 17.7**	**MIF**
		N/A	P4	0.2 ± 0.0	P4			0.4	P4**	**0.3 ± 0.1**	**P4**
	Embryo			15.8 ± 2.3	MIF			14.9 ± 1.3	MIF	**15.3 ± 0.6**	**MIF**
				0.3 ± 0.1	P4			0.2 ± 0.0	P4	**0.2 ± 0.1**	**P4**
High (400 ng/L MIF	Adult	22.61	MIF	89.2 ± 12.6	MIF			64.6 ± 4.4	MIF	**76.9 ± 17.4**	**MIF**
		N/A	P4	4.4 ± 0.2	P4			N/A	P4	**4.4**	**P4**
	Embryo			23.0 ± 1.8	MIF			28.6 ± 1.7	MIF	**25.8 ± 4.0**	**MIF**
				5.1	P4**			4.2 ± 0.1	P4	**4.7 ± 0.6**	**P4**
P4 (400 ng/L)	Adult	N/A	MIF	N/A	MIF			N/A	MIF	**N/A**	**MIF**
		N/A	P4	13.4 ± 2.0	P4			35.6 ± 7.8	P4	**24.5 ± 15.7**	**P4**
	Embryo			N/A	MIF			N/A	MIF	**N/A**	**MIF**
				275.8 ± 1.6	P4			231.9 ± 28.9	P4	**253.8 ± 31.1**	**P4**

*: Only one sampling point considered due to difficulties during sample preparation. **: Analytes measured in only one of 2-3 samples and thus no S.D. calculated. N/A: not analyzed (< LOD).

All pre-exposure water samples were free of MIF and P4, except for the mid and high MIF treatments, where MIF was detected up to 22.6 ng/L, most probably due to a contamination during sampling or sample preparation. The mean concentrations (intra-day variability) for the different sampling points (after 4 and 18 days exposure) and the inter-day mean concentrations are shown in Table 1, whereas samples after 10 days exposure were only

analyzed from water control and 4 ng/L MIF treatments to compensate for difficulties during extraction of samples after 4 and 18 days exposure (water control samples after 4 and 18 days exposure were accidently contaminated during preparation). No MIF was present in all other samples of water and solvent controls in adult and embryo tanks. The measured mean concentrations of MIF in the adult tanks were 4.5 ± 3.1, 39.4 ± 17.7 and 76.9 ± 17.4 ng/L. In embryo tanks, they were more than 50 % lower, being 2.8 ± 2.0, 15.3 ± 0.6 and 25.8 ± 4.0 ng/L MIF. The traces of P4 detected in control samples and MIF treatments are due to the high instrumental sensitivity and cannot be considered as real contamination. The P4 concentration in adult tanks was 24.5 ± 15.7 ng/L, which was unexpectedly about 10-times lower than in embryo tanks (253.8 ± 31.1 ng/L P4). Detailed data on measured concentrations of MIF and P4 in individual exposure water samples, including the intra-day and inter-day variability, are given in Table S6.

5.4.2. Macroscopic observations.

Effects on biological measures. Exposure of adult zebrafish to 5 - 77 ng/L MIF and 25 ng/L P4, respectively, did not affect the body weight and body length and thus the condition factor (CF) in both sexes (Fig. 1). We did not observe any exposure-related mortality or behavior alterations. Only one fish (male) died during the exposure period from the group exposed to 25 ng/L P4. Some fish jumped out from exposure tanks among all treatments and were not considered as dead fish. The gonad weight, and thus the gonadosomatic index (GSI), indicated a slightly decrease in females exposed to 5 - 77 ng/L MIF, and was significant in females exposed to 25 ng/L P4 (Fig. 1, Table S7). The GSI in males was slightly increased after MIF and P4 exposure, respectively, albeit not significantly (Fig. 1, Table S7).

At sampling we realized that the gender ratios were not always 4 + 6 females/males in each tank, which reflects the uncertainty of zebrafish gender selection at the beginning of the experiment. The correct gender ratios of each tank were considered for further calculations of biological measures and reproductive performance and are given in Table S7.

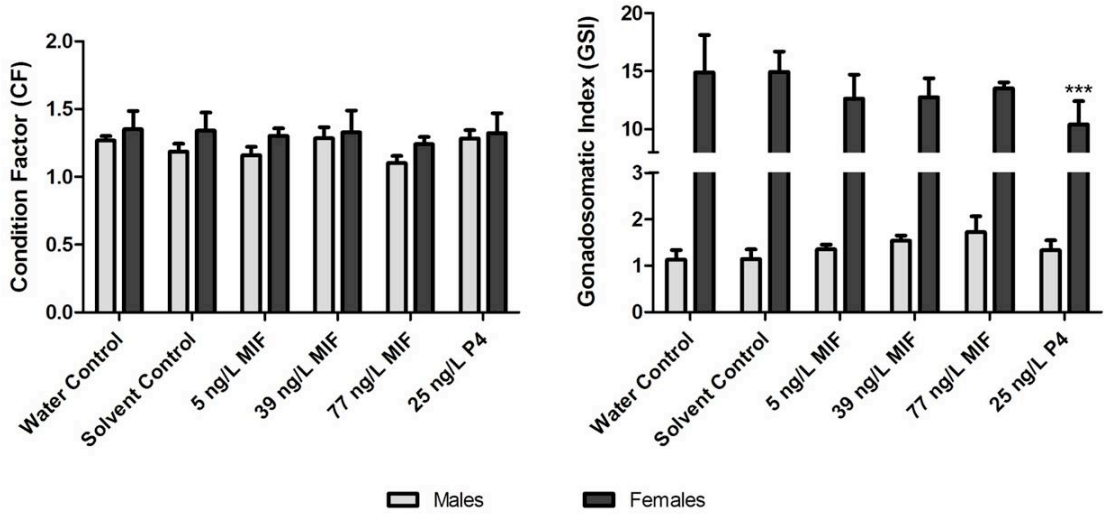

Fig. 1: Condition factor (CF) and gonadosomatic index (GSI) of adult female and male zebrafish exposed for 21 days to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. The CF was calculated as body wet weight [mg] / body length [mm] x 100 (*n*=16 females and 24 males, respectively, per treatment). The GSI was calculated as gonad weight [mg] / body weight [mg] x 100 (*n*=12 samples per gender and treatment). Asterisks indicate significantly different GSI compared to solvent control (*** $p < 0.0001$).

5.4.3. Reproduction.

Effects on reproductive performance. Breeding groups of fish in water control showed an increase in egg production in time. During the 21 day exposure period we observed an increased egg production in the 5 and 77 ng/L MIF dose groups. Fig. 2 shows the cumulative mean number of eggs per female per day. The slope of the curve of cumulative mean egg numbers in the pre-exposure and exposure is significantly different at 5 and 77 ng/L MIF, which was not the case at 39 ng/L MIF and in the solvent control. The cumulative egg production (and curve slope) at 25 ng/L P4 was similar as in the solvent control, and thus indicates no difference between pre-exposure and exposure.

By calculating a ratio of the average number of eggs per female per day laid during pre-exposure and exposure (ratio exposure vs. pre-exposure) we found a median (*n*=4 replicate tanks per treatment) ratio > 1 for fish exposed to 5 (ratio=1.5), 39 (ratio=1.4) and 77 (ratio=1.5) ng/L MIF and the water control (ratio=1.7), indicating an increase in average number of eggs per female during exposure in these treatments. There was one replicate

tank in the water control group with a very high ratio (3.2), which was not excluded for analysis. The ratio of fish exposed to the solvent control and 25 ng/L P4 were similar (ratio=1.2 and 1.1, respectively), indicating no or less increase in egg production. The ratios of individual tank replicates are shown in Fig. S1.

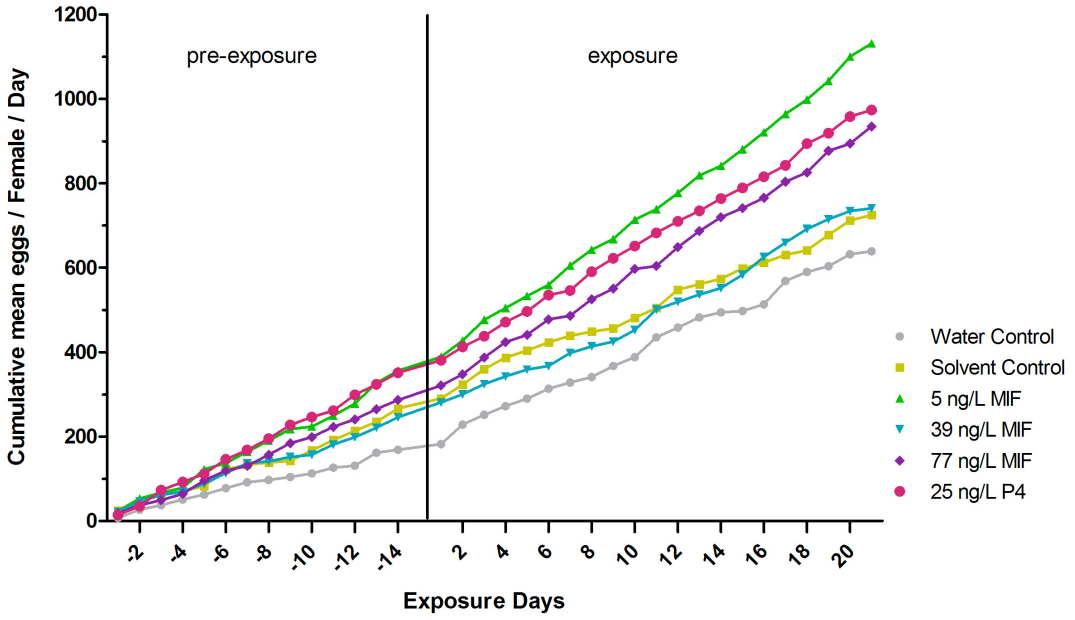

Fig. 2: Cumulative mean number of eggs per female per day after 21 day exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. The pre-exposure period is indicated by -1 to -14 days followed by the exposure period indicated by +1 to +21 days. Each curve represents the mean values of four replicate tanks per day and treatment.

Fig. 3 and Table S8 give the number of eggs per female per week (assuming seven days a week). At 5 ng/L MIF it is significantly increased during the whole exposure period, and after two weeks at 77 ng/L MIF and 25 ng/L P4. No or little effect was observed at 39 ng/L MIF and in the solvent control. Considering the number of eggs per female per day and comparing pre-exposure and exposure periods (Fig. S2), we found no difference in controls, but an increase in egg production after MIF and P4 treatment, which is significant at 5 ng/L MIF. By analyzing the spawning frequency (meaning how often the breeding groups spawned per week, assuming seven days a week) in each replicate tank in the pre-exposure and exposure, no obvious influence of MIF and P4 was observed on spawning events

(Fig. S3). No effects were observed on fertility success of laid eggs after exposure to 5 - 77 ng/L MIF and 25 ng/L P4, respectively (Fig. 4).

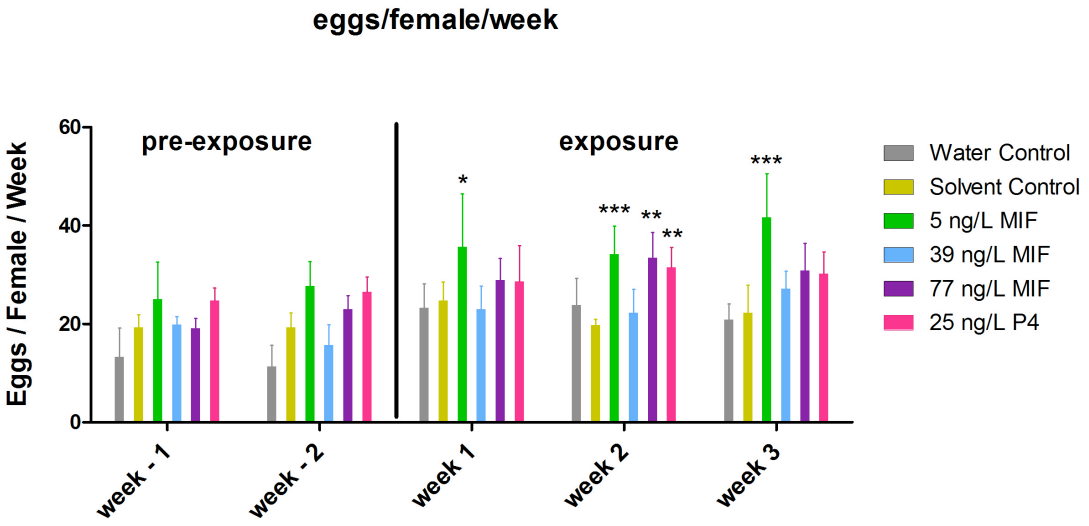

Fig. 3: Number of eggs per female per week after two weeks pre-exposure followed by three weeks exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Each bar represents the mean value ± S.D. of four replicate tanks per week and treatment. Asterisks indicate significantly different number of eggs per female and week compared to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

Effects on F1 generation. No abnormal development or behavior alterations were observed in zebrafish embryos (F1 generation) further exposed to 3-26 ng/L MIF and 254 ng/L P4, respectively, for 144 h post fertilization (*hpf*). The hatching success of embryos after 96 *hpf* were not different between the pre-exposure and exposure at each treatment (Fig. 4). No treatment-related effects occurred in embryo survival (considering hatched and non-hatched but alive embryos) (Fig. S4). Therefore, exposure of breeding groups to MIF and P4 did not negatively influence their fertilization success and early development of the F1 generation.

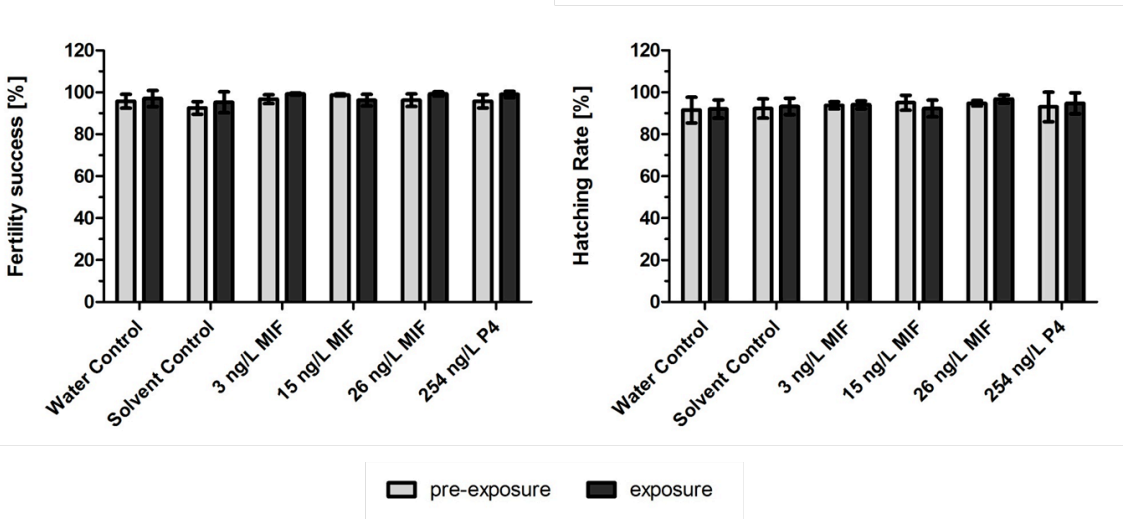

Fig. 4: Fertility success and hatching rate of embryos originating from adult zebrafish exposed to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. The hatchability was subsequently assessed in embryos after 96 *hpf* exposure to water control, solvent control (0.01 % DMSO), 3, 15, 26 ng/L MIF and 254 ng/L P4. The data compares pre-exposure and exposure and shows the mean values ± S.D. of four replicates per treatment, expressed in percentages.

5.4.4. Steroid hormone levels.

As shown in Fig. 5, MIF and P4 had no significant effect on plasma concentrations of 11-KT and E2 in adult males and females, respectively. Calculated mean concentrations of 11-KT in adult males were 8.9 ± 3.2, 8.8 ± 4.5, 6.8 ± 4.0, 9.2 ± 4.5, 5.7 ± 2.4 and 10.5 ± 8.9 ng/mL in water control, solvent control, 5, 39, 77 ng/L MIF and 25 ng/L P4, respectively. Calculated mean concentrations of E2 in adult females were 1.0 ± 0.4, 0.4 ± 0.2, 0.3 ± 0.1, 0.2 ± 0.1, 0.2 ± 0.1 and 0.2 ± 0.2 ng/mL in water control, solvent control, 5, 39, 77 ng/L MIF and 25 ng/L P4, respectively.

5.4.5. Gonad Histology.

Females. The ovaries showed no significant difference in the proportions of pre-vitellogenic oocytes (perinucleolar oocytes (PO) and cortical alveolar oocytes (CO)) after exposure to 5 - 77 ng/L MIF, in contrast to ovaries of females exposed to P4, which showed a lower frequency of CO (Fig. S5). Early vitellogenic ooyctes (EV) are present with significantly lower

frequency in females exposed to 39, 77 ng/L MIF and 25 ng/L P4 compared to the solvent and water control. Females exposed to 25 ng/L P4 showed a significant increase in mid-late vitellogenic oocytes (LV). A similar trend for an increase in LV was observed in females exposed to 5 - 77 ng/L MIF. Fig. 6 summarizes all investigated oocyte stages among all treatments. There was no difference in the total number of oocytes in females exposed to MIF and P4 (Fig. S6). All ovaries were in mid-late developmental stage (stage 2-3), meaning that majority of observed follicles were early-late vitellogenic (OECD, 2010; U.S. EPA, 2007). Representative images of ovaries are shown in Fig. 7.

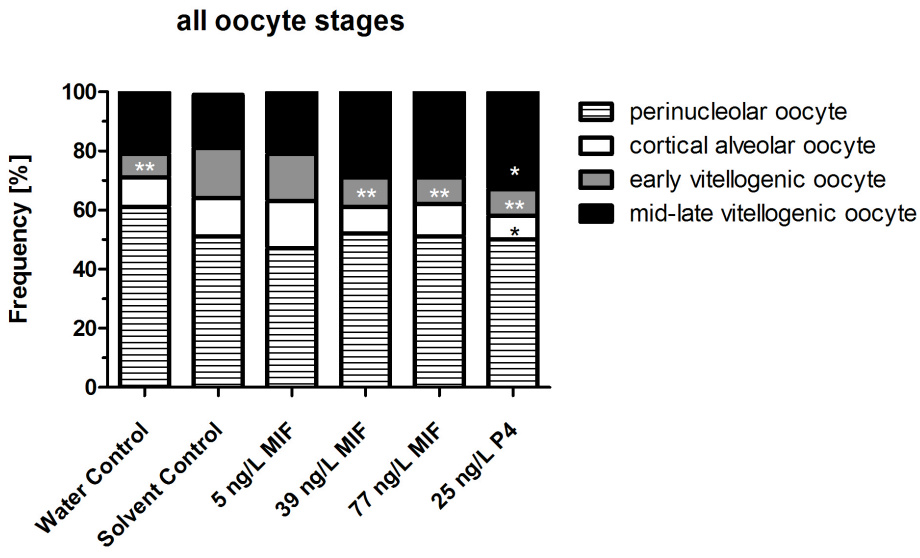

Fig. 6: Relative proportions of perinucleolar oocytes (PO), cortical alveolar oocytes (CO), early vitellogenic (EV) and mid-late vitellogenic oocytes (LV) in ovaries of adult zebrafish females after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Results are shown as mean values (n=6-8 ovaries per treatment). Asterisks indicate significantly different proportions to solvent control (* p<0.05), (** p<0.001), (*** p<0.0001).

Table 2 summarizes several histopathological alterations observed exclusively in ovaries of females exposed to MIF and P4. Post-ovulatory follicles are characterized by collapsed empty follicles, which have released their oocyte during spawning (spent post-ovulatory follicle). They had intact granulosa and theca cell layers, which were often enlarged. In contrast, atretic follicles are a sign of degeneration and characterized by clumping,

perforation of the chorion, disorganization of the ooplasm, irregular cell shapes formed by disintegration of granulosa cell structures, and the follicles contain oocyte debris (OECD, 2010; U.S. EPA, 2007).

Table 2: Histopathological observations from ovaries of female zebrafish after 21 day-exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Condition of oocytes includes an assessment of the prevalent of mature oocytes and the maturation stage of the ovaries; both are determined visually from each section. Presence of post-ovulatory follicles, atretic follicles and proteinaceous fluid is represented by number of fish affected per treatment, and appropriate severity degree in the individually affected fish. Severity degrees in individual fish and sections are represented as semi-quantitative observations based on visual scores: not present (-); present to minor (±), moderate (+) or strong (++) degree, respectively.

Treatment	**Condition of oocytes**		**Histopathological observations**		
	Mature oocytes present	Ovary stage	Post-ovulatory follicle *	Atretic follicle *	Proteinaceous fluid **
Water Control	+ to ++	2-3	-	-	1/3
Solvent Control	+	2	-	-	-
5 ng/L MIF	+ to ++	2	-	-	2/3
39 ng/L MIF	+ to ++	2-3	2/4 (± to +)	2/4 (±)	2/4
77 ng/L MIF	+ to ++	2	2/4 (± to +)	2/4 (+)	3/4
25 ng/L P4	+ to ++	2	2/4 (± to +)	2/4 (± to +)	2/4

Severity degree (-, ±, +, ++) was assessed in individual fish and individually for each section and is represented as the range occurring within one treatment.
* Number of fish affected within one treatment (e.g. 2/4 means two fish out of four within one treatment have shown these observations).
** Number of fish per treatment showing this observation (*n*=3-4 fish per treatment).

Females exposed to 39 and 77 ng/L MIF and 25 ng/L P4 showed both post-ovulatory and atretic follicles in two out of four investigated fish per treatment. Every section of each

female was analyzed and staged individually and showed the presence of post-ovulatory and atretic follicles ranging from a minor to moderate degree, which means that more than two occurrences were observed per section. Proteinaceous fluid, visible as homogenous dark pink material (presumably VTG) in interstitial spaces, was present in two or three out of four fish per treatment after exposure to MIF and P4. We also observed an increased presence of interstitial connective tissue and an increased appearance of blood vessels in ovaries after exposure to MIF and P4. Representative images of histopathological observations in females are shown in Fig. 8.

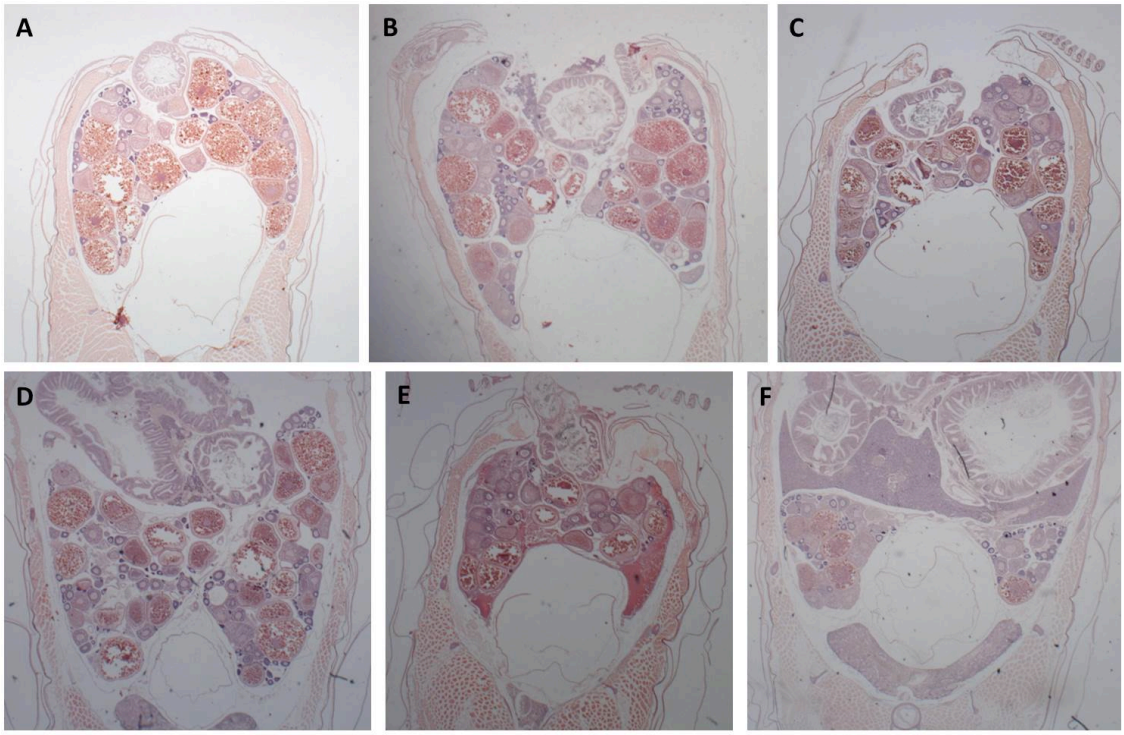

Fig. 7: Typical histological sections of ovaries of adult zebrafish females exposed for 21 days to water control (**A**), solvent control (**B**), 5 (**C**), 39 (**D**), 77 (**E**) ng/L MIF and 25 ng/L P4 (**F**). Ovaries were staged based on number of perinucleolar oocytes (PO), cortical alveolar (CO), early vitellogenic oocytes (EV) and mid-late vitellogenic oocytes (LV). All ovaries were in mid-late developmental stage (stage 2-3), meaning that the majority of observed follicles were early-late vitellogenic. (20x magnification)

Males. It was not possible to differentiate more than two major spermatogenic cell types at 100x magnification. Thus, to separate only immature (spermatogonia and spermatocytes)

and mature (spermatids and spermatozoa) spermatocytes was a compromise for the analysis of the total area of each gonad, instead of analyzing several randomly selected areas within each gonad. Fig. 9 and S7 show that the relative percentages of immature and mature spermatocytes were significantly altered after exposure to 5 ng/L MIF and 25 ng/L P4. The testes showed a lower percentage of mature spermatocytes, similar to the water control as compared to the solvent control. No significant differences in spermatocyte percentage occurred after exposure to 39 and 77 ng/L MIF. There was no difference in the total gonad size among all treatments (data not shown). All testes were in mid-spermatogenic stage (stage 2), meaning that spermatocytes, spermatids and spermatozoa are present in roughly equal proportions and that the germinal epithelium is thinner than stage 1 but thicker than stage 3 (OECD, 2010; U.S. EPA, 2007). Representative images of testes are shown in Fig. 10.

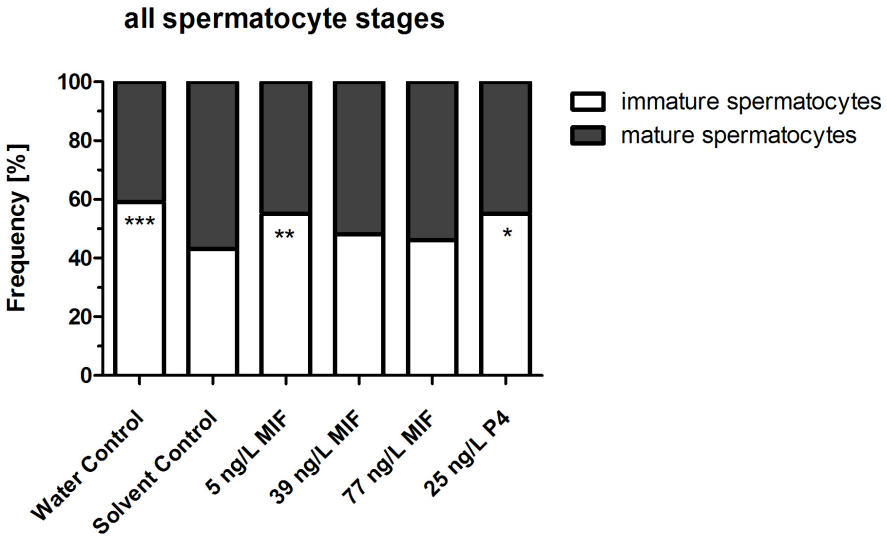

Fig. 9: Relative percentages of different testicular developmental stages, differentiated as immature (spermatogonia and spermatocytes) and mature (spermatids and spermatozoa) spermatocytes, in adult zebrafish males after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Results are shown as mean values (n=12-16 male gonads per treatment). Asterisks indicate significantly different percentages to solvent control (* p<0.05), (** p<0.001), (*** p<0.0001).

5.5. Discussion

5.5.1. Analytical chemistry.

The measured MIF concentrations in lower treatment groups matched the nominal concentrations (4 and 40 ng/L MIF). In contrast, the measured concentration of 77 ng/L MIF was significantly lower than nominal (400 ng/L MIF), and can be explained by the instability of MIF during storage at -20 °C and lower accuracy at higher concentrations. In a separate stability control experiment, we observed a decrease of 83 % at nominal 400 ng/L MIF after three months of storage, and found that the water composition plays a role. The water samples of our experiment were stored even longer (6 months) until analysis. The expected loss in the 40 ng/L group with measured concentrations of 39 ng/L MIF was not found, in part due to different water qualities and organic matter components (fish exposure water vs. reconstituted water without fish). Considering all quality control steps performed, including the accuracy of the QC samples with exposure water samples, and previously reported analytical results (Zucchi et al., 2012), we have several lines of evidence that the chemical properties of MIF are unstable at higher concentrations.

The lower MIF concentrations in embryo tanks compared to adult tanks can be explained by higher sorption-capability of stainless-steel tank walls in adult exposures. Other reasons could be the lower mixing-flow of fresh water within the 20 L-tank due to the presence of embryo glass containers and sorption to the glass. Unexpectedly, P4 concentrations were lower in adult than embryo tanks (confirmed by independent sampling days). The generally lower observed P4 concentration might in part be due the 6 months of storage, which is in alignment with previous results (Zucchi et al., 2012). The intra-day (T0, T1 and T2 (after 24 h)) and inter-day (after 4 and 18 days exposure) variability was generally low and suggested stability of MIF and P4 in exposure water using a flow-through system with one complete renewal per day.

5.5.2. Reproduction.

After analyzing the reproductive data in many different ways (eggs per day/ per week, cumulative mean eggs, ratios of exposure vs. pre-exposure) we have obtained some

evidence that egg production was significantly increased in zebrafish after 21 day exposure to 5 and 77 ng/L MIF. It is difficult to explain why no dose-dependent response occurred in egg production, but similar observations have been described (Giesy et al., 2000; Paulos et al., 2010; Skolness et al., 2013). When analyzing the cumulative mean number of eggs per female per day, it is important to compare the curve-slopes of the pre-exposure and exposure period within the same treatment, and not among other treatments, as individual fish produce an individual reproductive baseline. The increase in reproductive performance might be influenced by both the spawning frequency and the number of eggs per spawning and female. The increased egg production observed in the water control could be a sign that the adaptation period prior pre-exposure was not long enough. Moreover, probably higher spawning fish (more reproductive) were chosen in water control tanks by chance, although the selection of adult fish was randomized. However, all results were compared to the solvent control, with equal amounts of DMSO (0.01 %) present in all chemical exposure groups.

Exposure up to 77 ng/L MIF and 25 ng/L P4 did not affect the fertility of laid eggs, which is in accordance to results on P4 in fathead minnows (DeQuattro et al., 2012). Hatchability and survival of zebrafish embryos of the F1 generation (after 96 *hpf*) was not adversely affected at 3 - 26 ng/L MIF and 254 ng/L P4, respectively, similar to the results for P4 in fathead minnows (DeQuattro et al., 2012). Further impacts on zebrafish development of the F1 generation and gender determination were not assessed within this study. However, MIF and P4 alter gene expression during embryonic development (Blüthgen et al., 2013; Zucchi et al., 2012), so effects might become apparent later.

Our data indicate that MIF affects reproduction in fish at low ng/L concentrations. The increased egg production is difficult to explain. MIF acts at different steroid hormone receptors and exhibits multiple hormonal activities in mammals and *in vitro* (Blüthgen et al., 2013; Spitz et al., 1996). In mammals, MIF inhibits the activity of P4 in humans and stimulates uterine contraction, but does not necessarily stop ovulation (Spitz et al., 1996). We hypothesize a similar mechanism of action to account for the increased ovulation in zebrafish. An increase in egg production in fish was reported in fathead minnows after exposure to 16 ng/L norethindrone (Paulos et al., 2010).

Continuous spawning occurred during exposure to 25 ng/L P4, indicating no effect on egg production in zebrafish. The reported decrease in egg production by higher concentrations of P4 in fathead minnows (DeQuattro et al., 2012) is based on nominal concentrations with no comparison of egg production during pre-exposure and exposure. However, egg production during exposure should ideally only be compared to pre-exposure spawning of the same fish. Overall, although we assume that the concentration during our exposure was higher than 25 ng/L P4 (due to the factors discussed in the analytical chemistry section), we conclude that reproduction in zebrafish is not negatively affected at this concentration. Additional experiments, including more replicates should be performed to assess the effects of P4 on reproductive outcomes.

However, the results in this paper are novel and should be considered rather preliminary. Further research on the effects of MIF and P4 on fish reproduction is undoubtedly required. Although the study forming the basis of this and the accompanying paper (Blüthgen et al., 2013) was a significant one, including as it did both biology and chemistry, and involving the assessment of effects at many levels of organization (including apical, cellular, biochemical and molecular), nevertheless these results should not be considered as definite. A definitive study would need to include a higher number of fish in each treatment, more replication, and assessment of egg production (fecundity) for a longer period of time prior pre-exposure to distribute equally spawning fish among all treatments, and thus to ensure that egg production is initially consistent across all treatment groups. In addition, the histopathological assessment should include more fish in a definite study. Such an experiment would have greater statistical power, and hence the chance of detecting a reproducible effect would be increased, and the chances of avoiding false positive results (non-repeatable effects) would be decreased. Based on our results we strongly recommend to conduct such a comprehensive definitive study, which would also help to determine whether or not the apparent non-monotonic concentration-response relationship (the intermediate concentration did not show significant reproductive effects, whereas the lowest concentration did) is repeatable. Non-sigmoidal concentration-response relationships have also been reported for the effects of some synthetic progestogens on fish fecundity (Paulos et al., 2010; Runnalls et al., 2013). It is currently unclear whether these are repeatable observations or if they were a consequence of lack of statistical power due to the

high variability of egg production between individual fish, an argument that also applies to the fecundity data in this paper.

5.5.3. Steroid hormone analysis.

The overall plasma concentrations of 11-KT in males and E2 in females, respectively, did not significantly differ among treatments. Based on the sensitivity limitation of RIA, which is similar to other assays (e.g. ELISA), the small, apparent concentration changes of E2 should not be over-interpreted, as the variability of individual samples is higher for detections close to the LOD. This is in particular true for the water control, which showed an increased E2 concentration in females compared to all other treatments. This observation may also be due to solvent effects (Hutchinson et al., 2006). Nevertheless, we have confidence in the 11-KT level in males and E2 level in females, as steroid concentrations are in a similar range to those previously reported in adult zebrafish (Blüthgen et al., 2014; Baudiffier et al., 2012; Galus et al., 2013). Recognizable changes in steroid level occur only as a response to drastic reproductive effects, e.g. decrease of 11-KT, T and E2 level after fish stopped spawning (Runnalls et al., 2013; Paulos et al., 2010).

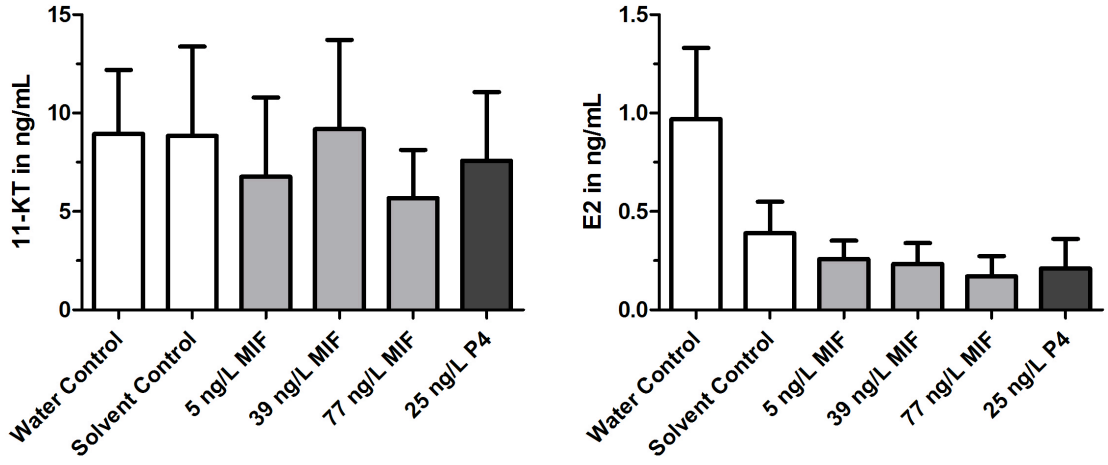

Fig. 5: Concentrations of 11-ketotestosterone (11-KT) (left) and 17β-estradiol (E2) (right) measured in blood plasma of adult zebrafish males and females, respectively, after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4 in ng/mL. Concentrations are given as mean values ± S.D. (*n*=8 samples per treatment).

Fish synthesize a combination of androgens and estrogens, with androgens (mainly 11-KT and testosterone (T)) predominant in males and estrogens (mainly E2) predominant in females. It would have been interesting to determine the ratio of T:E2 and 11-KT:E2, respectively, as physiological changes may have influenced different steroid levels after MIF and P4 exposure. Furthermore, it would have been interesting to measure the concentration of 17α,20β-dihydroxy-4-pregnen-3-one in females and males, but due to the very limited amount of blood plasma of individual zebrafish this was not possible.

5.5.4. Histology.

Females. The significant differences in EV should not be over-interpreted, as the differentiation of EV and LV is not very strict and rather subjective. The sum of pre-vitellogenic (PO and CO) and vitellogenic (EV and LV) ooctyes is more important. Fig. 6 (all oocyte stages) shows an increase of vitellogenic oocytes after 39 - 77 ng/L MIF and 25 ng/L P4 exposure, respectively. Obviously, the maturation of oocytes is stimulated in zebrafish females after exposure to MIF and P4, similarly as found for P4 in another study (Zucchi et al., 2013).

Even if we have not seen a pronounced alteration in different oocyte maturation stages, MIF and P4 caused some histopathological changes in female zebrafish. We found spent post-ovulatory follicles in ovaries of female fish exposed to 39 - 77 ng/L MIF and 25 ng/L P4, respectively, which are an indication of recent spawning and thus oocyte release (U.S. EPA, 2007), and are in accordance with our reproductive data showing an increased egg production. In addition, fish in these treatments spawned continuously, including the day before sampling (day 21), which might explain the decreased GSI.

The atretic follicles we found in ovaries of females exposed to MIF and P4 are rather the result of degradation and resorption of oocytes. Atresia is a degenerative process and can be seen as controlled removal of excess/damaged follicles, and their presence supports the reproductive performance of our study, although stress and environmental insults (e.g. overpopulation, poor nutrition, xenobiotics) may also increase the incidence of atresia (U.S. EPA, 2007). The presence of post-ovulatory and atretic follicles in reproductive tissue of fish are sensitive endpoints for endocrine disruption and have been reported after exposure

of fish to various progestins (Zeilinger et al., 2009; Zucchi et al., 2013) and anti-androgens (Jensen et al., 2004).

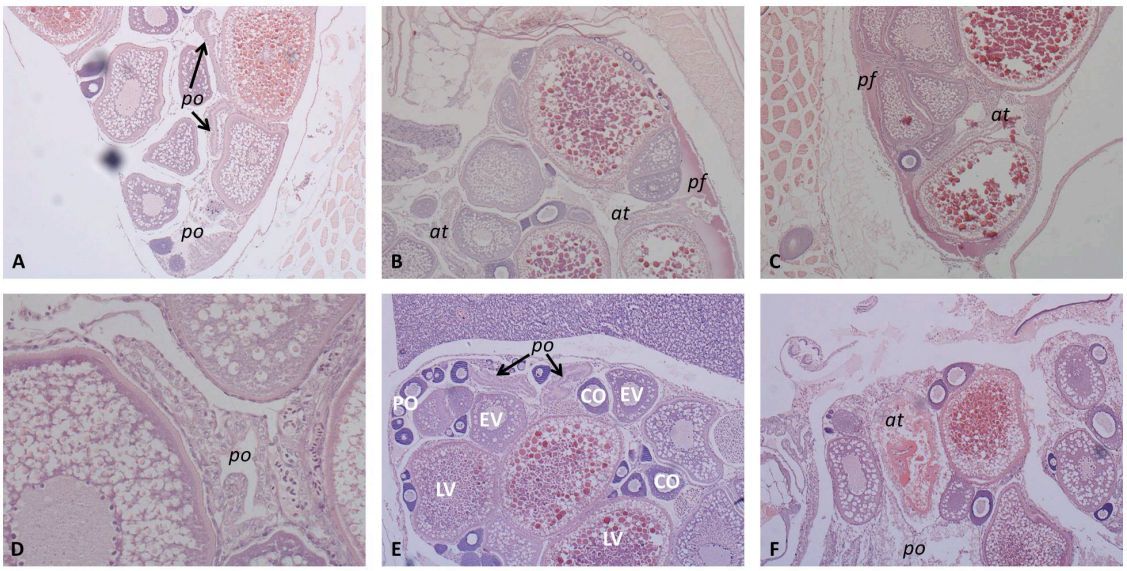

Fig. 8: Histopathological observations found in ovaries of female zebrafish after 21 day exposure to 39 (**A**), 77 ng/L MIF (**B-D**) and 25 ng/L P4 (**E-F**). The observed alterations included post-ovulatory follicles (*po*), atretic follicles (*at*) and proteinaceous fluid (*pf*). Examples of oocyte maturation stages shown in **E** include PO: perinucleolar oocyte, CO: cortical alveolar oocyte, EV: early vitellogenic oocyte, LV: mid-late vitellogenic oocyte. (100x and 400x (**D**) magnification)

The presence of interstitial proteinaceous fluid is usually associated with exposure to estrogenic substances (e.g. 17β-estradiol), especially in male fish. Also ovaries of female Japanese medaka showed this effect after exposure to 4-*tert*-octylphenol (OECD, 2010), as did fathead minnow after exposure to dutasteride (Margiotta-Casaluci et al., 2013), and zebrafish after exposure to P4 (Zucchi et al., 2013). The reason for the presence of proteinaceous fluid in ovaries of females exposed to 5 - 77 ng/L MIF and 25 ng/L P4, respectively, is not understood, but might be an indication of estrogenicity, as it was associated with increased egg production for MIF. In addition, it is difficult to understand the meaning of the presence of proliferated connective tissue and blood vessels observed in females exposed to MIF and P4. But it is likely that the presence of additional connective tissue resulted from the resorption activity of atretic and post-ovulatory follicles, whereas proliferation of blood vessels might be the result of an increased requirement of

late-vitellogenic oocytes to cause the observed increase in egg production. We conclude that the observed histological alterations in adult zebrafish ovaries could be due to a combination of several factors directly or indirectly induced by MIF and P4. Identifying the precise mechanism of action will not be easy.

Males. Few effects on spermatogenesis in reproductive tissue of adult zebrafish males were observed after exposure to 5 - 77 ng/L MIF and 25 ng/L P4. The decrease in proportion of mature spermatocytes at 5 ng/L MIF and 25 ng/L P4 is in contrast to a previous study which reported an increase of mature sperms after exposure to synthetic progestins while fish stopped spawning (Zeilinger et al., 2009). This could be an indication of the antiandrogenic activity of MIF and P4 in combination with observed increased spawning activity, in particular at 5 ng/L MIF. The overall gonad size (area and GSI), however, did not change among all treatments.

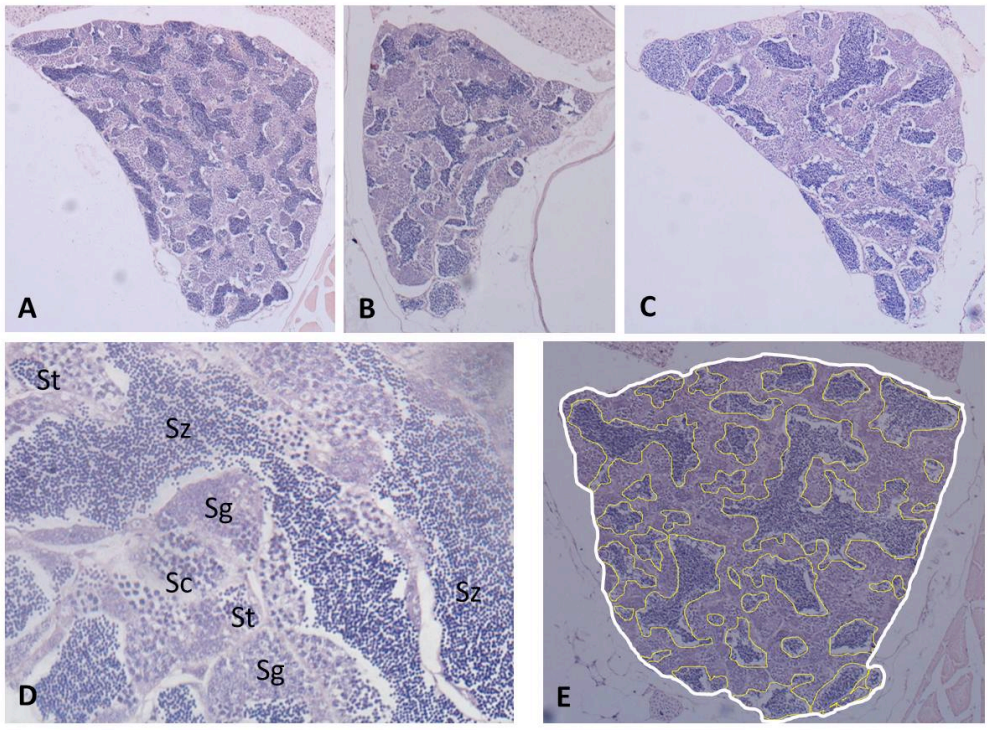

Fig. 10: Typical histological sections of testes of adult zebrafish males exposed for 21 days to solvent control (**A**) 5 (**B**, **D**), 77 (**C**) ng/L MIF and 25 ng/L P4 (**E**). Individual testes were evaluated by calculating the relative percentage of immature (spermatogonia and spermatocytes) and mature (spermatids and spermatozoa) spermatocytes. All testes were in mid-spermatogenic stage (stage 2), meaning that spermatocytes, spermatids and spermatozoa were present in roughly equal proportions. **A-C**, **E**: 100x magnification, **D**: 400x magnification, whereas **D** shows individual spermatocytes (Sg: spermatogonia, Sc: spermatocytes, St: spermatids, Sz: spermatozoa) and **E** the areas considered as mature spermatocytes within the total area of one testis.

5.6. Conclusions

Here we provide first insights into the reproductive effects of the antiprogestin MIF, demonstrating an increase in egg production after exposure to 5 and 77 ng/L MIF. Egg production was not altered after exposure to 25 ng/L P4. The increased reproductive performance of fish exposed to MIF was not reflected in levels of sex steroid hormones in blood plasma. However, the increase of mature vitellogenic oocytes in ovaries indicated acceleration of oocyte maturation by MIF and P4, including the occurrence of post-ovulatory and atretic follicles and proteinaceous fluid. Fertility of eggs and hatching success of further exposed embryos (F1 generation) was not affected by MIF and P4. Further evidence of the effects of MIF at environmentally realistic concentrations on gene expression is given in the associated report (Blüthgen et al. 2013). We conclude that our data should be regarded rather preliminary. Additional experiments should be performed including more biological replicates to increase the statistical power and thus to confirm and further substantiate the here reported reproductive outcomes of MIF and P4 in fish.

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Supporting Data

In Appendix A4 details of the analytes detection method and detailed results of analytical chemistry (validation, QC samples, stability test, individual analytes concentrations in exposure water) are provided. Tables and figures showing detailed results of biological parameter and reproductive performance (ratio exposure vs. pre-exposure, eggs/female/week, eggs/female/day, spawning frequency, embryo survival) are given. Figures showing detailed results of histological analysis (relative percentages of spermatocytes and oocytes in males and females, respectively, and total number of oocytes in females) are provided in Appendix A4.

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Chapter 6

Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 2. Gene expression analysis and *in vitro* activity

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6.1 Abstract

Here, we analyzed the transcriptional effects of the antiprogestin mifepristone (MIF, RU486) and progesterone (P4) in zebrafish as well as their *in vitro* activities in yeast-based reporter gene assays. This study is associated with the reproduction study in adult zebrafish and embryos exposed for 21 days to 5, 39, 77 ng/L MIF, and 25 ng/L P4 (Blüthgen et al., 2013). The *in vitro* activities of MIF and P4 were investigated using a series of recombinant yeast-based assays (YES, YAS, YPS) and compared to transcriptional alterations obtained in fish tissues and embryos from the exposure study. MIF elicited antiestrogenic, androgenic and progestogenic activities in recombinant yeast, similar to P4, and no antiprogestogenic activity *in vitro*. The transcriptional alterations of steroid hormone receptors were similar in adult males and females, and more pronounced in embryos. MIF tended to down-regulate the androgen (*ar*), progesterone (*pgr*) and glucocorticoid (*gr*) receptors in adult fish and embryos. Transcripts of the estrogen receptor (*esr1*) and vitellogenin (*vtg1*) were not significantly altered. A trend for down-regulation was observed for transcripts of genes belonging to steroidogenic enzymes including 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), P450 aromatase A (*cyp19a*) and 11β-hydroxylase (*cyp11b*). P4 resulted in similar transcriptional alterations as MIF. The data indicate that gene expression changes and *in vitro* activities match only in part including the lack of antiprogestogenic activity of MIF. Additionally, effects on reproduction and gonad histology described in the associated report (Blüthgen et al., 2013) can only partly be explained by gene expression data presented here.

Keywords: mifepristone, RU486, progesterone, antiprogestin, *in vitro* hormonal activity, recombinant yeast system, gene expression

6.2. Introduction

Mifepristone (MIF) (commercial name: Mifeprex™, RU486, Mifegyne™) is a synthetic antiprogestin used as an effective non-invasive method for medical termination of pregnancy (Spitz et al., 1996). MIF acts by a competitive interaction with the natural progesterone (P4) at the progesterone receptor (PR). MIF exhibits mixed agonistic and antagonistic activity to the PR *in vitro* and *in vivo*. Its activity as antiprogestin is strongly dependent on the presence of other steroid hormones (e.g. P4 and 17β-estradiol (E2)), the phase of the menstrual cycle and the predominant isoform of the PR (PRα, PRβ) (Spitz et al., 1996; Spitz, 2003). Furthermore, MIF exerts some antiestrogenic and weak antiandrogenic activity in mammals (rats), and it is well known as antiglucocorticoid (at high doses) (Spitz et al., 1996; Spitz, 2003).

MIF was detected in wastewater effluents at concentrations of 195 ng/L (Liu et al., 2011), and at similar concentrations in hospital effluents (Liu et al., 2010). Surface water concentrations have not yet been reported thus far, but expected in the lower ng/L-range. Environmental concentrations of P4 were reported in a similar range; 0.3-1.4 ng/L in rivers in Spain (Kuster et al., 2008) and 14-27 ng/L in freshwater rivers in the U.S.A. (Pal et al., 2010).

Recombinant yeast-based receptor assays can help to identify mechanism of action and are used to investigate hormonal activities of substances *in vitro*. These assays represent useful tools to detect agonistic and antagonistic hormonal activities of substances interacting with steroid receptors. These assays are specific for the receptor of interest (estrogen, androgen, progesterone receptor), very sensitive, reliable and robust (Sohoni and Sumpter, 1998). Some synthetic progestins were recently investigated in yeast screens showing strong androgenic and progestogenic activities, including levonorgestrel (LNG), norethindrone (NET), drospirenone (DRSP) and gestodene (Runnalls et al., 2013). Weak progestogenic activities of MIF have been reported by Chatterjee et al. (2008).

Transcriptomics (e.g. RT-qPCR and microarrays) are a useful technology to elucidate modes of action of substances *in vivo* (Fent and Sumpter, 2011). A recent study has demonstrated effects on gene expression in zebrafish embryos after exposure to progestins and MIF, indicating antiprogestogenic and androgenic activities of MIF, and androgenic and estrogenic activities of P4 (Zucchi et al., 2012). Antiandrogenic activity has also been reported in the

three-spined stickleback after exposure to low concentrations (ng/L) of LNG (Svensson et al., 2013).

In the present study, we investigated the *in vitro* activities of MIF and P4 in three different recombinant yeast systems to elucidate the agonistic and antagonistic interaction with human estrogen (ER), androgen (AR) and progesterone (PR) receptors. We then compared the *in vitro* activity with the *in vivo* activity in adult zebrafish and embryos on the transcriptional level to elucidate the molecular effects behind the effects on reproduction and gonad histology reported in the associated report (Blüthgen et al., 2013).

6.3. Materials and Methods

6.3.1. Chemicals. Chemicals used for *in vitro* assays, mifepristone (MIF, RU486, 11β-(4-dimethyl-amino)-phenyl-17β-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one, CAS no: 84371-65-3, purity ≥ 98 %), progesterone (P4, 4-pregnene-3,20-dione, CAS no: 57-83-0, purity ≥ 99 %), 17β-estradiol (E2, 3,17β-dihydroxy-1,3,5(10)-estratriene, CAS no: 50-28-2, purity ≥ 98 %), dihydrotestosterone (DHT, CAS no: 521-18-6), flutamide (FLU, 2-methyl-N-(4-nitro-3-[trifluoromethyl]phenyl)propanamide, CAS no: 13311-84-7), hydroxytamoxifen (OH-tamoxifen, (Z)-4-(1-[4-(dimethylaminoethoxy)phenyl]-2-phenyl-1-butenyl)phenol, CAS no: 68047-06-3, purity ≥ 98 %) and 17α,20β-dihydroxyprogesterone (DHP, 17α,20β-dihydroxy-4-pregnen-3-one, CAS no: 1662-06-2) and ethanol were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). The Kits used for RNA extraction (RNe*asy* Mini Kit 74104 and RN*ase*-Free DN*ase* Set 79254) and enzymes for cDNA synthesis (M-MLV Reverse Transcriptase (M1705)) were purchased from Qiagen (Basel, Switzerland) and Promega (Dübendorf, Switzerland), respectively.

6.3.2. *In vitro* assays. MIF and P4 were assayed for their hormonal activities using a series of *in vitro* screens. The estrogenic and antiestrogenic activity was assessed in the YES assay (Routledge and Sumpter, 1996), the androgenic and antiandrogenic activity in the YAS assay (Sohoni and Sumpter, 1998), and progestogenic and antiprogestogenic activity in the YPS assay (Gaido et al., 1997; Lorenzen et al., 2004). MIF and P4 were included in each of the

screens, and in addition in the YPS, also 17α,20β-dihydroxyprogesterone (DHP) was assessed as major natural progestin in fish. Estradiol (E2), dihydrotestosterone (DHT), flutamide (FLU), hydroxytamoxifen (OH-tamoxifen) and P4 were used as standards for the appropriate assays. Previously reported recombinant hER, hAR and hPR yeast strains were used (Runnalls et al., 2013). Details of the recombinant yeast-based assays (YES, YAS, YPS) were described previously and the assays were carried out according to their protocols (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998; Gaido et al., 1997; Lorenzen et al., 2004). Agonistic activities of chemicals used as standards were assessed first to determine the concentration used to assess the antagonistic activities.

Serial dilution series of all chemicals were prepared and chemical concentrations used in the agonistic/antagonistic YES, YAS and YPS assays are summarized in Table 1. The following agonist concentrations were added to the medium to produce a sub-maximal response (65 %) in the appropriate antagonist screens: 4 x 10^{-10} M E2 (YES antagonist screen), 2 x 10^{-9} M DHT (YAS antagonist screen) and 1 x 10^{-8} – 8 x 10^{-9} M of P4 (YPS antagonist screen).

Table 1: Molar concentrations of chemicals tested in the following *in vitro* screens: yeast estrogen screen (YES), yeast androgen screen (YAS) and yeast progesterone screen (YPS). All screens were assessed for agonistic and antagonistic activities of the chemicals.

	Chemical concentrations for *in vitro* screens [M]					
	YES		**YAS**		**YPS**	
	Agonist	**Antagonist**	**Agonist**	**Antagonist**	**Agonist**	**Antagonist**
MIF	5.0E-04 - 2.4E-07	5.0E-04 - 2.4E-07	5.0E-05 - 2.4E-08	5.0E-05 - 2.4E-08	5.0E-04 - 2.4E-07	5.0E-04 - 2.4E-09
P4	2.5E-07 - 1.2E-10	2.5E-05 - 1.2E-08	2.5E-07 - 1.2E-10	2.5E-05 - 1.2E-08	2.5E-07 - 1.2E-10	-
DHP	-	-	-	-	2.5E-04 - 1.2E-07	-
E2	1.0E-08 - 4.9E-12	-	-	-	-	-
OH-tamoxifen	-	2.5E-05 - 1.2E-08	-	-	-	-
DHT	-	-	5.0E-08 - 2.4E-11	-	-	-
FLU	-	-	-	5.0E-05 - 2.4E-08	-	-

Abbreviations: MIF: mifepristone, P4: progesterone, DHP: 17α,20β-dihydroxyprogesterone, E2: estradiol, OH-tamoxifen: hydroxytamoxifen, DHT: dihydrotestosterone, FLU: flutamide.

The assay development was followed by reading absorbance at 540 and 620 nm (YES and YAS) and at 420 and 600 nm (YPS), respectively. All chemicals were tested in triplicate and

each experiment was carried out two times. Solvent controls (ethanol) and blanks (medium only) were included on each 96-well plate.

6.3.3. *In vivo* exposure. The following results were obtained in the associated report (Blüthgen et al., 2013) after 21 day exposure of adult zebrafish breeding groups (males and females) to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4, with four replicate tanks each. Originating embryos (F1 generation) were further exposed to water control, solvent control (0.01 % DMSO), 3, 15, 26 ng/L MIF and 254 ng/L P4 for 120 h post fertilization (*hpf*). Adult tissues of brain, liver, gonads were collected and pooled independently (*n*=4 samples per tissue per gender and per treatment), and embryos were pooled and collected (*n*=4 samples per treatment) at the end of exposure and stored in RNA*later* at -80 °C for subsequent gene expression analysis.

6.3.4. Gene expression analyses.

RNA extraction. Brain, liver and gonad (testes and ovary, respectively) tissues of three females and three males per replicate tank were pooled independently. Embryos were pooled per egg-cup (*n*=25-30 embryos per egg-cup). Total RNA was extracted from adult tissue pools (*n*=4 pooled samples per gender/tissue/treatment) and embryo pools (*n*=4 pooled samples per treatment) using the RN*easy* Mini Kit (Qiagen, Basel, Switzerland). RNA was purified and DN*ase* and divalent cations were removed by further treated with RN*ase*-Free DN*ase* (Qiagen, Basel, Switzerland). RNA concentration and quality was analyzed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington DE, USA) by measuring the absorbance at 260 and 280 nm. Only RNA samples with purity between 1.8 and 2.0 for both ratios 260/280 nm and 260/230 nm were subjected to RT-qPCR analyses. In addition, the purity of RNA samples was assessed by electrophoresis (rRNA intensity ratio for 28S/18S about 2:1) (Taylor et al., 2010). RNA samples were stored at -80 °C for subsequent RT-qPCR analysis.

RT-qPCR analysis. The isolated total RNA of pooled adult brain, liver, testes and ovary samples as well as from pooled embryos was subjected to RT-qPCR analysis. In total, the

number of samples were *n*=4 replicates per tissue/gender/treatment for adult tissues (after 21 days exposure) and *n*=4 replicates per treatment for embryos (after 120 *hpf*). Total RNA (1 µg) was reverse-transcribed using the cDNA Synthesis Kit (Promega, Dübendorf, Switzerland). The total volume of 14 µL (RNA + RN*ase*-free water) was incubated with 1 µL of random hexamers (Roche, Switzerland) at 70 °C for 5 min to melt secondary structures within template. A volume of 10 µL of a master-mix solution, containing 5 µL M-MLV 5x- reaction buffer, 1.25 µL dNTPs (Sigma-Aldrich, Switzerland), 0.4 µL M-MLV reverse transcriptase and 3.35 µL RN*ase*-free water, were added to each sample. The complete reaction mixture was incubated at 37 °C for 60 min, following 5 min at 95 °C to stop the reaction. The cDNA was stored at -20 °C.

Finally, 50 ng of cDNA per reaction were used for RT-qPCR based on SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland). The following two-step real-time PCR profile was used: enzyme activation step at 95 °C (10 min) and 40 cycles of 95 °C (30 s), 57.5-59 °C (60 s) depending on the target transcript as shown in Table 2, followed by a melting curve analysis post run (65-95 °C) which confirmed specificity of chosen primers as well as absence of primer dimers.

For RT-qPCR gene-specific primers from published zebrafish primer sequences (Table 2) were obtained from Microsynth (Basel, Switzerland) and analyzed specifically in the following tissues. The glucocorticoid receptor (*gr*), progesterone receptor (*pgr*), estrogen receptor alpha (*esr1*) and androgen receptor (*ar*) were investigated in adult brain, vitellogenin 1 (*vtg1*) in adult liver, *gr, pgr*, P450 aromatase A (*cyp19a*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*) and 11β-hydroxylase (*cyp11b*) in adult testes and ovaries. A similar targeted gene expression approach was used in embryos. The ribosomal protein L13a (*RpL13a*) was selected as housekeeping gene for normalization due to stable expression among treatments and tissues. PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled embryo cDNA (R^2 > 0.98 for all primers). A no template control reaction was included in every run for each primer pair to test for potential contamination and to assess the presence of primer-dimers (Taylor et al., 2010).

Table 2: Primer sequences for quantitative RT-PCR analysis, sequence sources and annealing temperature. The glucocorticoid receptor (*gr*), progesterone receptor (*pgr*), estrogen receptor alpha (*esr1*) and androgen receptor (*ar*) were investigated in adult brain, vitellogenin 1 (*vtg1*) in adult liver, *gr*, *pgr*, P450 aromatase A (*cyp19a*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*) and 11β-hydroxylase (*cyp11b*) in adult testes and ovary. All target genes were analyzed in embryos. The ribosomal protein L13a (*RpL13a*) was selected as housekeeping gene for normalization due to stable expression among treatments and tissues.

Target gene	GeneBank number	Sense primer (5' - 3')	Antisense primer (5' - 3')	Product size (bp)	Annealing temperature [°C]
gr [a]	EF567112	ACAGCTTCTTCCAGCCTCAG	CCGGTGTTCTCCTGTTTGAT	116	59
pgr [b]	NM_001166335	GGGCCACTCATGTCTCGTCTA	TCTCCACTCTGAAAATATGTGGACTTT	96	59
vtg1 [c]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94	57.5
esr1 [d]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163	59
ar [e]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237	59
cyp19a [f]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92	58.5
hsd3b [g]	AY279108	GCAACTCTGGTTTTCCACACTG	CAGCAGGAGCCGTGTAGCTT	102	59
hsd17b3 [g]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121	57.5
cyp11b [a,h]	NM_001080204	GCTCATGCACATTCTGAGGA	TGTGCTGAAGGTGATTCTCG	115	59
RpL13a [i]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100	57.5 - 59

Data sources: [a] (Alsop et al., 2008), [b] (Chen et al., 2010), [c] (Hoffmann et al., 2006), [d] (Martyniuk et al., 2007), [e] (Hossain et al., 2008), [f] (Arukwe et al., 2008), [g] (Hoffmann et al., 2008), [h] (Fuzzen et al., 2011), [i] (Oggier et al., 2010).

All reactions were run in duplicate using the Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland). Normalization and calculation of expression levels was performed as previously (Blüthgen et al., 2012). The mRNA expression levels of different target genes are expressed as fold change (log2) according to the formula: $2^{-(\Delta Ct(\text{treated sample})-\Delta Ct(\text{untreated sample}))}$ and referred to solvent control.

6.3.5. Data analysis and statistics. Data were graphically illustrated and statistically analyzed by GraphPad Prism 5 (GraphPad Software, San Diego, USA). Significant differences in transcript levels (RT-qPCR) were analyzed by one way analysis of variance (1-way ANOVA) followed by a Tukey post-hoc test (95 % confidence interval). Results are shown as mean ± standard deviation (S.D.). Significant differences were considered when $p \leq 0.05$.

6.4. Results

6.4.1. *In vitro* assays.

The yeast screen results obtained from two independent experiments for each assay are presented as mean values (*n*=6 replicates per chemical and assay). Neither MIF nor P4 showed estrogenic activity in the YES (data not shown), but antagonistic activity (Fig. 1). MIF was a partial hER-antagonist and about 100-times less potent than OH-tamoxifen. P4 showed full antagonistic activity, with 10-times higher potency than MIF and 10-times lower potency than OH-tamoxifen. MIF and P4 showed full agonistic dose-response curves in the YAS (Fig. 1). MIF was a weak hAR-agonist, being about 1000-times less potent than DHT, in contrast to P4, which was about 10-times less potent than DHT. Neither MIF nor P4 showed antagonistic activity to the hAR, but rather additive agonistic activity (Fig. 1). MIF showed sub-maximal agonistic activity in the YPS and was about 1000-times less potent than P4 (Fig. 1). DHP showed full agonistic activity with similar potency as MIF. No antagonistic activity of MIF or P4 was observed (data not shown).

6.4.2. Gene expression analysis.

Selected target genes were investigated in brain, liver and gonads of exposed adult fish and compared to expression levels in exposed embryos of the F1 generation. No difference occurred between water and solvent control for all investigated transcripts and thus mRNA alterations were referred to the solvent control.

Fig. 2 depicts the transcripts of *pgr*, *gr*, *ar* and *esr1*. They indicate a slight tendency for down-regulation, similarly in males and females after exposure to 5 - 77 ng/L MIF and 25 ng/L P4. The transcripts of the *ar* were down-regulated similarly in the brain of males and females, being significant in females at 39 ng/L MIF (log2=-0.8) and 25 ng/L P4 (log2=-0.8), respectively. Transcripts of *esr1* in males were not significantly altered by MIF and P4 and were not completely investigated in females due to low expression levels. Fig. 3 shows that MIF and P4 did not significantly alter the expression of *vtg1* in the liver of adult females and males.

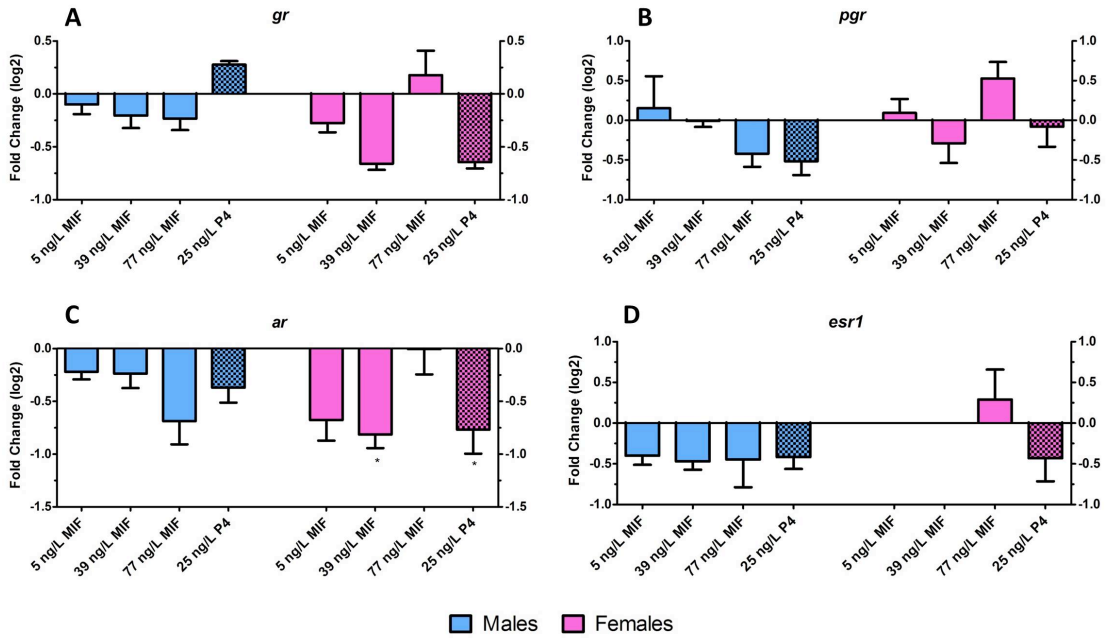

Fig. 2: Relative gene expression in brain of adult zebrafish males and females after 21 day exposure to 5, 39, 77 ng/L MIF and 25 ng/L P4. All expression levels are referred to solvent control (0.01 % DMSO) and are expressed as fold change log2 using the $2^{-\Delta\Delta Ct}$ method. Transcripts of the following target genes are shown: **A:** glucocorticoid receptor (*gr*), **B:** progesterone receptor (*pgr*), **C:** androgen receptor (*ar*), **D:** estrogen receptor (*esr1*). Results are given as mean ± S.D. (*n*=4 per treatment and tissue and gender). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$).

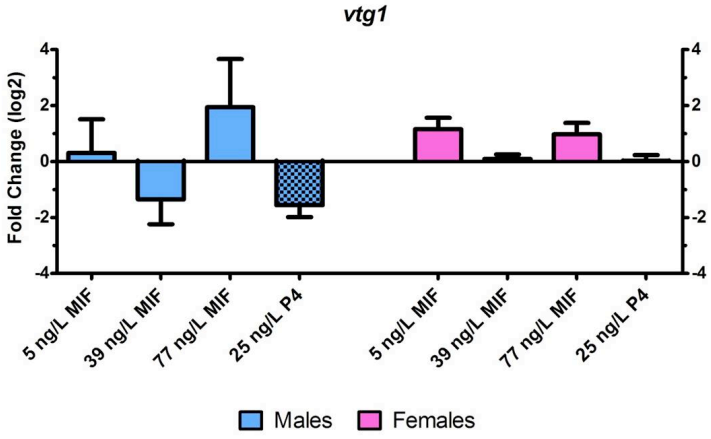

Fig. 3: Relative gene expression of vitellogenin (*vtg1*) in liver of adult zebrafish males and females after 21 day exposure to 5, 39, 77 ng/L MIF and 25 ng/L P4. Expression levels are referred to solvent control (0.01 % DMSO) and are expressed as fold change log2 using the $2^{-\Delta\Delta Ct}$ method. Results are given as mean ± S.D. (*n*=4 per treatment and tissue and gender).

An overall down-regulation of transcripts involved in steroidogenesis was observed by MIF and P4 in testes and ovaries, although significantly only in females (Fig. 4). A tendency for down-regulation of the *gr* was observed in gonads similarly as in the brain. Transcripts of the *pgr* were significantly down-regulated at 39 ng/L MIF (log2=-0.9) and 25 ng/L P4 (log2=-1.0) in females. The aromatase *cyp19a* transcript as well as the *hsd17b3* were significantly down-regulated in females at 39 ng/L MIF (log2=-1.7 and log2=-1.2, respectively). The transcripts of *hsd3b* were down-regulated in all treatments, albeit not significantly. P4 also led to down-regulation of these transcripts, being significant for *hsd17b3* (log2=-1.2) in females. Transcripts of *cyp11b* also showed a down-regulation in males and females, being significant in females (up to log2=1.3 for MIF and log2=-1.5 for P4).

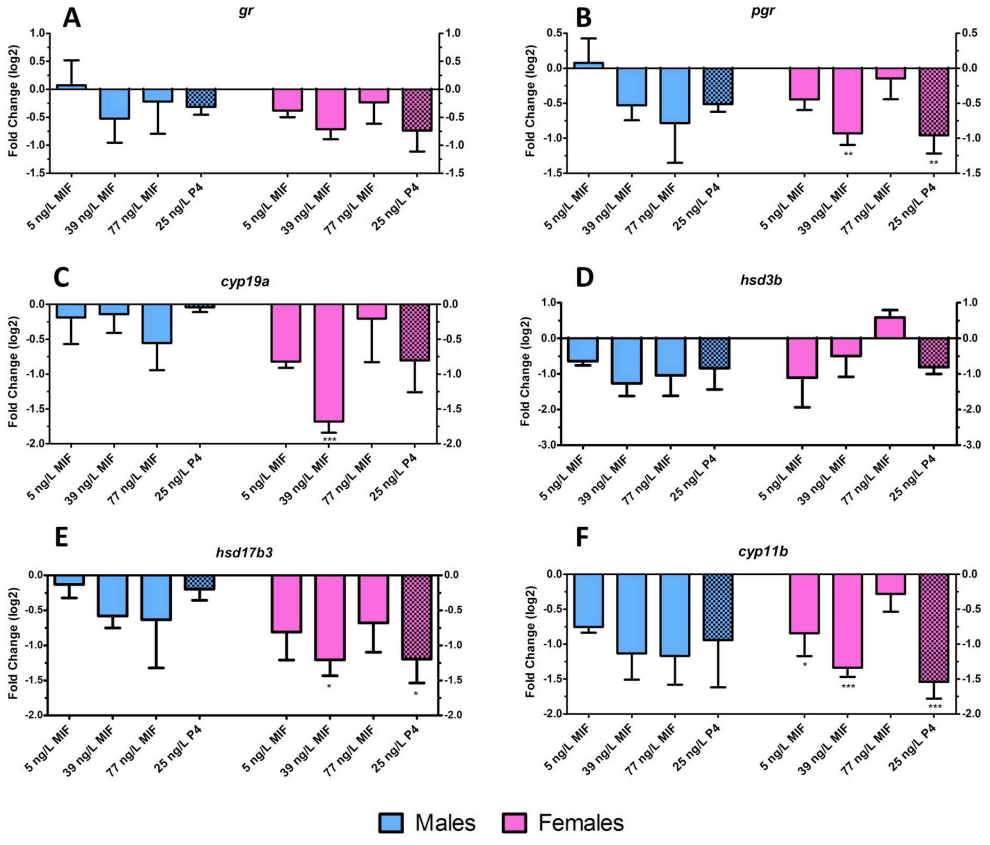

Fig. 4: Relative gene expression in gonads of adult zebrafish males (testes) and females (ovaries) after 21 day exposure to 5, 39, 77 ng/L MIF and 25 ng/L P4. All expression levels are referred to solvent control (0.01 % DMSO) and are expressed as fold change log2 using the $2^{-\Delta\Delta Ct}$ method. Transcripts of the following target genes are shown: **A:** glucocorticoid receptor (*gr*), **B:** progesterone receptor (*pgr*), **C:** P450 aromatase A (*cyp19a*), **D:** 3β-hydroxysteroid dehydrogenase (*hsd3b*), **E:** 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), **F:** 11β-hydroxylase (*cyp11b*). Results are given as mean ± S.D. (*n*=4 per treatment and tissue and gender). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

Similar targeted transcripts were investigated in embryos (F1 generation) and are depicted in Fig. 5. A significant down-regulation of the *gr* was observed after exposure to 15 and 26 ng/L MIF (up to log2=-0.9), and 254 ng/L P4 (log2=-1.1), respectively, similarly to transcripts of *pgr* (up to log2=-0.9) at 15 ng/L MIF and 254 ng/L P4. Both transcripts showed a stronger responsiveness than in adult brain and gonads. Transcripts of *esr1* and *vtg1* were not significantly altered, except at 3 ng/L MIF with a significant up-regulation (log2=1.5). While *cyp19a* and *hsd3b* showed no significant transcriptional alterations (similar as in adults), the *hsd17b3* transcripts were significantly induced at 3 ng/L MIF only (log2=0.9). Similar as in adult gonads, *cyp11b* was down-regulated, being significant at 15 ng/L MIF and 254 ng/L P4.

6.5. Discussion

6.5.1. *In vitro* assays.

In the YES, MIF and P4 showed exclusively antiestrogenic activity *in vitro* with 10 - 100 times less potency to the estrogen antagonist OH-tamoxifen. Neither MIF nor P4 showed estrogenic activity *in vitro*, similar to other synthetic progestins (Runnalls et al., 2013). This is reflected by the gene expression analysis, where none of the genes related to estrogenic activity (*esr1*, *vtg1*) were induced. MIF and P4 showed activation of the hAR *in vitro*, whereby MIF is indicated to be weak AR agonist. This androgenic activity was not reflected in our *in vivo* gene expression data, where we found rather a down-regulation of the *ar* transcript and transcripts involved in androgen synthesis. P4 shows a similar potency to the hAR as other synthetic progestins (gestodene and norethindrone) but is less potent than levonorgestrel (Runnalls et al., 2013). MIF and P4 did not show any antagonistic activity, they rather enhanced the androgenic activity, similar to other compounds (Christen et al., 2010).

P4 and DHP activated the hPR in the YPS assay as other synthetic progestins although at lower rate (Runnalls et al., 2013). That MIF showed partially progestogenic activity was reported before in mammals (Spitz et al., 1996; Spitz, 2003). The binding affinity of DHP, the main endogenous progestin in fish, to the hPR is much lower compared to P4, which was expected, as each recombinant receptor shows highest affinity for the appropriate

physiological important hormone in the species (Thomas, 2012). The similar binding affinity of DHP and MIF suggests that both compounds may compete similarly with the zebrafish PR.

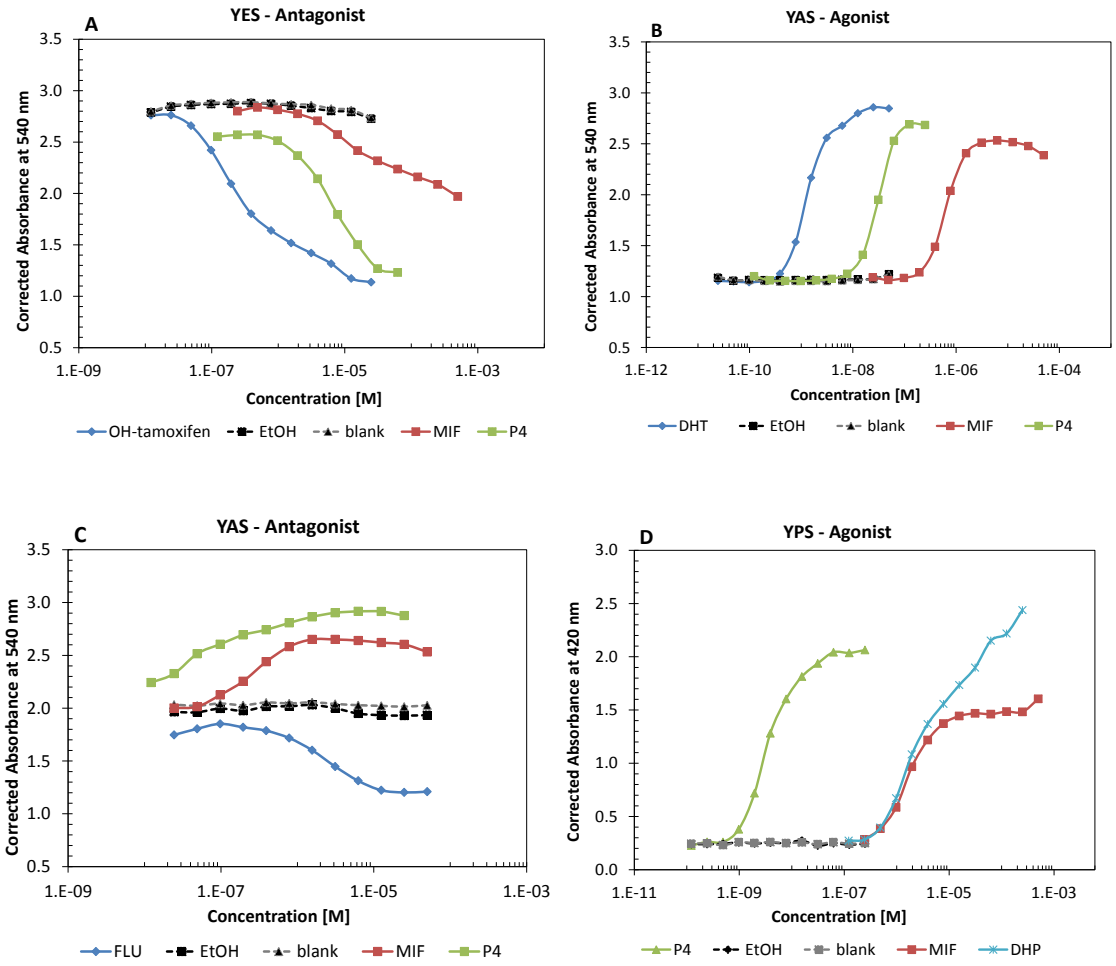

Fig. 1: *In vitro* activities of MIF, P4 and DHP (E2, DHT, FLU, OH-tamoxifen used as standards) were assessed in recombinant yeast estrogen (YES), androgen (YAS) and progestogenic (YPS) assays. **A:** Antagonistic YES assay in the presence of 4 x 10^{-10} M E2; **B:** Agonistic YAS assay; **C:** Antagonistic YAS assay in the presence of 2 x 10^{-9} M DHT; **D:** Agonistic YPS assay. Each assay was carried out in two independent experiments including three replicates each. Solvent controls (ethanol) and blanks (medium only) were included on each 96-well plate. Data are represented as mean values (*n*=6 replicates per chemical).

Surprisingly, we were not able to show the antiprogestogenic activity of MIF *in vitro*, in contrast to gene expression data. The absent antiprogestogenic activity *in vitro* may probably be based on the recombinant yeast system that lacks specific coactivators and/or corepressors that are essential for the function of human PR. In HEK-293 cells, MIF showed weak agonist and potent antagonist effects on hPRβ (data not shown). Nevertheless, partial

antagonism (about 50 % response) of MIF to hPR was reported in yeast (Chatterjee et al., 2008). Another reason could be that MIF alone does not exert antiprogestogenic activity in zebrafish and that it requires rather a combination of (natural and synthetic) steroid hormones, similarly as shown in mammals (Spitz et al., 1996; Spitz, 2003). This would also explain why MIF led to down-regulation of *pgr* transcripts in the brain and gonads of adult fish and embryos, but not *in vitro*.

6.5.2. Gene expression analysis and link to reproductive data.

In general, the regulation of investigated target genes was similar in adult males and females and embryos (F1 generation). The data showed reduced mRNA receptor expression (*gr*, *pgr*, *ar*) after exposure to MIF and P4, with embryos being more sensitive. Zucchi et al. (2012) investigated transcriptional alterations of these receptors in zebrafish embryos, and did not find any alterations of the *gr*, *pgr* and *ar* after exposure to 104 ng/L P4 for 144 *hpf*, whereas *ar* and *pgr* were induced after 96 *hpf*, indicating also the variable responsiveness at different developmental stages and exposure times (Blüthgen et al., 2014).

In contrast to the investigated receptors, the F1 generation (embryos) was less sensitive to transcript alterations of steroidogenic enzymes, probably based on the fact that embryos (after 120 *hpf*) have not reached yet the reproductive cycle, although steroidogenesis in embryonic development is not yet fully elucidated (Tokarz et al., 2013).

The down-regulation of the *gr* reflects the antiglucocorticoid activity of MIF in mammals (Spitz et al., 1996). The response of the *pgr* is much stronger in gonads than in the brain and thus supports that the reproductive tissues (testes and ovaries) are the main targets for the action of MIF and P4 on the *pgr* (Thomas, 2012). The down-regulation of the *pgr* after progestin P4 exposure was surprising, but it might be explained by the reproductive results of these fish. The P4 treated fish were continuously spawning (even the day before sampling), and we observed many post-ovulatory follicles in the ovaries, which are representative for spent oocytes (ovulation) (Blüthgen et al., 2013). It might be reasonable to combine these observations with the decreased *pgr* receptor levels in ovulated oocytes (Thomas, 2012). The observed inhibition of the *ar* in adult brain and embryos indicated a similar antiandrogenic activity in both developmental stages.

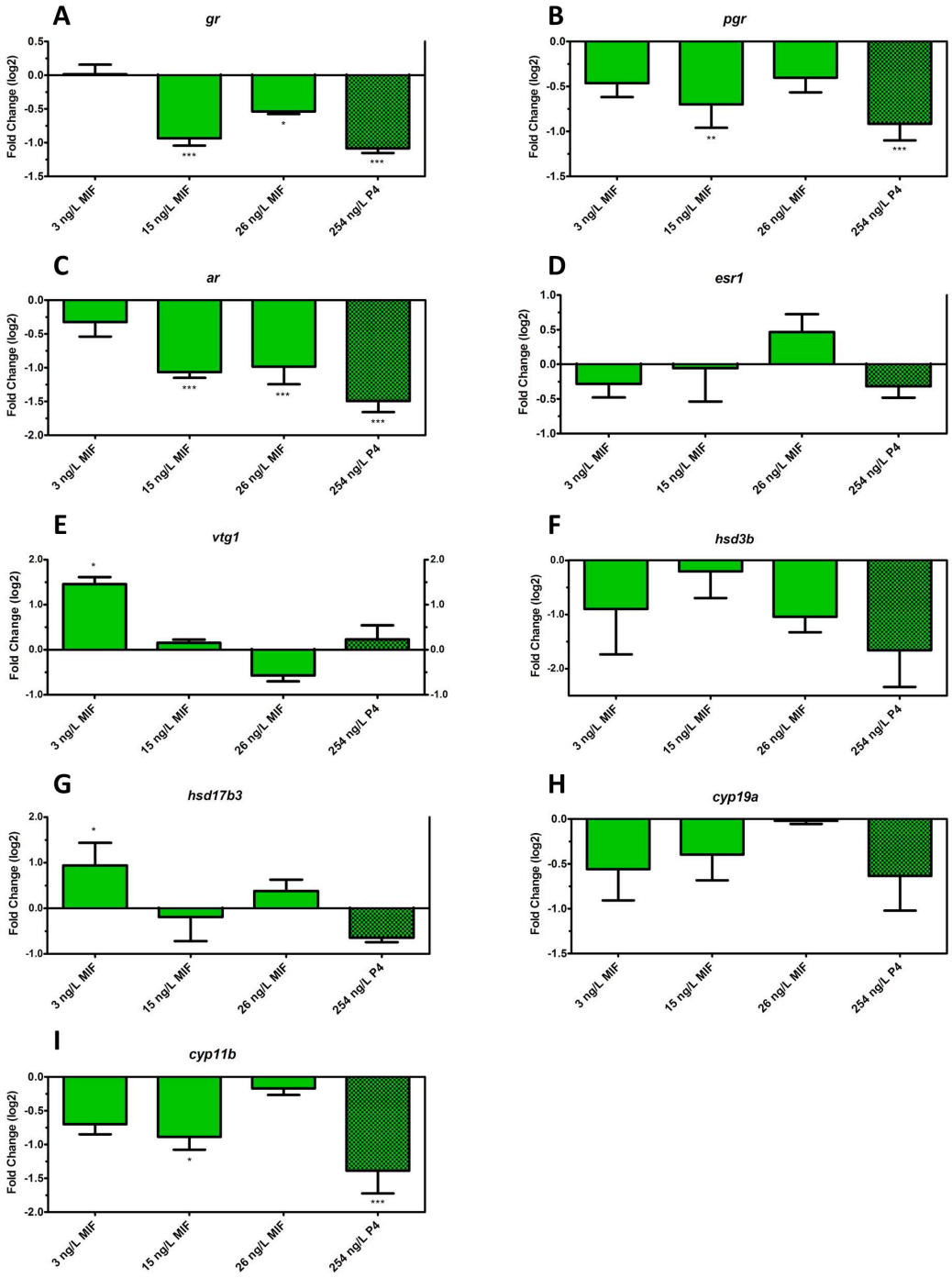

Fig. 5: Relative gene expression in embryos (F1 generation) after 120 *hpf* exposure to 3, 15, 26 ng/L MIF and 254 ng/L P4. All expression levels are referred to solvent control (0.01 % DMSO) and are expressed as fold change log2 using the $2^{-\Delta\Delta Ct}$ method. Transcripts of the following target genes are shown: **A:** glucocorticoid receptor (*gr*), **B:** progesterone receptor (*pgr*), **C:** androgen receptor (*ar*), **D:** estrogen receptor (*esr1*), **E:** vitellogenin (*vtg1*), **F:** 3β-hydroxysteroid dehydrogenase (*hsd3b*), **G:** 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*), **H:** P450 aromatase A (*cyp19a*), **I:** 11β-hydroxylase (*cyp11b*). Results are given as mean ± S.D. (*n*=4 per treatment). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

Transcripts of steroidogenic enzymes tended to be down-regulated; *hsd3b*, which is important in the formation of the precursor sex steroids; *hsd17b3*, which is involved in production of main androgens (testosterone (T), 11-ketotestosterone (11-KT)) and estrogens (estradiol (E2)) and *cyp19a*, which is mainly responsible for the estrogen formation. These transcription data suggest that MIF and P4 possessed an antiandrogenic and antiestrogenic activity *in vivo* in adult zebrafish and embryos, similar to mammals (Breton et al., 2012). The inhibition of *cyp19a* in females at 39 ng/L MIF came along with no increase in egg production, compared to other treatments (Blüthgen et al., 2013).

In our associated study we found an increased egg production after exposure to 5 - 77 ng/L MIF (Blüthgen et al., 2013). We hypothesized that the overall tendency for inhibition of steroidogenic enzymes might be a feedback response on the molecular level to the observed "excess" presence of sex steroid hormones in terms of increased egg production, considering that sex steroids are synthesized in perifollicular cells and incorporated in ovulated eggs. However, we did not find an increase in sex steroid hormones (11-KT in males and E2 in females) measured in blood plasma of the same fish used for gene expression analysis (Blüthgen et al., 2013). Another hypothesis could be that the overall observed inhibition of steroidogenic enzymes is reflected in the presence of degenerative processes (atretic and post-ovulatory follicles) observed in ovaries of females exposed to MIF and P4 (Blüthgen et al., 2013). Overall, it is difficult to explain how the observed effects on gene regulation are related to the observed physiological and reproductive responses in adult fish considering that we investigated only at one time point and expression patterns differ in time (Blüthgen et al., 2014). In addition, as mentioned in the associated report (Blüthgen et al., 2013), the statistical power of the *in vivo* study is relatively low, as a consequence of having too low numbers of fish per treatment, which is in turn also reflected in gene expression data (standard deviations). Although gene expression is a very sensitive endpoint after chemical exposure, the biological variability can led to less obvious results.

Another action of the *gr* antagonist MIF in zebrafish is the corticosteroid stress axis, revealed by transcripts of *gr* and *cyp11b*. Although the literature does not clearly distinguish between *cyp11b* (postulated to synthesize 11β-hydroxytestosterone from testosterone) and *cyp11c* (postulated to synthesize cortisol) and their functionality is unproven (Tokarz et al., 2013), we hypothesize that the observed down-regulation of *cyp11b* might come along with an

inhibition of androgen formation and the cortisol stress axis by MIF and P4. A stress-induced synthesis and secretion of cortisol, regulated by the *gr*, results in increased glucose production by hepatocytes through induction of the hepatic glucose-6-phosphatase (G6Pase) and phosphoenol pyruvate carboxykinase (PEPCK), which are rate-limiting enzymes in gluconeogenesis (Aldermann et al., 2012). Thus, we hypothesize that MIF, a *gr* antagonist in zebrafish, as confirmed by gene expression changes, could lead to an attenuation of stress-induced cortisol level and thus diminished stress response, based on transcriptional inhibition of *cyp11b*, similar as observed in rainbow trout (Aldermann et al., 2012). An investigation of glucose metabolism markers, such as G6Pase and PEPCK, would further support this hypothesis (Elo et al., 2007).

6.6. Conclusions

Our results on gene expression after 21-day exposure of paired adult zebrafish males and females with continuing embryo exposure to low concentrations of MIF and P4, confirm our *in vitro* data only for the antiestrogenic activity. The reduced *ar* and *pgr* expression by MIF and P4 observed *in vivo* suggests antiandrogenic and antiprogestogenic effects of these compounds, which is in contrast to our *in vitro* results. Based on overall down-regulation of investigated steroidogenic enzymes and steroid hormone receptors, we conclude that MIF and P4 may show antiandrogenic and antiestrogenic activities in addition to their antiprogestogenic activities at concentrations in the lower ng/L range in zebrafish. Differences between *in vivo* and *in vitro* activities may result from lacking coactivators/corepressors and lacking interaction of MIF with other steroid hormones and steroid hormone receptors in the yeast assays. Recombinant yeast assays based on zebrafish steroid hormone receptors would be necessary to explore species differences in receptor responses. In summary, it remains elusive how effects of MIF and P4 on reproduction, gonad histology and plasma sexual hormone levels can be linked to transcriptional alterations.

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Chapter 7

General Discussion, Conclusions and Outlook

As this thesis addresses ecotoxicological effects of two main groups of compounds, UV filters (Chapter 2 – 4) and synthetic steroid hormones (Chapter 5 - 6), the general discussion and conclusions are split consequently into two sections, the effects obtained by UV filters and by (anti-) progestins.

7.1 Discussion and conclusions to UV filters

This thesis provides novel results on effects of UV filters in zebrafish. The UV filters BP-3 (Chapter 2), BP-4 (Chapter 3) and OC (Chapter 4) were selected because they are heavily used and little knowledge about their ecotoxicological and toxicological profile. The results provide first insights into the ecotoxicology of these compounds in zebrafish. The exposure studies were performed at concentrations in the lower µg/L-range, which is lower than in previous studies on benzophenone-2 (Weisbrod et al. 2008), 3-benzylidene camphor (Kunz et al. 2006a) and 2-ethylhexyl-4-methoxycinnamate (Christen et al. 2011). However, they are still above concentrations to be encountered in the aquatic environment. A variety of different endpoints on molecular and physiological levels were included in all studies. The main purpose was the elucidation of the modes of action of these UV filters based on transcriptomics. As with every technique, this is, however, associated with limitations, thus final conclusions on the environmental risks of BP-3, BP-4 and OC cannot be drawn. However, the output of transcriptomic data could be improved by combination of other "omics" technologies, such as proteomics and metabolomics, although this approach is very expensive (Fent and Sumpter 2011). These issues are discussed in more detail in the next paragraphs.

Benzophenone-3 (BP-3)

Considering the metabolism of BP-3 by measuring potential benzophenone-type transformation products in exposure water and bodies of fish represented a straightforward approach. Interestingly, we found that BP-3 is partly metabolized to BP-1 in adult fish, similar to the situation in mammals (Kunisue et al. 2012). The metabolite BP-1 was not found in exposure water of embryos, and its accumulation in embryos was not considered. In general,

analytical chemistry of UV filters is challenging due to their ubiquitous presence in many different consumables (e.g. solvents, gloves, plastics). Also contamination by experimenters due to their use of personal care products should always be considered. In Chapter 2 we hypothesized that BP-3 is mainly transformed to BP-1 by metabolism in adult fish and that embryos lack this capability due to probably not yet fully active metabolizing enzymes. But further experiments are necessary to prove whether BP-3 is really metabolized by P450 enzymes in zebrafish and that photodegradation or microbial degradation can be excluded. One straightforward possibility would be to expose adult zebrafish to isotopically labeled BP-3 (BP-3-d_5), which is commercially available, and to measure potential transformation products, e.g. BP-1, by analytical chemistry. Furthermore, exposure of adult zebrafish to radioactive BP-3 (^{14}C-BP-3), once available, would allow to track the bioconcentration of BP-3 and metabolites in different tissues. The induction of the P450 enzyme *cyp1a1* transcripts was hypothesized to play a role in the metabolism of BP-3 to BP-1, but measurement of the enzyme activities, for example using the ethoxyresorufin-deethylase enzyme assay, would further support our hypothesis.

The transcriptional alteration of genes involved in the endocrine system and steroidogenesis indicated an antiestrogenic and antiandrogenic activity of BP-3 in adult males and embryos, similar to previously reported *in vitro* results (Kunz et al. 2006b). Our data indicated that it is not likely that BP-3 exhibits estrogenic activities in males up to 312 µg/L based on the absence of transcriptional alterations of target genes, VTG protein levels and histological changes in testes. We hypothesized that these effects are rather due to BP-3, and the estrogenic BP-1 may contribute. More clarity would be gained when transcriptional alterations would be investigated in additional experiments where adult males were exposed to BP-1. On the other hand, it is unclear whether similar or different effects occur in adult females.

Regarding the microarray analysis, the liver was expected to be the main target organ of BP-3 with regard to xenobiotic metabolism, and hence chosen for global gene expression analysis. This analysis did not provide any new information about affected biological or physiological pathways, which may be regarded as an example that microarrays are not always useful to elucidate the mode of action of a chemical. But to notice, the target organ of BP-3 at our tested concentrations could be different to liver, and hence analysis of

multiple tissues (e.g. gonads, brain, kidney) should be considered in further transcriptomics experiments. Based on our data, we can conclude that BP-3 does not pose a risk with respect to hormonal activities at concentrations 2 - 312 µg/L, which are about 10 times higher than encountered in the environment. However, we cannot exclude that other environmental risks to fish are posed by BP-3.

Benzophenone-4 (BP-4)

The study investigated effects of the UV filter BP-4 on zebrafish (Chapter 3) and highlights the importance of analyzing transcription profiles in multiple tissues to elucidate the molecular effects of environmental chemicals. We compared alterations of transcripts in different tissue of adult zebrafish (brain, liver and testes) and embryos. Based on a targeted gene expression approach focusing on genes which play essential roles in different hormonal pathways (estrogen and androgen signaling, steroidogenesis and thyroid hormone system), we conclude that BP-4 can be considered as having an antiestrogenic activity. But this should further be proven in additional experiments considering also physiological endpoints, protein levels (e.g. VTG) and enzyme activities (e.g. P450 aromatase A/B *cyp19a/b*). This study illustrates that transcripts are altered differently in different tissues and that the response is not always strongest in the expected target organ in which the transcript is known to be predominant. For example, transcripts of the androgen receptor (*ar*) and the 17β-hydroxysteroid dehydrogenase type 3 (*hsd17b3*) were significantly down-regulated in the liver of adult fish, but both showed unexpected low responses in their main target organs, brain (for *ar*) and testes (for *hsd17b3*). Transcripts related to estrogenicity, estrogen receptor (*esr1*) and vitellogenin (*vtg1*) were down-regulated in the liver and up-regulated in the brain, whereas *esr1* is mainly expressed in the brain. Transcripts of the P450 aromatase B (*cyp19b*) were similarly up-regulated in adult brain and embryos, in contrast to the isoform *cyp19a*. The fact that *cyp19b* is considered to be related to estrogenicity this would imply that BP-4 is rather estrogenic than antiestrogenic. This illustrates that conclusions about hormonal activities of chemicals based on transcriptional alterations of steroid hormone receptors and steroidogenic enzymes alone are limited, because the molecular mechanism and feedback regulations behind are much more complex. Measurement of enzyme activities of cyp19a/b or the usage of transgenic zebrafish (Brion et al. 2012) would provide

additional evidence. Similarly, this holds for effects on the thyroid hormone synthesis by analyzing expression levels of only two genes (*hhex* and *pax8*) (Elsalina et al. 2003). Further experiments should be considered including the measurement of the thyroid hormones (T3 and T4) and/or dejodinase enzyme activity, which converts T4 to T3. Despite these limitations gene expression analysis is a powerful and sensitive tool for first characterization of toxicological profiles of chemicals.

Octocrylene (OC)

The main focus of this study was to elucidate the modes of action of the UV filter OC in different developmental stages of zebrafish (Chapter 4). This study includes two major improvements compared to the previous studies (Chapter 2 and 3), namely different exposure time points for gene expression analysis and the measurement of sex steroid hormone levels. The study confirms that transcription profiles are time-dependent (Fent and Sumpter 2011). This highlights also some of the limitation of transcriptomics as expressional changes can vary in time, and thus influence their interpretation. Despite this limitation our microarray analysis in the brain and liver of adult fish revealed new and unexpected modes of action of OC. On this basis we hypothesized that OC may affect the formation of blood vessels, blood circulation and blood flow, developmental processes and haematopoiesis. Although the biological meaning of these transcriptomics results have to be further investigated, in particular how these alterations may translate into physiological changes in fish, this study illustrates how useful the microarray technology can be to discover new and unexpected modes of action of environmental chemicals. There are advanced possibilities to study the blood circulation, heart development and blood vessel fusion in zebrafish, for example by using optical imaging techniques and transgenic fluorescent zebrafish cell-lines (Watkins et al. 2012, Herwig et al. 2011, Korzh et al. 2008, Weinstein 1999). These investigations are preferably performed on zebrafish embryos by using transgenic cell-lines which express fluorescent proteins and thus can visualize parts of the vascular system. Thus, microarray data lead to hypotheses about potential effects of OC that should subsequently be confirmed by forthcoming studies focusing on these physiological endpoints.

The transcriptional alterations were found to be similar in adult brain and liver and embryos, indicating a similar mode of action at different developmental stages. Interestingly, no indication of hormonal activity of OC was found, based on transcriptional alterations of endocrine targets and sex steroid hormone levels in adult fish. These *in vivo* findings are in contrast to *in vitro* data previously reported (Kunz and Fent 2006), and illustrates that *in vitro* systems (e.g. yeast-based) have their limitations (due to lack of cofactors, different uptake and metabolism) to predict effects in organism.

Another notice to the microarray data interpretation is that the selection of transcripts of revealed biological pathways is very challenging and depends on the expertise and knowledge of the scientist as well as on the used software for data analysis. This was done based on microarray data and knowledge obtained for other UV filters. It is possible that prioritization of affected pathways and transcripts may be more focused based on a broader physiological and biochemical knowledge. However, the complete information of altered genes and pathways are delivered for further data interpretation (Chapter 4 and Appendix A3). Additional software tools for pathway analysis related to zebrafish (e.g. DAVID software) than to humans, as done in our studies (MetaCore software), should be considered in forthcoming studies to achieve more focused biological processes in zebrafish.

Another interesting observation was the depigmentation of embryos exposed to OC, which is mentioned in the publication (Chapter 4) only briefly, because we do not have an explanation for this. This was a physiological response, observed in two independently performed experiments, and should be further investigated. It is known that adverse effects of chemicals such as *p*-tert-butylphenol, anilines and amines result in depigmentation of zebrafish embryos (Nagel 2002). Therefore, this parameter is routinely measured in the zebrafish early life stages test (DarT test) (Nagel 2002). More clarity on pigmentation effects of OC could be provided for example by investigation of a dose-response curve (e.g. DarT test) and quantification of pigmentation, imaging and histology of zebrafish embryos exposed to OC in multiwell-plates.

Nevertheless, these findings are novel and provide first insights into potential modes of action of the UV filter OC. The results of this study should be taken as motivation for further experiments to investigate the toxicological profile of this UV filter.

7.2. Discussion and conclusions to (anti-) progestins

The two generation reproduction assay performed using mixed genders of adult zebrafish constitutes a complex study that included reproductive, biological, physiological, histological and transcriptional changes. Additionally, the *in vivo* results of the anti-progestin mifepristone (MIF) and progesterone (P4) were compared with *in vitro* hormonal activities. Adult zebrafish and embryos were exposed to low and environmental relevant concentrations of MIF and P4 (ng/L), which constituted also a challenge with respect to analytical chemistry. Although the best available resources and sensitive analytical instruments were used, these pharmaceuticals, in particular MIF, posed considerable challenges. Due to the instability of MIF water samples should be stored for only very short periods of time, or even better avoid storage and analyze the samples directly after sampling. The bioconcentration of MIF and P4 in adult fish and embryos was not considered in this thesis, but would be of interest. It is recommended to use a flow-through system for *in vivo* exposure instead of static water renewal if possible to reduce stress for the fish and achieving more stable exposure concentration condition and the adaption phase of fish to exposure conditions should be long enough.

Regarding the reproductive performance of adult fish, we observed an increase in egg production at 5 and 77 ng/L MIF and no effect after exposure to 25 ng/L P4. These data cannot be compared with other studies. So far, only one study reported reproductive effects of P4 (DeQuattro et al. 2012). This study highlights that data comparison can be very difficult, when different standards for the reproductive data interpretation are applied. We found remarkable histopathological changes in the ovaries of adult females, including post-ovulatory follicles, atretic follicles and proteinaceous fluid. The meaning of these histological changes is not yet completely understood. We also do not know how this relates to reproduction, physiology and (sexual) behavior. However, these findings represent a response at an organ level as compared to time-dependent transcriptional alterations, which may be transient. Due to different responsiveness of organism to chemical exposure we suggest to analyze more biological replicate samples in further experiments to diminish the variability and to increase the statistical power of the experiment. It would also be interesting to investigate whether the histopathological effects in females are reversible and whether effects in males appear later.

We found any changes of plasma levels of the main sexual hormones (11-ketotestosterone in males and 17β-estradiol in females) in adult fish after exposure to MIF and P4. This might partly be due to the insensitivity of the radioimmunoassays (RIA). An alternative for measuring sex steroid hormone concentrations in blood plasma would be a sensitive HPLC-Mass Spectrometry method, which would provide also more specificity compared to immunoassays. Furthermore, it would have been interesting to measure concentrations of other steroid hormones in blood plasma, as fish synthesize a combination of androgens and estrogens, with androgens (11-ketotestosterone, testosterone) being predominant in males and estrogens (17β-estradiol) being predominant in females. Changes may have also occurred at levels of the main natural progestin in fish (17α,20β-dihydroxy-4-pregnen-3-one) after MIF and P4 exposure. But the small sample volumes originating from zebrafish are a limitation.

We did not find any effects of MIF and P4 on fertility of laid eggs, hatching and survival of embryos. However, other effects, such as gender determination, sexual maturation or development of the F1 generation, might become apparent later. More clarity would be provided by a full-life-cycle assessment up to F2 generation. The observed reproductive effects could only partly be linked to transcriptional alterations in different tissues of adult fish and embryos (F1 generation). This emphasizes the limitation of the time-dependent transcriptional profiles used to elucidate the mode of action of a chemical and the challenge to link them to physiological and biological responses in organism. It is rather speculative to conclude an antiprogestogenic and antiglucocorticoid activity of MIF and P4 *in vivo* based on transcriptional down-regulation of the receptors only, because complex feedback mechanism may influence the expression. We hypothesized in the publication (Chapter 6) that MIF could also led to an attenuation of stress induced cortisol levels and thus a diminished stress response based on transcriptional data, which should be corroborated by measuring cortisol levels in adult and embryonic fish.

Finally, the comparison of our transcriptomics data to *in vitro* hormonal activities revealed that yeast-based systems are an elegant tool to screen for hormonal activities of environmental chemicals, but they are not fully representatives of complex biological responses in organism. In particular in the case of MIF, this might be due to the lack of specific coactivators and/or corepressors in the yeast-based system. Moreover, recombinant

yeast systems with zebrafish-specific receptors are lacking. In the case of UV-filters, indeed significant differences between yeasts expressing human or fish-specific hormone receptors were found (Kunz et al. 2006b).

Nevertheless, the results of this reproduction assay, which included many different endpoints, stimulate further research on effects of MIF and P4 on fish. Based on our data, we strongly suggest to perform further experiments to get more confidence on the reproductive effects of MIF and P4 on fish. For example, fathead minnow (*Pimephales promelas*) and the three-spined stickleback (*Gasterosteus aculeatus*) would provide more information about the androgenic potential of MIF and P4. Sticklebacks produce the protein spiggin in the kidneys of females exclusively as response to exogenous androgens. Fathead minnow females show specific secondary sex characteristics as response to androgenic activities, such as tubercles, dorsal fin spots and fatpads and thus would further corroborate reproductive effects and highlight potential changes in (sexual) behavior (aggressiveness of fish) due to exposure to MIF and P4.

7.3. Outlook

This thesis provides novel and first insights into effects of UV filters (BP-3, BP-4 and OC), and the synthetic steroids (MIF and P4) in different developmental stages of zebrafish. Overall, the results in this thesis are important, but need extension in forthcoming studies to reach final conclusions to the environmental risks of these compounds. Open questions need to be addressed in further investigations. The following research questions and suggestions should help to provide a better understanding of the mode of action and which reproductive effects these chemicals may pose to fish.

- **Benzophenone-3 (BP-3):**
  - Is BP-3 really metabolized to BP-1 by P450 enzymes in zebrafish and is it possible to detect other metabolites of BP-3 in fish?

- Which role plays photodegradation and microbial degradation of BP-3?
- Do zebrafish embryos really lack the capability of transformation of BP-3 to BP-1 or are both compounds accumulated in embryos and failed to be detected in this thesis in exposure water, because embryos did not excrete them?
- Are the observed transcriptional alterations of BP-3 reflecting its modes of action at different exposure times and are they originating from the parent compound or the transformation product BP-1?

These research questions could be assessed by *in vivo* studies using isotopically labeled BP-3 (BP-3-d_5) or radioactive BP-3 (^{14}C-BP-3). This would also allow to investigate tissue-dependent bioaccumulation and to calculate mass balances of BP-3 and transformation products. Determination of a transformation rate (BP-3 to BP-1) would be helpful to estimate potential estrogenic activities at certain mixture-concentrations. Uptake and bioconcentration of BP-3 and metabolites should be considered in exposed embryos. An *in vivo* study using BP-1 under similar conditions and including similar endpoints would provide more information whether (transcriptional) effects are arising from BP-3 or BP-1. Measurement of cyp1a1 enzyme activities in tissues and embryos could clarify whether this P450 enzyme is involved in these metabolic processes. Microarray analysis in other tissues than liver could provide further information on the modes of action of BP-3.

- **Benzophenone-4 (BP-4):**

- Does BP-4 really pose antiestrogenic activity in fish?
- Does BP-4 really affect the thyroid hormone synthesis?

Potential estrogenic or antiestrogenic activity of BP-4 should be investigated in further experiments with focus on enzyme activities (e.g. cyp19a/b, hsd17b3), protein levels of estrogenic biomarkers and eventually by using transgenic zebrafish. Effects on the thyroid hormone system should be investigated by measuring thyroid hormone

concentrations in adult and maybe juvenile fish after exposure to BP-4 and the measurement of the dejodinase could be included.

- **Octocrylene (OC):**

  - How do transcriptional alterations affect the physiology and development of zebrafish?
  - Do we observe concentration-related effects of OC at lower (more environmental relevant) concentrations?
  - In which organs is OC mainly bioconcentrated and how is the situation in embryos?
  - The depigmentation in embryos could indicate an impact on development. Is this observation in embryos reproducible and how can it be explained? Accordingly, do similar effects occur in juvenile or adult zebrafish?

  The transcriptomic analysis of OC provided interesting and unexpected new modes of action for an UV filter. They are worth to be addressed in further more specific experiments. For example, an elegant way of studying the circulatory system and cardiac development would be the use of fluorescent zebrafish cell-lines. OC did not show a dose-response in transcriptional alterations in zebrafish, which could indicate that the maximal transcription response was already achieved at 383 µg/L. Investigation of concentration-related effects below 100 µg/L OC would provide more clarity. In further exposure studies the bioconcentration of OC in multiple tissues of adult fish and embryos should be considered. Effects on pigmentation should be further investigated in early life-stage tests including quantification of pigmentation, imaging and eventually histology. Transgenic zebrafish cell-lines, which express fluorescent proteins, could be used to visualize the vascular development. When reliable and reproducible observations would be obtained it would probably be also interesting to consider a life-cycle study (starting exposure from embryos (F0 generation) up to embryos of F1 generation) to investigate whether depigmentation results in long-lasting effects such as disturbed mating behavior, development or survival.

- **Mifepristone (MIF):**

  - Are the reproductive effects of MIF reproducible in zebrafish and other fish species?
  - Does MIF cause adverse reproductive effects in fish after chronic exposure?
  - What are the consequences of the observed histopathological observations in females and are they reversible?
  - What are long-term effects of our observed increased egg production?

  Reproductive effects of MIF should be investigated using fathead minnows, including secondary sex characteristics, sex steroid hormone level and histology, to confirm our observations in zebrafish. A longer exposure of adult zebrafish (F0 generation) could show whether the increased reproductive performance reaches a "plateau" or is steadily increasing and whether other effects are observed after chronic exposure, for example histological changes in males. Including a "depuration phase" of adult fish after exposure to MIF would unravel whether histopathological observations are reversible. Performing a full-life-cycle study (up to F2 generation) would provide more information how reproduction and development of fish is influenced by MIF, for example, which effects MIF pose to the sex ratio and sexual development and behavior, maturation and reproduction and fertility in F1/F2 generations. In addition, such a study is also more representative for the chronic exposure situation in the environment. Complementary to the study performed in this thesis, the exposure of embryos (F1 generation) should be prolonged to juveniles to investigate whether adverse effects appear later in development.

- **Progesterone (P4):**

  - How is the dose-response of P4 on fertility and reproduction of zebrafish and other fish species? Is the result observed in this thesis reproducible?
  - Does P4 cause reproductive effects in fish at environmentally relevant concentrations?

It was not the main focus of this thesis to study reproductive effects of P4 and it is necessary to include a minimum of three different concentrations in further studies to claim potential reproductive effects of P4. Basically, similar suggestions can be followed as mentioned above for MIF, because the knowledge on potential effects of P4 in fish is still very limited.

As a final conclusion, the results of this thesis show that the combination of different techniques and the consideration of relevant endpoints, such as physiological and biological changes (e.g. enzyme activities, protein levels, histology) strengthen the outcome of *in vivo* studies in the ecotoxicological assessment of chemicals. Further investigations are necessary to draw final conclusions on environmental risks of BP-3, BP-4, OC, MIF and P4. In this thesis we investigated effects of these compounds on zebrafish as single chemicals, which should be extended to complex mixture experiments of UV filters and (anti-) progestins, respectively, as this represents the actual situation in the aquatic environmental.

Appendices

Supporting Information to Chapter 2 - 5

Appendix A1

Supporting Information to Chapter 2

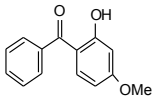
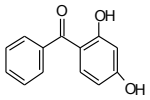
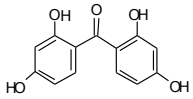
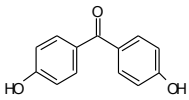
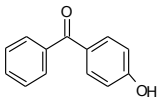
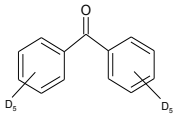
Effects of the UV-filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*)

A1.1. Materials and Methods

A1.1.1. Analytical methods

LC-MS/MS analysis

Table S1: Summary of detected analytes by LC-MS/MS in water and fish samples. Chemical structure, molecular mass (g/mol), octanol-water partitioning coefficient ($logK_{OW}$) as well as chromatographic properties as retention time t_R (min) and detected ions (m/z) are given for each analyte.

Analyte	Chemical structure	Molecular mass (g/mol)	$logK_{OW}$	t_R in min	Precursor ion (m/z)	Product ion (m/z)
BP-3		228.25	3.64	15.2	229 $[M+H]^+$	151 $[M-C_6H_6]^+$
BP-1		214.22	3.17	12.3	213 $[M-H]^-$	213 $[M-H]^-$
BP-2		246.22	3.16	9.5	245 $[M-H]^-$	245 $[M-H]^-$
4,4'-DHB		214.22	2.55	8.7	213 $[M-H]^-$	213 $[M-H]^-$
4-HB		198.20	2.95	11.3	197 $[M-H]^-$	197 $[M-H]^-$
d_{10}-BP		192.28	3.18	14.2	193 $[M+H]^+$	110 $[M-C_6D_5+H]^+$

A1.1.2. Molecular and biochemical analyses

Microarray analysis. Three groups of adult fish (solvent control, 2.4 and 312 µg/L BP-3) were selected for microarray analysis, which was performed at the FGCZ (Functional Genomics Center Zurich). The integrity of extracted total RNA of individual liver samples was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples containing 260/280 nm ratios > 1.8, 28S/18S ratios > 1.4 and RNA integrity numbers (RIN) > 8

were processed further. A total of 12 arrays (Agilent 1-Color, 4x44 K zebrafish microarray) were used, including four arrays for every group. Total RNA samples were reverse-transcribed into double-stranded cDNA, Cy3 labeled and hybridized according to manufacturer's manual at the FGCZ. The raw microarray data obtained were analyzed using GeneSpring GX 11.5 software (Agilent Technologies).

Data analysis and statistics. Raw microarray data were analyzed using the GeneSpring GX 11.5 software (Agilent Technologies). All data were quantile normalized. Several quality control steps (e.g. volcano plots, correlation plots and correlation coefficients, quality metric plots and PCA) were performed using the quality control tool of GeneSpring to ensure the good quality of the data. Differentially expressed genes from microarrays were determined using a *t*-Test (unpaired), followed by a Benjamini-Hochberg multiple correction-ANOVA test (*p*-value < 0.3 (FDR)). To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneSpring GX 11.5 was used. Moreover, MetaCore^{TM} (GeneGo, San Diego, CA) was used to identify the involvement of the differentially expressed genes in specific pathways (FDR=0.1).

A1.2 Results and Discussion

A1.2.1. General toxicity

Exposure of adult males

Table S2: Biological parameters of adult study: survival of fish after 14 days of exposure to 2.4, 84 and 312 µg/L BP-3 and 5 ng/L EE2 respectively, body length, body weight (wet) and condition factor CF (CF = body weight [g]/body length [mm] x 100). Results for biological parameters are given as mean ± standard deviation (*n*=60 male zebrafish per dose group).

	Dose groups	survival [%]	body length [cm]	body weight [mg]	CF
controls	water	100	3.82 ± 0.2	489.6 ± 83.6	1.28 ± 0.04
	solvent (0.01% DMSO)	100	3.83 ± 0.2	475.8 ± 71.6	1.24 ± 0.03
	EE2 (5 ng/L)	100	3.83 ± 0.2	511.4 ± 99.6	1.33 ± 0.10
BP-3	10 µg/L	100	3.85 ± 0.2	499.0 ± 82.5	1.29 ± 0.07
	200 µg/L	100	3.81 ± 0.2	481.2 ± 80.2	1.26 ± 0.05
	600 µg/L	97	3.89 ± 0.2	510.4 ± 82.4	1.31 ± 0.06

Exposure of eleuthero-embryos

Table S3: Summary of eleuthero-embryo exposure study: survival of eleuthero-embryos after 120 h exposure to 8.2, 113.7 and 437.7 µg/L BP-3, sample pool size range (*n*=4 pools, 1 pool for every replicate and dose-group) used for RNA extraction.

	Dose groups	survival [%]	pool size
controls	water	84	20
	solvent (0.01% DMSO)	72	20 - 24
BP-3	10 µg/L	63	20 - 24
	200 µg/L	74	20
	600 µg/L	71	19 - 23

A1.2.2. Analytical chemistry

Quality assurance

Water analysis. A detailed summary of quality assurance for the exposure water analysis of the adult and eleuthero-embryo study is shown in Table S4, including linearity (R^2), standard deviation for system suitability (SST), limit of detection/quantification (LOD/LOQ) and recovery ranges of individual analytes for the spiked samples.

Fish analysis. Table S5 summarizes all data of quality assurance for the detection (analyte retention time, linear regression coefficient, standard deviation of SST, LOD and LOQ) and extraction method (recovery ranges of spike samples) for all detected analytes in the fish samples.

Table S4: Quality assurance for water analysis of adult and eleuthero-embryo study. The analyte retention times (t_R) are shown for BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB for both detection methods, LC-MS/MS (adult study) and LC-DAD (eleuthero-embryo study) at 290 nm. The linear regression coefficient (R^2) and the standard deviation for system suitability (SST) as well as the limit of detection (LOD) and limit of quantification (LOQ), respectively, are shown for every measured analyte. The recovery ranges include the recoveries of individual spike samples for every treatment group.

	Detection method	t_R in min	R^2	SST sd in %	LOD, LOQ	Recovery range in [%]
BP-3	LC-MS/MS	15.2	0.999	1.63	0.15 ng (S/N > 20)	71 - 80
BP-1		12.4	1.000	7.68		77 - 88
BP-2		9.6	0.992	8.61		103 - 112
4,4'-DHB		8.8	0.993	3.22		102 - 116
4-HB		11.3	0.992	3.20		106 - 119
BP-3	LC-DAD	11.8	1.000	0.91	1.0 ng (S/N > 4)	64 - 79
BP-1		9.7	1.000	1.09		79 - 115

Table S5: Quality assurance for fish analysis of the adult study. The analyte retention times (t_R) are shown for BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP, detected by LC-MS/MS (at 290 nm), as well as the linear regression coefficients (R^2) and the standard deviation for system suitability (SST). The limit of detection (LOD) is set equal to the limit of quantification (LOQ) for every analyte. The recovery ranges include the recoveries of individual spike samples for every treatment group. d_{10}-BP is detected as internal extraction standard (IS) in exposed fish samples (d_{10}-BP as IS) and in unexposed-spike samples (d_{10}-BP in spike samples).

	t_R in min	R^2	SST sd in %	LOD, LOQ	Recovery range in [%]
BP-3	15.2	0.998	2.42	0.03 ng (S/N > 20)	48 - 70
BP-1	12.3	0.999	8.82		37 - 48
BP-2	9.5	0.989	9.16		59 - 129
4,4'-DHB	8.7	0.987	2.87		56 - 107
4-HB	11.3	0.986	2.48		48 - 67
d_{10}-BP as IS	14.2	0.999	2.87		65 - 74
d_{10}-BP in spike samples					58 - 86

Analytical results

Adult fish water analysis. Detailed data of measured exposure concentrations in water samples of the adult study are shown in Table S6. In addition, the recoveries of same analytes detected in spiked samples are shown for every treatment.

Table S6: Measured concentrations of BP-3, BP-1, BP-2, 4,4'-DHB and 4-HB in exposure water of the adult study. The actual water concentrations (µg/L) were detected in water samples collected three times randomly from different replicate tanks (*n*=3 samples per time point and treatment) after 0, 24 and 48 h of BP-3 treatment. In addition, the median water concentrations are shown from 0 to 48 h. The mean recoveries of spiked samples are shown for every group and analyte (*n*=2 spike samples per treatment).

Dose groups	**measured median conc. in µg/L** (n=3)	**BP-3**	**BP-1**	**BP-2**	**4,4-DHB**	**4-HB**
Control	0h	< LOQ	n.d.	n.d.	n.d.	n.d.
	24h	< LOQ	n.d.	< LOQ	n.d.	n.d.
	48h	< LOQ	n.d.	n.d.	n.d.	n.d.
	0-48h	-	-	-	-	-
	Recovery for Spike samples in % (n=2)	*75*	*86*	*107*	*116*	*119*
Solvent control DMSO	0h	< LOQ	n.d.	n.d.	n.d.	n.d.
	24h	< LOQ	n.d.	n.d.	n.d.	n.d.
	48h	< LOQ	n.d.	n.d.	n.d.	n.d.
	0-48h	-	-	-	-	-
	Recovery for Spike samples in % (n=2)	*80*	*88*	*112*	*113*	*118*
Low 10 µg/L BP-3	0h	4.8	n.d.	n.d.	n.d.	n.d.
	24h	2.4	0.3	n.d.	n.d.	n.d.
	48h	1.7	0.5	n.d.	n.d.	n.d.
	0-48h	**2.4**	**0.4**	-	-	-
	Recovery for Spike samples in % (n=2)	*73*	*80*	*103*	*106*	*106*
Mid 200 µg/L BP-3	0h	124	n.d.	n.d.	n.d.	n.d.
	24h	84	6	n.d.	n.d.	n.d.
	48h	80	10.4	n.d.	n.d.	n.d.
	0-48h	**84**	**8.2**	-	-	-
	Recovery for Spike samples in % (n=2)	*73*	*83*	*107*	*107*	*106*
High 600 µg/L BP-3	0h	312	n.d.	n.d.	n.d.	n.d.
	24h	366	17.4	n.d.	n.d.	n.d.
	48h	294	23.3	n.d.	n.d.	n.d.
	0-48h	**312**	**20.4**	-	-	-
	Recovery for Spike samples in % (n=2)	*71*	*77*	*106*	*102*	*108*

(n.d.: not detected; < LOQ: below limit of quantification (0.15 ng))

Eleuthero-embryo water analysis. Table S7 shows a detailed representation of measured concentrations in water samples of the eleuthero-embryo study after 0 and 24 h of exposure to BP-3. The recoveries of spiked samples are given for every treatment group.

Table S7: Measured concentrations of BP-3 and BP-1 in exposure water of the eleuthero-embryo study. The actual concentrations (µg/L) are detected in water samples collected three times randomly from different replicate tanks (*n*=3 samples per time point and treatment) after 0 and 24 h of exposure to BP-3. In addition, the median water concentrations are shown from 0 to 24 h. The mean recoveries of spiked samples are shown for every group and analyte (*n*=2 spike samples per treatment).

Dose groups	**measured median conc. in µg/L** (n=3)	**BP-3**	**BP-1**
Control	0h	n.d.	n.d.
	24h	n.d.	n.d.
	0-24h	-	-
	Recovery for Spike samples in % (n=2)	*77*	*94*
Solvent control DMSO	0h	n.d.	n.d.
	24h	n.d.	n.d.
	0-24h	-	-
	Recovery for Spike samples in % (n=2)	*64*	*79*
Low 10 µg/L BP-3	0h	8.1	n.d.
	24h	8.3	n.d.
	0-24h	**8.2**	-
	Recovery for Spike samples in % (n=2)	*74*	*90*
Mid 200 µg/L BP-3	0h	117.4	n.d.
	24h	109.9	n.d.
	0-24h	**114**	-
	Recovery for Spike samples in % (n=2)	*77*	*115*
High 600 µg/L BP-3	0h	449.9	n.d.
	24h	425.6	n.d.
	0-24h	**438**	-
	Recovery for Spike samples in % (n=2)	*79*	*103*

(n.d.: not detected)

Adult fish analysis. Detailed results of analyzed analyte concentrations measured in adult fish samples are shown in Table S8. The recoveries of every analyte for the unexposed spike samples are shown as well as the recoveries of d_{10}-BP, used as internal extraction standard (IS) for exposed samples.

Table S8: Measured concentrations of BP-3, BP-1, BP-2, 4,4'-DHB, 4-HB and d_{10}-BP in adult fish samples after exposure to 2.4, 84 and 312 µg/L BP-3 (*n*=10 for every treatment). The measured concentrations are given as mean ± standard deviation in ng/g wet body weight (bw). Recoveries of spike samples are given as mean for every analyte (*n*=2 for every treatment) as well as the internal standard (IS) d_{10}-BP.

Dose groups	**measured mean conc. in ng/g bw** (n=10)	**BP-3**	**BP-1**	**BP-2**	**4,4-DHB**	**4-HB**	**IS d_{10}-BP [%]**
Control	fish samples (n=10)	n.d.	n.d.	n.d.	n.d.	n.d.	71
	Recovery for Spike samples in % (n=2)	*54*	*44*	*120*	*77*	*59*	*62*
Solvent control DMSO	fish samples (n=10)	n.d.	n.d.	n.d.	n.d.	n.d.	65
	Recovery for Spike samples in % (n=2)	*48*	*37*	*59*	*56*	*48*	*58*
Low 10 µg/L BP-3	fish samples (n=10)	44.5 ± 25.7	43.2 ± 26.9	n.d.	n.d.	n.d.	74
	Recovery for Spike samples in % (n=2)	*59*	*48*	*103*	*93*	*62*	*68*
Mid 200 µg/L BP-3	fish samples (n=10)	7918 ± 2194	217 ± 228	n.d.	< LOD	n.d.	71
	Recovery for Spike samples in % (n=2)	*70*	*37*	*122*	*107*	*67*	*86*
High 600 µg/L BP-3	fish samples (n=10)	20686 ± 8551	300 ± 166	n.d.	< LOD	n.d.	70
	Recovery for Spike samples in % (n=2)	*48*	*39*	*129*	*85*	*63*	*65*

(n.d.: not detected; < LOD: below limit of detection (0.03 ng))

A1.2.3. Molecular and biochemical analyses

qRT-PCR analysis. Figure S1 graphically summarizes relative gene expression level of *hsd3b*, *cyp11b2* and *hsd11b2* in testes of adult zebrafish males. Figure S2 graphically summarizes relative gene expression level of *ar*, *cyp19b*, *vtg1* and *hsd3b* in eleuthero-embryos.

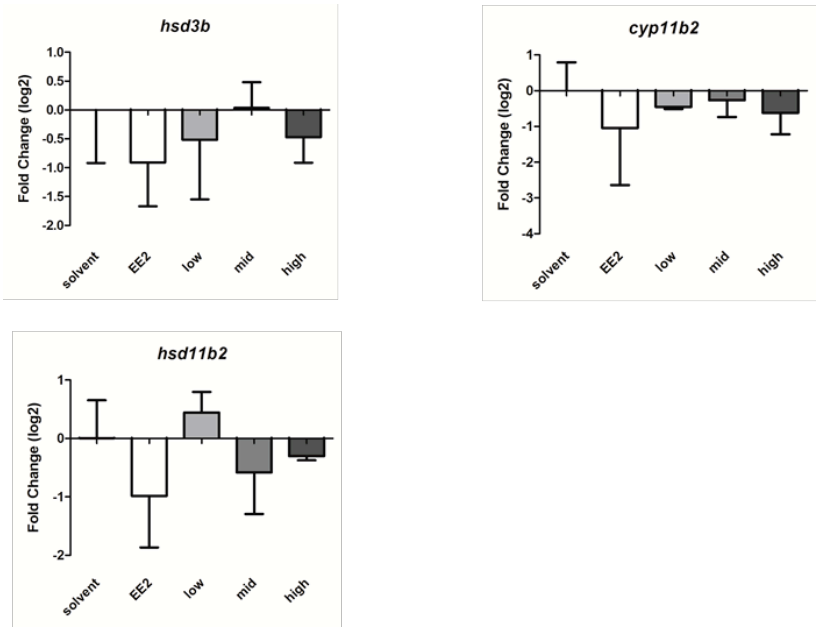


Figure S1: Relative gene expression in testes of adult zebrafish males of *hsd3b*, *cyp11b2* and *hsd11b2* after exposure to 2.4 (low), 8.4 (mid), 312 (high) µg/L BP-3 and 5 ng/L EE2 (positive control). Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. Results are given as mean ± standard deviation (*n*=4-5 replicates per treatment).

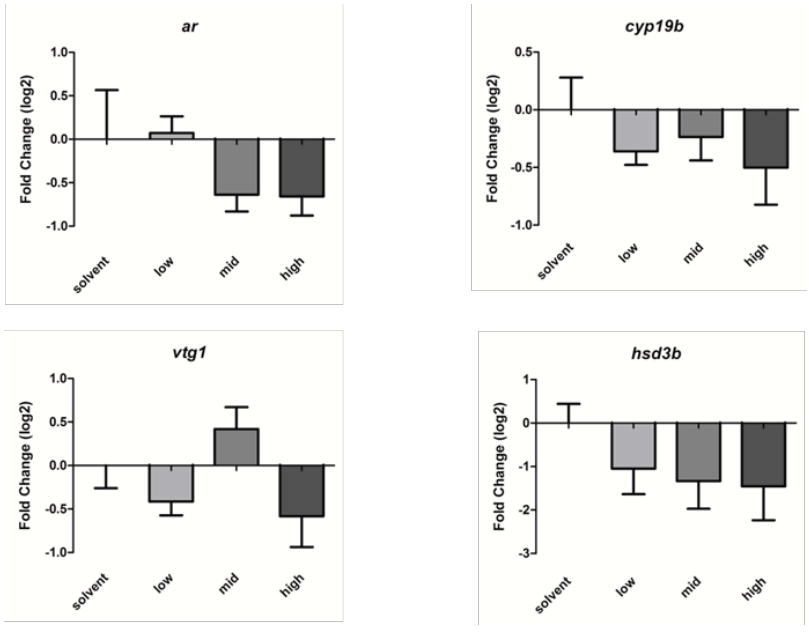


Figure S2: Relative gene expression in eleuthero-embryos of *ar*, *cyp19b*, *vtg1* and *hsd3b* after exposure to 8.2 (low), 114 (mid) and 438 (high) µg/L BP-3. Relative transcript abundance was quantified by real-time reverse transcription PCR; fold changes (log2) were determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance is referred to solvent control. Results are given as mean ± standard deviation (*n*=3-4 replicates per treatment).

Global gene expression (microarrays)

Global gene expression profiles can reveal unknown modes of action of a compound (Zucchi et al., 2011; Lam et al., 2008), as the net sum of all transcriptional alterations are assessed at once. We used this approach to elucidate the unknown modes of action of BP-3. In contrast to many studies, where pooled tissue samples are used for microarray analysis (Oggier et al., 2011; Oggier et al., 2010), we assessed liver samples of individual fish to analyze for inter-individual variability in the effect of BP-3, using Agilent 1-Color zebrafish microarray for three exposure groups, solvent control, 2.4 and 312 µg/L BP-3, respectively (*n*=4 samples per group). The advantage of using individual liver samples is that within pools bias is increased and biological outliers cannot be discriminated beyond an increased biological variability (Kendziorski, 2003). Four samples per group were found to be sufficient for hybridization and statistical analysis. Comparison of the global gene expression profiles of fish exposed to 312 µg/L BP-3 versus solvent control, show 9 differentially expressed entities for a false discovery rate (FDR) < 0.1, whereas 118 entities out of 35470 genes were observed for a FDR = 0.3 (Table S9). The genes were considered differentially expressed for FDR < 0.3 and a fold change (FC) > 1. In contrast, exposure to 2.4 µg/L BP-3 no entities were observed for a FDR < 0.98 in comparison to the solvent control.

To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneSpring GX 11.5 was used. Moreover, MetaCoreTM (GeneGo, San Diego, USA) was used to identify differentially expressed genes in specific pathways (FDR = 0.1). However, no specific pathways were affected by BP-3. In contrast to our previous experience with pooled samples (Oggier et al., 2011; Oggier et al., 2010), in the present study a relatively high variability between replicates within one group occurred, probably due to the analysis of individual fish liver. As a consequence no correlation among the four analyzed arrays derived from individual fish, and the differential exposure groups, can be ruled out. Therefore, we hypothesized that the lack of pathway identification upon exposure to 312 µg/L BP-3, is due to variable reaction of individual zebrafish, rather than the lack of any affected physiological pathway after exposure to BP-3.

Table S9: Significantly altered genes, expressed as fold changes (log2) in individual liver samples of adult male zebrafish after exposure to 312 µg/L BP-3, referred to solvent control and determined by cDNA microarrays (FDR=0.3, FC=1).

Agilent Probe ID	Genbank Accession number	Gene symbol	Fold change (log2)	Regulation
A_15_P179221	BC152129	si:dkeyp-81f3.3	1.7	Up
A_15_P194036	NM_001013314	apobec2a	2.6	Down
A_15_P444135	NM_001042775	abcg2a	3.0	down
A_15_P527887	BC124236	LOC100151464	4.6	up
A_15_P212811	NM_001042775	abcg2a	2.4	down
A_15_P140796	NM_200912	mad1l1	1.5	up
A_15_P715336	AB267908	mylka	2.3	up
A_15_P203771	XM_001331982	ccr8.1	2.0	up
A_15_P396610	XM_001333237	LOC794477	2.0	up
A_15_P104502	NM_212439	per1b	19.9	down
A_15_P118856	NM_001104945	si:dkey-15h8.11	52.5	up
A_15_P102923	NM_201301	fxr1	1.3	down
A_15_P404605	CK028591	id:ibd5038	4.0	up
A_15_P673566	CK027957	wu:fb49f03	2.8	up
A_15_P109811	NM_001007313	ppm1bb	1.3	down
A_15_P632231	NM_001039513	cnpy4	1.9	up
A_15_P100620	NM_001017900	zgc:110602	2.2	down
A_15_P150946	NM_212829	chst14	2.2	up
A_15_P161881	NM_001083826	ryk	1.5	up
A_15_P402190	NM_001114883	zgc:171553	3.0	up
A_15_P465395	CD759780	si:ch211-214c7.4	3.0	up
A_15_P116826	NM_131867	hsf2	12.5	down
A_15_P173191	NM_131792	cry2b	4.8	up
A_15_P620666	NM_194425	mhc1ze	151.5	up
A_15_P671311	NM_001004536	rnf113a	1.3	up
A_15_P244176	XM_679609	LOC556735	1.7	up
A_15_P102267	NM_001002696	zgc:92630	2.9	up
A_15_P628496	NM_001144045	kifap3	3.5	up
A_15_P201291	NM_001033721	itgav	1.8	up
A_15_P189641	NM_001123246	si:ch211-103n10.4	3.3	up
A_15_P690196	NM_001123012	aplp2	2.2	up

A_15_P238461	XM_001920310	LOC100150713	4.1	up
A_15_P101566	NM_200584	gstt1b	4.2	up
A_15_P620006	NM_131089	her2	4.7	up
A_15_P119540	CK015579	si:dkey-109n11.1	11.4	down
A_15_P622916	NM_001020778	pcdh1gb9	3.1	up
A_15_P133209	AY648784	nop10	1.7	down
A_15_P630656	NM_001110414	ppp4ca	1.3	up
A_15_P338985	NM_201064	zgc:66475	1.6	down
A_15_P187316	NM_205729	nr1d1	10.7	down
A_15_P103946	NM_131788	cry5	5.0	down
A_15_P113423	NM_194425	mhc1ze	373.6	up
A_15_P114897	NM_194396	xiap	1.7	down
A_15_P141561	NM_199917	synj2bp	1.8	down
A_15_P206076	NM_001082826	rhot1b	2.2	up
A_15_P245901	XM_001924012		1.3	up
A_15_P355555	XM_695117		1.3	up
A_15_P114240	NM_201302	sec22bb	1.6	up
A_15_P772801	CK015579	si:dkey-109n11.1	6.7	down
A_15_P591017	XM_002664139	mgea5	1.9	up
A_15_P112842	NM_212603	anp32b	1.5	up
A_15_P110041	NM_199541	id2b	4.6	up
A_15_P182041	NM_001197060	si:dkey-109n11.1	5.7	down
A_15_P366130	NM_001007352	rnf24	6.8	up
A_15_P601187	NM_001045055	si:ch211-132b12.7	8.9	down
A_15_P141096	NM_001039107	bhlhe41	6.8	down
A_15_P133821	BC060943	si:dkey-52h23.1	18.5	up
A_15_P169151	NM_001045265	zgc:136584	1.3	up
A_15_P673586	CF549786	wu:fb81c07	5.3	down
A_15_P104768	NM_200907	vps26a	1.5	up
A_15_P221951	XM_691258	LOC567944	1.6	up
A_15_P199221	XM_693188	LOC569779	2.3	up
A_15_P117410	NM_131089	her2	4.4	up
A_15_P159166	NM_001002215	zgc:92357	3.8	up
A_15_P473965	NM_001161601	amd1	1.8	down
A_15_P656731	BC063995	mhc1ze	52.0	up
A_15_P107762	NM_001007294	ankrd9	40.8	down
A_15_P665461	NM_213363	uba2	1.5	up

A_15_P725141	NM_001080565	zgc:158404	6.4	up
A_15_P188306	NM_001160022	sh3pxd2aa	1.9	up
A_15_P177056	BC107500	si:ch211-284a13.1	14.8	down
A_15_P263411	BC096780	cry2b	8.1	up
A_15_P104163	NM_194416	mmp14a	4.7	up
A_15_P110290	NM_205729	nr1d1	65.4	down
A_15_P675046	CK676891		1.7	up
A_15_P200721	XM_684096	si:ch211-214c7.4	2.4	up
A_15_P103407	NM_213075	flot2a	1.8	up
A_15_P264991	EE716419		2.4	down
A_15_P475200	NM_201581	gtf2h2	1.8	up
A_15_P207306	NM_001045314	slc25a47a	6.7	down
A_15_P658736	NM_212439	per1b	18.5	down
A_15_P101039	NM_131577	arntl1a	2.2	up
A_15_P119881	NM_212772	tuba8l	1.7	up
A_15_P722141	NM_212560	bcl7a	1.9	up
A_15_P629056	NM_131400	tef	7.6	down
A_15_P629531	NM_001122710	irf4a	1.9	down
A_15_P433820	BC131854	bhlhe41	8.4	down
A_15_P664221	BC044204	cry5	5.9	down
A_15_P111110	NM_194418	sepw2b	6.5	up
A_15_P216081	BC159185		18.2	up
A_15_P744906	NM_001030183	per1a	3.8	down
A_15_P105775	NM_001005957	mgst1	3.0	up
A_15_P278591	NM_131435	snap25a	1.6	down
A_15_P410375	NM_001080582	mxra8a	2.5	up
A_15_P169726	NM_001080565	zgc:158404	3.1	up
A_15_P749236	NM_131400	tef	9.0	down
A_15_P120707	NM_205686	cry-dash	3.7	down
A_15_P734801	NM_001199766	macc1	2.8	up
A_15_P173596	NM_001080072	zgc:158267	1.4	down
A_15_P161566	NM_131786	cry3	4.7	down
A_15_P734651	NM_201083	fus	3.2	up
A_15_P112147	NM_001110414	ppp4ca	1.5	up
A_15_P376210	NM_001080034	zgc:158387	4.5	up
A_15_P103418	NM_001098758	ppwd1	1.3	up
A_15_P734756	NM_001004649	zgc:101035	1.8	up

A_15_P671391	NM_001003589	ccdc12	1.8	up
A_15_P104739	NM_131789	cry1a	2.5	down
A_15_P448815	NM_001128381	traf1	1.9	down
A_15_P166596	NM_131400	tef	8.3	down
A_15_P685026	XM_001332159	LOC792628	3.3	down
A_15_P164271	NM_001030100	noxa1	1.3	down
A_15_P155586	BC056795		1.6	up
A_15_P103364	NM_131584	per3	5.8	down
A_15_P402990	XM_001334914		1.7	down
A_15_P193691	NM_001077519	plekhh1	3.4	up
A_15_P108054	NM_131400	tef	8.0	down
A_15_P576707	BC124236	LOC100151464	4.6	up
A_15_P356245	NM_001199491	foxp4	3.0	up

A1.2.4. Histology

The Frequencies of different testicular developmental stages (spermatogonia, spermatocytes and spermatids) in adult male zebrafish are shown in Fig. S3.

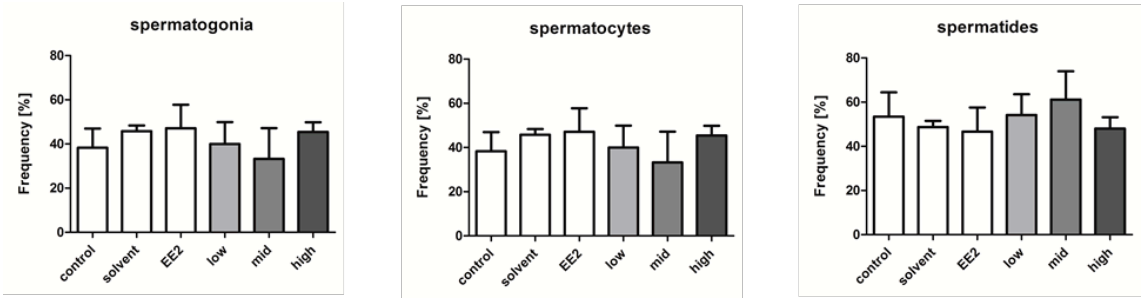


Figure S3: Relative frequencies of spermatogonia (left), spermatocytes (middle) and spermatids (right) in adult male zebrafish after exposure to 2.4 (low), 8.4 (mid) and 312 (high) µg/L BP-3 and 5 ng/L EE2 (positive control). Results are given as mean ± standard deviation (*n*=5 fish including both gonads per treatment).

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Appendix A2

Supporting Information to Chapter 3

The UV-absorber benzophenone-4 alters transcripts of genes involved in hormonal pathways in zebrafish (*Danio rerio*) eleuthero-embryos and adult males

A2.1. Results

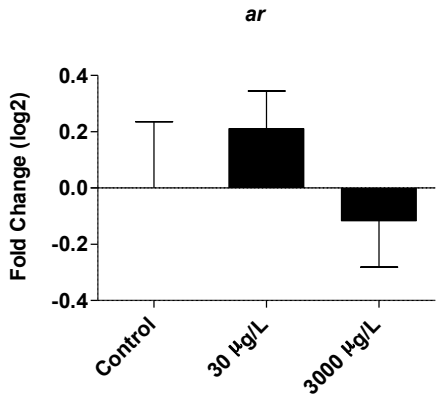

Figure S1: Relative gene expression of *ar* in zebrafish eleuthero embryos after exposure to 30 and and 3000 µg/L of BP-4. Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log2) in *ar* abundance as compared to control values were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± standard deviation (*n*=6 replicates for eleuthero embryos).

Appendix A3

Supporting Information to Chapter 4

Accumulation and effects of the UV-filter octocrylene in adult and embryonic zebrafish

A3.1. Results and Discussion

A3.1.1. Exposure experiments.

Exposure of adult males. At the end of experiment all fish (*n*=15 per replicate, *n*=75 per treatment) were anesthetized in KoiMed Sleep and body length and weight of individual fish (wet weight) were measured in order to determine the condition factor (CF = weight [g] / length [mm] x 100) (Table S1). The individual testes were weighed immediately after dissection before pooling for further RNA extraction, to determine the gonadosomatic index (GSI = testes weight [mg] / body weight [mg] x 100). The biological parameters of adult exposed fish are shown in Table S1.

Table S1: Biological parameters of adult study: survival of fish after 16 days of exposure to 22, 209 and 383 µg/L OC, respectively, body length, body weight, condition factor CF (CF = body weight [g] / body length [mm] x 100) and gonadosomatic index (GSI = testes weight [mg] / body weight [mg] x 100). Total number of exposed male fish considered for calculations were *n*=75 and *n*=45 per treatment for CF and GSI, respectively.

Treatment	Survival [%]	Body length [cm]	Body weight [mg]	CF	GSI
water	100	3.9 ± 0.3	441.0 ± 89.4	1.12 ± 0.03	0.86 ± 0.16
solvent (DMSO)	93	3.9 ± 0.2	451.6 ± 78.0	1.16 ± 0.03	0.99 ± 0.20
100 µg/L OC	87	3.8 ± 0.3	436.2 ± 87.4	1.14 ± 0.06	0.90 ± 0.12
1000 µg/L OC	100	3.9 ± 0.2	426.0 ± 75.8	1.09 ± 0.00	1.11 ± 0.25
2000 µg/L OC	87	3.9 ± 0.2	425.1 ± 80.0	1.09 ± 0.03	1.16 ± 0.20

Exposure of embryos. During the exposure of embryos, mortality and behavior were recorded daily and coagulated eggs or dead eleuthero-embryos were removed immediately. The highest mortality was observed within the first 24 h, but did not significantly differ between treatments. Subsequently, the mortality was negligible. Embryos are very sensitive for water quality and fungus development, however no relation of mortality among treatments and controls was observed. Embryos attached to fungus were counted as "dead" and removed immediately. Almost all embryos hatched after 96 h, with the remaining hatching by 144 h at latest. Eleuthero-embryos displayed normal swimming behavior by the end of the 144 h exposure. Table S2 shows the overall survival of each treatment (*n*=4 replicates per treatment, A-D) after 24, 48, 96 and 144 *hpf* as well as the hatch rate after

96 and 144 *hpf*. The sample pool size range (for replicate A-D) is shown in Table S2 (*n*=4 pools per treatment and minimum 1 pool for every replicate) for the different sampling points (48, 96 and 144 *hpf*), which were used for RNA extraction and subsequent RT-qPCR analysis.

Table S2: Summary of embryo exposure study: individual (per replicate A-D) and mean survival of eleuthero-embryos is given for every treatment after 24, 48, 96 and 144 h exposure to 69, 293 and 925 µg/L OC. The individual (per replicate A-D) and mean hatch rate for every treatment is shown after 96 and 144 h exposure as well as the larvae pool size range for the three sampling points (after 48, 96 and 144 *hpf*) used for RNA extraction.

group	treatment	replicate	survival [%] after				hatch rate [%] after		larvae pool size (*n*=)		
			24 *hpf*	48 *hpf*	96 *hpf*	144 *hpf*	96 *hpf*	144 *hpf*	48 *hpf*	96 *hpf*	144 *hpf*
1	control	A	59	59	55	55	100	100	45	45	20
		B	57	57	56	56	98	100	45	45	21
		C	64	64	63	60	96	100	50	45	24
		D	53	51	50	49	97	100	40	40	18
		mean	**58**	**58**	**56**	**55**	**98**	**100**	**40-50**	**40-45**	**18-24**
2	dmso	A	59	59	58	58	100	100	45	45	26
		B	62	60	59	59	99	100	45	45	28
		C	66	64	64	63	99	100	50	45	31
		D	70	69	68	68	100	100	50	45	25, 15
		mean	**64**	**63**	**62**	**62**	**99**	**100**	**45-50**	**45**	**15-31**
3	100 µg/L OC	A	67	66	63	62	100	100	50	45	29
		B	63	63	63	63	100	100	45	45	20, 15
		C	41	38	37	37	100	100	30	30	14
		D	51	50	49	49	100	100	40	40	18
		mean	**55**	**54**	**53**	**53**	**100**	**100**	**30-50**	**30-45**	**14-29**
4	1000 µg/L OC	A	38	30	30	29	100	100	30	15	14
		B	59	48	45	45	100	100	40	30	20
		C	60	56	54	53	100	100	45	40	21
		D	64	64	63	63	99	100	50	50	25
		mean	**55**	**49**	**48**	**47**	**100**	**100**	**30-50**	**15-50**	**14-25**
5	2000 µg/L OC	A	61	54	52	52	100	100	45	40	18
		B	66	50	47	47	100	100	40	35	18
		C	58	53	51	51	100	100	45	40	17
		D	58	55	54	54	100	100	45	40	22
		mean	**60**	**53**	**51**	**51**	**100**	**100**	**40-45**	**35-40**	**17-22**

A3.1.2. Analytical chemistry.

Quality Control. Recoveries from previously performed validations for extraction of OC in water and fish are given in Table S3 and S4. Recoveries of spike samples, which were conducted in each extraction series, both in water and fish analysis, and served as quality control for the extraction method, are given in Table S5 and S6. For the water analysis the recovery rates of the spike samples are 59 - 97 % (adult study) and 54 - 91 % (embryo study), respectively (Table S5). The recoveries of spike samples conducted for the chemical properties test (degradation study) are 69 - 90 % (Table S5). For the fish analysis the recovery rates are 89 - 107 % (Table S6). All recovery rates are in an acceptable range and

are reproducible (see Tables S5 and S6). The linear regression coefficient (R^2) was always > 0.999 within the calibration range and the SST was calculated as 0.5 %. The limit of quantification (LOQ) was set equal to the limit of detection (LOD) at 1 µg/mL for a signal to noise ratio (S\N) > 7 derived from visual inspection.

Table S3: Recoveries from previously performed **validation of the water** extraction method (*n*=2 samples per treatment).

Dose [µg/L]	OC Spike level [µg/L]	Recovery sample 1 [%]	Recovery sample 2 [%]	Recovery (mean) [%]
blank	-	n.d.	n.d.	n.d.
Low (100)	100	71	67	69
Mid (1000)	1004	82	80	81
High (2000)	2008	51	45	48

n.d.: not detected

Table S4: Recoveries from previously performed **validation of the fish** extraction method (*n*=2 samples per treatment).

Treatment	OC Spike level [ng]	Recovery sample 1 [%]	Recovery sample 2 [%]	Recovery (mean) [%]
solvent-blank	-	n.d.	n.d.	n.d.
matrix-blank	-	n.d	n.d.	n.d.
low	1260	88.9	61.1	75
high	5040	96.1	89.9	93

n.d.: not detected

Table S5: Mean recovery rates of **spike samples from water** analysis obtained during extraction of adult and embryo exposure water samples and chemical property test (degradation study). Number of replicates for each treatment are *n*=2.

		adult study		**embryo study**		**degradation study**	
group	dose [µg/L]	OC Spike level [µg/L]	Recovery (mean) [%]	OC Spike level [µg/L]	Recovery (mean) [%]	OC Spike level [µg/L]	Recovery (mean) [%]
1	control	107	59	100	76	-	-
2	dmso	107	59	100	91	-	-
3	Low (100)	107	60	100	67	107	69
4	Mid (1000)	1074	97	1002	54	-	-
5	High (2000)	2045	88	2004	83	2136	90

Table S6: Mean recovery rates of spike samples from fish analysis obtained during extraction of exposed fish (*n*=2 samples per treatment).

Group	Dose [µg/L]	Spike samples	
		spike level [ng]	recovery (mean) [%]
1	control	1039	90
2	dmso	1039	101
3	Low (100)	1039	89
4	Mid (1000)	5197	101
5	High (2000)	5197	107

Water analysis. Detailed data of measured exposure concentrations in water samples of the adult study are shown in Table S7. The actual OC concentrations in water samples collected during the eleuthero-embryo exposure study are shown in Table S8. Table S9 shows the results of the chemical properties test, performed under simulated exposure conditions and excluding organic matter. The test shows similar results to exposure water samples and high standard deviations for triplicate analysis. The overall low concentrations at 0 h (about 25-50 % of nominal) are an indication that OC is not stable during storage at -20°C. OC

concentrations at 0 h in embryo water are generally higher compared to others, whereas these samples were stored for the shortest period of 3 months. In conclusion, the chemical properties of OC discovered within this study, such as sorption to exposure-container material, inhomogeneous distribution and instability in water during storage at -20°C makes it a rather challenging chemical to work with and suggests other arrangements for forthcoming analysis.

Table S7: Measured OC concentrations in **exposure water of the adult study**. The actual water concentrations (µg/L) were detected in water samples collected three times randomly from different replicate tanks (*n*=3 samples per time point and treatment) after 0, 24 and 48 h of OC treatment. In addition, the mean water concentrations are shown from 0 to 48 h. These exposure water samples were extracted after about 5-6 months storage at -20°C.

Group	Dose [µg/L]	Measured OC concentrations				
		time 0h (mean ± sd) [µg/L]	**time 24h** (mean ± sd) [µg/L]	**time 48h** (mean ± sd) [µg/L]	average over time 0-48h (mean) [µg/L]	Percentage of nominal concentration [%]
1	control	n.d.	n.d.	n.d.	-	-
2	dmso	n.d.	n.d.	n.d.	-	-
3	Low (100)	42 ± 8	14 ± 3	10 ± 1	22	22
4	Mid (1000)	259 ± 52	69 ± 26	298 ± 200	209	21
5	High (2000)	803 ± 864	132 ± 29	215 ± 225	383	19

n.d.: not detected

Table S8: Measured OC concentrations in **exposure water of the eleuthero-embryo** study. The actual OC concentrations (concentrations (µg/L) were detected in water samples collected three times randomly from different replicate tanks (*n*=3 samples per time point and treatment) after 0 and 24 h. In addition, the mean water concentrations are shown from 0 to 24 h. These exposure water samples were extracted after about 3 month storage at -20°C. OC detected in control samples can be explained by potential contamination during thawing of water samples due to broken glass bottles.

Group	Dose [µg/L]	Measured OC concentrations			
		time 0h (mean ± sd) [µg/L]	**time 24h** (mean ± sd) [µg/L]	average over time 0-48h (mean) [µg/L]	Percentage of nominal concentration [%]
1	control	24 ± 23	49 ± 11	36	-
2	dmso	n.d.	n.d.	-	-
3	Low (100)	92 ± 70	46 ± 25	69	69
4	Mid (1000)	433 ± 167	153 ± 76	293	29
5	High (2000)	710 ± 73	1140 ± 1352	925	46

(n.d.: not detected)

Table S9: Measured OC concentrations obtained from the chemical properties test. Two different materials were tested, stainless steel tanks and glass, based on container materials used during adult and eleuthero-embryo exposure, respectively. Nominal concentrations of 100 and 2000 µg/L OC were tested under exposure simulated conditions (light/dark rhythm, oxygen), but excluding organic matter. Each concentration and material was tested in triplicate and samples were taken at 0, 24 and 48 h (*n*=3 for every treatment and time point). The water samples were extracted after about 10 month storage at -20°C.

Material	Dose [µg/L]	Measured OC concentrations				
		time 0h (mean ± sd) [µg/L]	**time 24h** (mean ± sd) [µg/L]	**time 48h** (mean ± sd) [µg/L]	average over time 0-48h (mean) [µg/L]	Percentage of nominal concentration [%]
Glass	Low (100)	34	13 ± 11	14 ± 8	20	18
	High (2000)	439	161 ± 99	163 ± 36	254	12
Stainless steel	Low (100)	58 ± 14	7 ± 2	10 ± 4	25	22
	High (2000)	859 ± 64	327 ± 221	131 ± 60	439	21

A.3.1.3. Molecular and biochemical analyses

Microarray data. The list of transcripts significantly altered after exposure to 383 µg/L OC are shown for brain (Table S10) and liver (Table S11) samples. For liver, it is notable that the enrichment analysis shows similar results for the gene entity list setting the FC ≥ 2.0 ($p < 0.05$), but GO processes and pathway maps appear with different priority as lower number of genes are imported in pathway analysis. As microarray analysis is not in any case more sensitive than RT-qPCR, which will be used for transcript validation, we preferred the larger entity list using FC ≥ 1.0 for the pathway analysis of liver samples. A summary of GO processes (Table S12) and pathway maps (Table S13) affected by 383 µg/L OC in brain and liver are given, including the complete list of altered transcripts involved in these processes. Table S14 shows the complete list of process networks (human based) affected by 383 µg/L OC in adult brain and liver, including the involved genes, which are obtained from MetaCore analysis.

Table S10: Significantly altered transcripts **in brain** of adult zebrafish males are shown after exposure to 383 µg/L OC revealed by microarray analysis (Agilent 1-color) and GeneSpring GX 12.0. In brain, 628 transcripts are significantly altered over dmso by setting the threshold at FC ≥ 2 and $p \leq 0.05$. Expression levels are expressed as absolute fold-change (FC abs).

ProbeName	GeneSymbol	GenbankAccession	*p*-value	FC (abs)	Regulation
A_15_P110013	ifng1-2	NM_212864	**0.021**	**3.2**	**down**
A_15_P235812			**0.014**	**3.4**	**down**
A_15_P120412	Bgn	NM_001002227	**0.018**	**2.4**	**down**
A_15_P160641	si:ch211-264f5.2	NM_001098253	**0.017**	**4.3**	**down**
A_15_P623196	si:ch211-244b2.2	NM_001114929	**0.022**	**2.1**	**down**
A_15_P750771	crabp2a	NM_182859	**0.013**	**2.1**	**down**
A_15_P170506	gpr39	NM_200417	**0.021**	**3.0**	**down**
A_15_P194351	zgc:64114	NM_200107	**0.015**	**2.5**	**down**
A_15_P208391	LOC100002730	NM_001098261	**0.016**	**2.1**	**down**
A_15_P332394		CK028151	**0.013**	**8.8**	**down**
A_15_P100199	bin1	NM_001004602	**0.016**	**2.1**	**down**
A_15_P634781	slc35b4	NM_212652	**0.020**	**2.2**	**down**
A_15_P112832	Ctgf	NM_001015041	**0.015**	**2.2**	**down**
A_15_P655091	crfa31.2	EF523378	**0.018**	**2.4**	**down**

A_15_P109818	Pbk	NM_001002387	**0.014**	**2.0**	**down**
A_15_P173581	cxcr3.1	NM_001089430	**0.016**	**2.1**	**down**
A_15_P513862		XM_002665462	**0.014**	**2.2**	**down**
A_15_P269726	cd40lg	NM_001144809	**0.020**	**2.3**	**down**
A_15_P205561	slc43a2b	NM_001008585	**0.025**	**2.6**	**down**
A_15_P116681	zgc:85866	NM_001001826	**0.013**	**2.0**	**down**
A_15_P100631	b3gnt5b	BC083259	**0.022**	**3.3**	**down**
A_15_P153831	lcn15	NM_001045415	**0.023**	**2.3**	**down**
A_15_P103885	mat1a	NM_199871	**0.024**	**2.7**	**down**
A_15_P629241	zgc:111821	NM_001002308	**0.018**	**3.7**	**down**
A_15_P664121		AY070267	**0.022**	**5.7**	**down**
A_15_P162421	coch	NM_001003823	**0.018**	**2.8**	**down**
A_15_P159336		XM_001919346	**0.020**	**2.0**	**down**
A_15_P761756		BC091916	**0.019**	**3.8**	**down**
A_15_P171271	zgc:154164	BC122463	**0.014**	**2.4**	**down**
A_15_P106877	hsp47	NM_131204	**0.014**	**2.6**	**down**
A_15_P743901		BC056304	**0.024**	**2.0**	**down**
A_15_P141436	alcamb	NM_212634	**0.023**	**2.5**	**down**
A_15_P119426		CO927351	**0.018**	**2.1**	**down**
A_15_P669096	sc:d0632	CU638766	**0.019**	**2.1**	**down**
A_15_P762351		XM_692217	**0.023**	**2.1**	**down**
A_15_P759696	si:dkey-23c22.6	NM_001082997	**0.024**	**3.1**	**down**
A_15_P175581	zgc:165555	NM_001098761	**0.015**	**2.2**	**down**
A_15_P299401			**0.013**	**2.3**	**down**
A_15_P665016	si:dkeyp-118h3.6	BC109457	**0.023**	**2.3**	**down**
A_15_P554172		BC155831	**0.017**	**2.9**	**down**
A_15_P100835	slc13a2	NM_213452	**0.019**	**2.3**	**down**
A_15_P658536	hamp2	NM_001023579	**0.013**	**2.9**	**down**
A_15_P166161	gdf2	NM_001171586	**0.021**	**2.3**	**down**
A_15_P537297	adssl1	NM_214820	**0.016**	**2.6**	**down**
A_15_P366150	dgat2	NM_001030196	**0.013**	**5.0**	**down**
A_15_P403175		BC129230	**0.018**	**3.1**	**down**
A_15_P592937		BC063249	**0.023**	**2.1**	**down**
A_15_P640358	c6	NM_200638	**0.013**	**2.5**	**down**

A_15_P104378	lpar6l	NM_199606	**0.018**	**2.1**	**down**
A_15_P102050	zgc:63599	NM_200672	**0.019**	**2.2**	**down**
A_15_P421545		CK028151	**0.014**	**6.9**	**down**
A_15_P394370		BC139857	**0.024**	**5.0**	**down**
A_15_P120840	col9a2	NM_212579	**0.017**	**2.0**	**down**
A_15_P444130		XM_002667724	**0.022**	**3.9**	**down**
A_15_P678691		XM_002661352	**0.013**	**2.1**	**down**
A_15_P266416	olig1	EH608531	**0.013**	**7.7**	**down**
A_15_P287511	cyp1c2	NM_001114849	**0.017**	**2.5**	**down**
A_15_P658791	col2a1a	NM_131292	**0.017**	**4.0**	**down**
A_15_P100804	aspn	NM_131713	**0.014**	**2.1**	**down**
A_15_P206851	slc43a1b	NM_001083000	**0.013**	**2.7**	**down**
A_15_P115782	prdm1a	NM_199515	**0.013**	**2.2**	**down**
A_15_P103825		AY293624	**0.025**	**2.0**	**down**
A_15_P259916		EH579308	**0.015**	**2.5**	**down**
A_15_P100087	msgn1	NM_182882	**0.022**	**2.2**	**down**
A_15_P105305	rspo3	NM_001017358	**0.013**	**2.0**	**down**
A_15_P109108	vil1l	NM_200238	**0.022**	**2.1**	**down**
A_15_P410840		XM_003199247	**0.022**	**2.8**	**down**
A_15_P736411	col2a1a	NM_131292	**0.024**	**3.0**	**down**
A_15_P108634	crygm2d8	NM_001002581	**0.018**	**2.2**	**down**
A_15_P663826	cd8a	AB186400	**0.017**	**2.1**	**down**
A_15_P138611		XM_003201045	**0.023**	**2.4**	**down**
A_15_P116725	col11a1a	BC090452	**0.017**	**2.0**	**down**
A_15_P736641		XM_693161	**0.015**	**2.2**	**down**
A_15_P401370		XM_003200989	**0.024**	**3.2**	**down**
A_15_P628085	c6	NM_200638	**0.013**	**2.2**	**down**
A_15_P153751	si:dkey-91f15.6	NM_001045047	**0.015**	**5.2**	**down**
A_15_P176776	cx30.3	NM_212825	**0.017**	**4.0**	**down**
A_15_P118350	chrne	NM_001003772	**0.014**	**3.3**	**down**
A_15_P760093	LOC100536130	XR_117782	**0.019**	**2.0**	**down**
A_15_P119415	socs3a	NM_199950	**0.022**	**2.1**	**down**
A_15_P646056	fabp1b.1	NM_001024651	**0.018**	**2.3**	**down**
A_15_P110900	xpnpep2	NM_201032	**0.018**	**2.1**	**down**

A_15_P743711	zgc:158846	NM_001083023	**0.024**	**2.1**	**down**
A_15_P408005	odf3l2	NM_001130670	**0.019**	**3.3**	**down**
A_15_P100991	pdia2	NM_201048	**0.015**	**2.4**	**down**
A_15_P114154	mmp9	NM_213123	**0.020**	**2.4**	**down**
A_15_P109352	sh3gl1a	NM_001105606	**0.017**	**3.9**	**down**
A_15_P687906	farp2	AI641740	**0.023**	**2.3**	**down**
A_15_P623321	sc:d217	NM_001122619	**0.013**	**2.7**	**down**
A_15_P366285	zgc:56376	NM_201095	**0.018**	**2.5**	**down**
A_15_P150381	capn2l	NM_001017807	**0.017**	**4.1**	**down**
A_15_P759666			**0.015**	**6.2**	**down**
A_15_P153701	wu:fu71h07	NM_001083852	**0.017**	**2.2**	**down**
A_15_P764151			**0.014**	**2.0**	**down**
A_15_P142231	tyrobp	NM_001100103	**0.022**	**2.1**	**down**
A_15_P758731			**0.016**	**2.1**	**down**
A_15_P658531	hamp2	NM_001023579	**0.016**	**2.6**	**down**
A_15_P112684	loxl2b	NM_001080626	**0.013**	**2.0**	**down**
A_15_P103427	f5	NM_001007208	**0.022**	**2.3**	**down**
A_15_P726901		BC159225	**0.016**	**4.2**	**down**
A_15_P148971	Lox	NM_207089	**0.013**	**2.0**	**down**
A_15_P221446	pdgfra	NM_131459	**0.020**	**2.7**	**down**
A_15_P622746	fabp1b.1	NM_001024651	**0.016**	**3.3**	**down**
A_15_P137611		DY564697	**0.025**	**4.4**	**down**
A_15_P110293	wu:fb12g12	AI384675	**0.013**	**2.0**	**down**
A_15_P116200	g2e3	NM_001003822	**0.013**	**2.4**	**down**
A_15_P141341	Ghrl	NM_001083872	**0.019**	**2.8**	**down**
A_15_P625306	LOC100000126	NM_001130075	**0.022**	**2.4**	**down**
A_15_P627276	neu3.4	NM_001109723	**0.018**	**2.2**	**down**
A_15_P625856	cfhl1	NM_001037703	**0.023**	**6.0**	**down**
A_15_P288796		XM_688178	**0.024**	**2.5**	**down**
A_15_P139771	si:ch211-87e4.2	NM_001083824	**0.020**	**4.8**	**down**
A_15_P568877		AM941343	**0.018**	**3.9**	**down**
A_15_P182696	si:ch211-197n10.2	NM_001102676	**0.016**	**5.6**	**down**
A_15_P108358	serpinf1	NM_001004539	**0.013**	**2.5**	**down**
A_15_P186046	zgc:194658	NM_001128750	**0.018**	**2.8**	**down**

A_15_P104104	hbbe2	NM_212846	**0.021**	**2.0**	**down**
A_15_P698786	notch1a	CT734181	**0.019**	**2.1**	**down**
A_15_P660586	cyp1c1	NM_001020610	**0.014**	**2.1**	**down**
A_15_P755196	slc43a1b	NM_001083000	**0.016**	**2.3**	**down**
A_15_P678959	tectb	NM_001008594	**0.024**	**2.1**	**down**
A_15_P179011	hamp1	NM_205583	**0.018**	**2.4**	**down**
A_15_P627222	zgc:173587	NM_001109836	**0.014**	**3.2**	**down**
A_15_P399525		EH505792	**0.019**	**2.5**	**down**
A_15_P103912	zgc:111893	NM_001039986	**0.019**	**2.3**	**down**
A_15_P138426	si:dkey-78l4.12	NM_001083067	**0.018**	**2.7**	**down**
A_15_P637416	Lox	NM_207089	**0.013**	**2.2**	**down**
A_15_P109228	zgc:111821	NM_001002308	**0.015**	**4.6**	**down**
A_15_P621916	lect2l	NM_001048055	**0.023**	**3.6**	**down**
A_15_P654848	isl2b	NM_130964	**0.017**	**7.4**	**down**
A_15_P297311			**0.017**	**6.1**	**down**
A_15_P269846		BC163037	**0.024**	**3.0**	**down**
A_15_P195506	fabp1b.1	NM_001024651	**0.022**	**2.8**	**down**
A_15_P106989	crygm2d6	NM_001002138	**0.020**	**2.1**	**down**
A_15_P116563	adam8a	NM_200637	**0.016**	**2.7**	**down**
A_15_P291486		EH487284	**0.021**	**2.1**	**down**
A_15_P639306	pmp22b	NM_001040355	**0.019**	**2.4**	**down**
A_15_P410800	cd248b	CT733915	**0.021**	**2.6**	**down**
A_15_P674406	xirp2a	XM_683113	**0.021**	**2.5**	**down**
A_15_P115720	adh8a	NM_001001946	**0.025**	**3.8**	**down**
A_15_P109326	LOC100007087	BC124510	**0.018**	**5.0**	**down**
A_15_P134341	cobl	BC090747	**0.022**	**2.1**	**down**
A_15_P111261	irf7	NM_200677	**0.017**	**2.2**	**down**
A_15_P666671	msgn1	NM_182882	**0.017**	**2.7**	**down**
A_15_P109699	dlgap5	NM_001004592	**0.023**	**2.0**	**down**
A_15_P725416	LOC100003983	NM_001083878	**0.022**	**2.3**	**down**
A_15_P170946		BC090264	**0.016**	**2.7**	**down**
A_15_P632141	zgc:85838	NM_212934	**0.024**	**2.3**	**down**
A_15_P755026	acp5a	NM_214773	**0.015**	**2.7**	**down**
A_15_P138626		XM_003201046	**0.023**	**2.2**	**down**

A_15_P102677	itpk1	NM_213017	**0.013**	**2.5**	**down**
A_15_P630281	fosb	NM_001007312	**0.016**	**2.1**	**down**
A_15_P102211	c6	XR_084426	**0.013**	**2.6**	**down**
A_15_P721631	gpx7	NM_001020501	**0.019**	**2.0**	**down**
A_15_P400625	LOC561161	NM_001135973	**0.013**	**2.0**	**down**
A_15_P105497	meox1	NM_001002450	**0.022**	**4.0**	**down**
A_15_P104030	lect1	NM_001126448	**0.013**	**2.5**	**down**
A_15_P101067	itgb1b.2	NM_212928	**0.014**	**2.4**	**down**
A_15_P196421	LOC100003983	NM_001083878	**0.021**	**3.0**	**down**
A_15_P103003	chia.1	NM_213050	**0.023**	**2.8**	**down**
A_15_P737476	si:ch1073-192f24.1	BC124681	**0.016**	**2.3**	**down**
A_15_P628651	lypla1	NM_001017616	**0.014**	**2.0**	**down**
A_15_P756031	cd248b	CT733915	**0.013**	**2.2**	**down**
A_15_P206781	p2rx3b	NM_198986	**0.018**	**2.8**	**down**
A_15_P217221	si:ch211-241e15.2	NM_001114686	**0.016**	**2.5**	**down**
A_15_P147706	si:dkey-251i10.2	BC122114	**0.017**	**2.2**	**down**
A_15_P762326		XM_691755	**0.024**	**2.1**	**down**
A_15_P441670		EH443854	**0.020**	**2.5**	**down**
A_15_P109764	nr4a1	NM_001002173	**0.015**	**2.8**	**down**
A_15_P396295		BC128878	**0.017**	**2.3**	**down**
A_15_P752816		CO927351	**0.022**	**2.9**	**down**
A_15_P663691	npsn	NM_001077779	**0.015**	**2.9**	**down**
A_15_P261151			**0.016**	**2.1**	**down**
A_15_P171636	rhogb	NM_200040	**0.024**	**2.2**	**down**
A_15_P119010	cxcr3.2	NM_001007314	**0.018**	**2.1**	**down**
A_15_P120422	itln1	NM_001076622	**0.022**	**2.2**	**down**
A_15_P113559	pck2	NM_213192	**0.021**	**3.1**	**down**
A_15_P163456	zgc:77799	NM_200868	**0.024**	**2.2**	**down**
A_15_P105745	bcan	NM_001083813	**0.014**	**2.3**	**down**
A_15_P632376	hamp2	NM_001023579	**0.018**	**3.0**	**down**
A_15_P341865	timp2b	NM_213296	**0.015**	**2.3**	**down**
A_15_P624181	crygm2d16	NM_001103134	**0.017**	**2.3**	**down**
A_15_P343075	il21r	NM_001113510	**0.016**	**2.3**	**down**
A_15_P378491		CO918741	**0.017**	**4.0**	**down**

A_15_P662896	p2rx3b	NM_198986	**0.015**	**3.1**	**down**
A_15_P168006		EH445822	**0.014**	**3.0**	**down**
A_15_P677801		XM_001922952	**0.014**	**4.0**	**down**
A_15_P737986		NM_001045014	**0.014**	**2.1**	**down**
A_15_P113791	rbp2a	NM_153004	**0.025**	**2.8**	**down**
A_15_P179516	Hp	BC152295	**0.025**	**3.9**	**down**
A_15_P225551	zgc:73311	NM_001193525	**0.013**	**2.1**	**up**
A_15_P101026	im:7151270	BC090302	**0.017**	**2.0**	**up**
A_15_P565462	sumo3a	NM_213124	**0.024**	**2.2**	**up**
A_15_P587192	sncgb	NM_001020652	**0.019**	**2.2**	**up**
A_15_P494577	zgc:63614	NM_200608	**0.023**	**2.3**	**up**
A_15_P222006		XM_679323	**0.014**	**2.1**	**up**
A_15_P723451	psmc4	NM_199750	**0.020**	**2.0**	**up**
A_15_P115194	pax7a	NM_131325	**0.015**	**2.5**	**up**
A_15_P113161	Fpgt	NM_001020754	**0.023**	**3.0**	**up**
A_15_P418145	mppe1	NM_001017701	**0.013**	**2.2**	**up**
A_15_P533757	cdh11	NM_131214	**0.015**	**2.1**	**up**
A_15_P173336	cwc25	NM_213462	**0.014**	**2.0**	**up**
A_15_P104013		DV594869	**0.015**	**2.1**	**up**
A_15_P659551	ndfip1	NM_199666	**0.017**	**2.1**	**up**
A_15_P498482		CN838190	**0.014**	**2.3**	**up**
A_15_P104532	mtnr1bb	NM_001201565	**0.018**	**2.6**	**up**
A_15_P682198	si:busm1-79m10.1	AI877793	**0.016**	**2.1**	**up**
A_15_P721826	rpl5a	NM_199756	**0.015**	**2.1**	**up**
A_15_P734446	cct6a	NM_201290	**0.017**	**2.0**	**up**
A_15_P156866	vipr2	NM_131779	**0.020**	**2.2**	**up**
A_15_P716016	gpr107	NM_001080046	**0.025**	**2.1**	**up**
A_15_P174681	cct7	NM_173248	**0.016**	**2.1**	**up**
A_15_P590662		BC154269	**0.021**	**2.4**	**up**
A_15_P737616	ube2i	NM_131351	**0.016**	**2.1**	**up**
A_15_P540962	ppp2r3c	NM_200131	**0.013**	**2.5**	**up**
A_15_P602647	uck1	NM_001004666	**0.014**	**2.2**	**up**
A_15_P109304	eif1b	NM_199588	**0.020**	**2.2**	**up**
A_15_P495697	Mpz	NM_194361	**0.013**	**2.5**	**up**

A_15_P739016		XM_678216	**0.020**	**2.2**	**up**
A_15_P721926	hmga1a	NM_213168	**0.021**	**2.0**	**up**
A_15_P763261	eif3ea	NM_200839	**0.017**	**2.0**	**up**
A_15_P131216		DQ851808	**0.017**	**2.2**	**up**
A_15_P515467	zgc:63674	NM_200616	**0.014**	**2.4**	**up**
A_15_P561732		XM_685812	**0.017**	**2.1**	**up**
A_15_P765766	psmd6	NM_200291	**0.015**	**2.2**	**up**
A_15_P206306	zgc:110791	NM_001013482	**0.016**	**2.2**	**up**
A_15_P115582	h3f3a	NM_212996	**0.015**	**2.2**	**up**
A_15_P747116	si:dkey-181m9.10	NM_001111188	**0.020**	**2.0**	**up**
A_15_P678601			**0.024**	**5.6**	**up**
A_15_P639156	sncgb	NM_001020652	**0.016**	**2.1**	**up**
A_15_P750951	cct5	NM_212613	**0.015**	**2.1**	**up**
A_15_P111744	zgc:101614	NM_001007452	**0.019**	**2.1**	**up**
A_15_P144611	scube2	NM_001014813	**0.022**	**2.3**	**up**
A_15_P580132	pm20d1.2	NM_001077301	**0.022**	**2.6**	**up**
A_15_P149956	map3k7 (TAK1)	NM_001020750	**0.013**	**2.1**	**up**
A_15_P187481	slc1a9	NM_001190759	**0.024**	**2.1**	**up**
A_15_P634356	fzd10	NM_131136	**0.024**	**2.5**	**up**
A_15_P628981	scn1ba	NM_001077539	**0.015**	**2.1**	**up**
A_15_P140626	zgc:113516	NM_001013574	**0.022**	**2.1**	**up**
A_15_P208731	fgfr1a	NM_152962	**0.019**	**2.2**	**up**
A_15_P143636	atp1a3b	NM_131685	**0.015**	**2.1**	**up**
A_15_P612862	wu:fk26h02	CN511429	**0.021**	**2.1**	**up**
A_15_P655731	atp6v1g1	NM_199934	**0.015**	**2.0**	**up**
A_15_P306756	pknox1.1	NM_131891	**0.022**	**2.2**	**up**
A_15_P543987	asmtl	NM_213511	**0.017**	**2.3**	**up**
A_15_P165406	LOC567650	XM_690949	**0.015**	**2.1**	**up**
A_15_P149121	zgc:153096	NM_001077287	**0.024**	**2.2**	**up**
A_15_P120839	zgc:112221	NM_001020763	**0.013**	**2.2**	**up**
A_15_P671966		DV586180	**0.013**	**2.2**	**up**
A_15_P406630	arl15	NM_001100033	**0.013**	**2.4**	**up**
A_15_P243511		DN600185	**0.014**	**2.7**	**up**
A_15_P278311	pnrc2	NM_001130192	**0.018**	**2.2**	**up**

A_15_P197851	map4k5	NM_199913	**0.014**	**2.0**	**up**
A_15_P228141	zgc:175274	NM_001114572	**0.013**	**3.0**	**up**
A_15_P268731	capn3	XM_693240	**0.015**	**2.1**	**up**
A_15_P727886	hnrnpl2	NM_213383	**0.015**	**2.2**	**up**
A_15_P105458	st3gal3l	NM_214785	**0.015**	**2.1**	**up**
A_15_P147956	golt1b	NM_001031671	**0.024**	**2.3**	**up**
A_15_P720901	pdcd2	NM_001045138	**0.016**	**2.2**	**up**
A_15_P598847		EG568693	**0.017**	**2.3**	**up**
A_15_P328481	irf5	NM_212875	**0.023**	**2.4**	**up**
A_15_P168476		XM_684403	**0.015**	**2.3**	**up**
A_15_P752966	Ctps	NM_199211	**0.015**	**2.3**	**up**
A_15_P136986	eif3ja	NM_201214	**0.013**	**2.7**	**up**
A_15_P621151	zgc:162824	NM_001083574	**0.023**	**4.2**	**up**
A_15_P308876		XM_002665194	**0.022**	**2.3**	**up**
A_15_P759716	LOC558654	NM_001099992	**0.019**	**3.0**	**up**
A_15_P621681	zgc:110152	NM_001017853	**0.015**	**2.7**	**up**
A_15_P413930	prkab1b	NM_214713	**0.015**	**2.1**	**up**
A_15_P563717		BC154804	**0.015**	**2.1**	**up**
A_15_P117207	timm23	NM_001105598	**0.016**	**2.1**	**up**
A_15_P747525	si:dkeyp-39e9.2	NM_001045100	**0.013**	**2.0**	**up**
A_15_P736837	Seta	NM_201475	**0.015**	**2.4**	**up**
A_15_P646161	nras	NM_131145	**0.013**	**2.1**	**up**
A_15_P220491	zgc:113115	NM_001013567	**0.013**	**2.1**	**up**
A_15_P307436		EE705781	**0.013**	**2.1**	**up**
A_15_P113930		XM_003200901	**0.015**	**3.3**	**up**
A_15_P726331		EE690470	**0.015**	**3.2**	**up**
A_15_P654651	chst11	NM_212824	**0.018**	**2.0**	**up**
A_15_P265136	LOC561960	XM_700969	**0.013**	**2.3**	**up**
A_15_P184076	si:dkeyp-117h8.1	NM_001199990	**0.016**	**2.0**	**up**
A_15_P741866	gpm6aa	NM_213200	**0.020**	**2.0**	**up**
A_15_P722061	mrps22	NM_001098735	**0.013**	**2.1**	**up**
A_15_P745166	ppt1	NM_213339	**0.017**	**2.0**	**up**
A_15_P714344			**0.018**	**2.3**	**up**
A_15_P344765	fbxl14a	NM_201482	**0.014**	**2.0**	**up**

A_15_P196076		BC115182	**0.013**	**2.3**	**up**
A_15_P198396	dpp6b	NM_001115122	**0.015**	**2.2**	**up**
A_15_P239856	zgc:65827	NM_213155	**0.017**	**2.2**	**up**
A_15_P105744	eif4a1b	NM_201510	**0.018**	**2.5**	**up**
A_15_P439430	rhoad	NM_001002445	**0.014**	**2.1**	**up**
A_15_P735936		EH557288	**0.021**	**2.1**	**up**
A_15_P637871	atg10	NM_001037124	**0.013**	**2.0**	**up**
A_15_P610642		CN013384	**0.013**	**2.3**	**up**
A_15_P761906			**0.019**	**3.2**	**up**
A_15_P687661	itm1	NM_201458	**0.018**	**2.1**	**up**
A_15_P408595	txndc9	NM_200021	**0.014**	**2.5**	**up**
A_15_P138901	LOC565106	NM_001144795	**0.015**	**2.0**	**up**
A_15_P747161	h3f3c	NM_200003	**0.021**	**2.1**	**up**
A_15_P659386	pvalb6	NM_205573	**0.015**	**2.8**	**up**
A_15_P749066	ldhba	NM_131247	**0.017**	**2.1**	**up**
A_15_P741886	zgc:162909	NM_001089583	**0.018**	**2.4**	**up**
A_15_P575887		DN903654	**0.015**	**2.2**	**up**
A_15_P761916		XM_001345430	**0.015**	**2.5**	**up**
A_15_P552892	ppp1r3da	NM_001110412	**0.018**	**2.2**	**up**
A_15_P550182			**0.025**	**3.3**	**up**
A_15_P656201	rtn1a	NM_001029967	**0.014**	**2.2**	**up**
A_15_P752266	zgc:92763	NM_001002560	**0.015**	**2.4**	**up**
A_15_P201401	mphosph8	NM_001126433	**0.016**	**2.2**	**up**
A_15_P659146	plrg1	NM_213440	**0.025**	**2.3**	**up**
A_15_P745621	prkcq	NM_001089370	**0.013**	**2.1**	**up**
A_15_P203466		XM_693718	**0.016**	**2.2**	**up**
A_15_P491267			**0.013**	**2.2**	**up**
A_15_P116496	zgc:112018	NM_001013467	**0.015**	**2.3**	**up**
A_15_P116445	atp1a3a	NM_131684	**0.017**	**2.2**	**up**
A_15_P195176	zgc:153955	NM_001080001	**0.017**	**2.0**	**up**
A_15_P663378		BC153310	**0.014**	**3.0**	**up**
A_15_P379445	aldh7a1	NM_212724	**0.023**	**2.1**	**up**
A_15_P733631			**0.013**	**2.0**	**up**
A_15_P756166	cct6a	NM_201290	**0.016**	**2.1**	**up**

A_15_P323351	cotl1	NM_200012	**0.018**	**2.1**	**up**
A_15_P192046	vamp4	NM_200735	**0.024**	**2.1**	**up**
A_15_P465185	bmpr1bb	NM_001114924	**0.019**	**2.3**	**up**
A_15_P726316	ldhba	NM_131247	**0.016**	**2.0**	**up**
A_15_P213451		XM_003197905	**0.020**	**3.1**	**up**
A_15_P490247		XM_685709	**0.015**	**2.3**	**up**
A_15_P675621		EV758136	**0.022**	**2.4**	**up**
A_15_P198736	acadm	NM_213089	**0.018**	**2.2**	**up**
A_15_P265807	zgc:174574	NM_001115070	**0.015**	**3.1**	**up**
A_15_P399000	snx12	NM_001145892	**0.014**	**2.1**	**up**
A_15_P765831	zgc:173624	NM_001109725	**0.015**	**2.5**	**up**
A_15_P739656	zgc:66484	NM_199827	**0.016**	**2.0**	**up**
A_15_P623706	hdgfrp2	NM_001002037	**0.014**	**2.1**	**up**
A_15_P722861	zgc:110626	NM_001017889	**0.013**	**2.2**	**up**
A_15_P175541	gad2	NM_001017708	**0.016**	**3.1**	**up**
A_15_P278361	ndrg3a	NM_199517	**0.017**	**2.0**	**up**
A_15_P281621			**0.016**	**5.1**	**up**
A_15_P174481	LOC100004819	NM_001082956	**0.015**	**2.5**	**up**
A_15_P135896	st6gal2	NM_001159914	**0.017**	**2.1**	**up**
A_15_P634506	zgc:172101	NM_001113644	**0.015**	**2.4**	**up**
A_15_P749161	smyd2a	NM_001013550	**0.017**	**2.4**	**up**
A_15_P745006	ppp2cb	NM_213293	**0.018**	**2.0**	**up**
A_15_P131441	hsp90ab1	NM_131310	**0.014**	**2.1**	**up**
A_15_P671996		XM_685771	**0.015**	**2.4**	**up**
A_15_P772056			**0.013**	**2.2**	**up**
A_15_P182886		XM_688741	**0.015**	**2.0**	**up**
A_15_P602667	tnfrsf19	NM_001044904	**0.014**	**2.3**	**up**
A_15_P450725		CO916938	**0.015**	**2.1**	**up**
A_15_P726631	clk4a	BC099995	**0.024**	**2.2**	**up**
A_15_P654741	zgc:198329	NM_001098246	**0.024**	**2.8**	**up**
A_15_P505107	zgc:153953	NM_001080005	**0.020**	**2.4**	**up**
A_15_P748686	rpl9	NM_001003861	**0.015**	**2.2**	**up**
A_15_P708141	tmem107	NM_213211	**0.016**	**2.2**	**up**
A_15_P223541			**0.013**	**2.2**	**up**

A_15_P188251		CU468794	**0.020**	**2.0**	**up**
A_15_P319706	zgc:152953	NM_001080057	**0.013**	**2.2**	**up**
A_15_P461690	eif5a	NM_213185	**0.017**	**2.3**	**up**
A_15_P741351	zgc:162431	NM_001089533	**0.013**	**2.1**	**up**
A_15_P596857		XM_692987	**0.021**	**2.6**	**up**
A_15_P720601	ppt1	NM_213339	**0.017**	**2.0**	**up**
A_15_P342510	ywhabl	NM_199562	**0.018**	**2.0**	**up**
A_15_P723936	elovl1b	NM_213416	**0.015**	**2.0**	**up**
A_15_P730256	LOC100150082	NM_001128401	**0.023**	**2.4**	**up**
A_15_P745956	vldlr	NM_200923	**0.024**	**2.3**	**up**
A_15_P288936		BC151973	**0.015**	**2.1**	**up**
A_15_P521252	wu:fj19g03	EH438865	**0.023**	**3.4**	**up**
A_15_P552142	gad2	NM_001017708	**0.013**	**2.0**	**up**
A_15_P208636	mapk15	NM_001020745	**0.021**	**2.9**	**up**
A_15_P752931	vps28	NM_200590	**0.016**	**2.2**	**up**
A_15_P745357	hmgb1a	NM_199555	**0.013**	**2.1**	**up**
A_15_P104924	slc4a4a	NM_001034984	**0.016**	**2.2**	**up**
A_15_P380440	nutf2	NM_001006000	**0.016**	**2.1**	**up**
A_15_P748626	hmga1a	NM_213168	**0.020**	**2.0**	**up**
A_15_P724601	Fibp	NM_200409	**0.016**	**2.6**	**up**
A_15_P518812		XM_688003	**0.018**	**2.5**	**up**
A_15_P507892	fam175a	NM_001005993	**0.021**	**2.1**	**up**
A_15_P541902	LOC795984	NM_001171031	**0.018**	**3.9**	**up**
A_15_P377355		BC059440	**0.015**	**2.1**	**up**
A_15_P730831		BC095810	**0.015**	**2.2**	**up**
A_15_P115423	zgc:110307	NM_001017591	**0.014**	**2.1**	**up**
A_15_P746641	LOC100005937	NM_001202477	**0.013**	**2.4**	**up**
A_15_P625389	LOC100006761	NM_001128816	**0.013**	**4.8**	**up**
A_15_P268502	stox1	NM_001123010	**0.016**	**2.0**	**up**
A_15_P598932	si:dkey-222k23.4	NM_001122617	**0.014**	**2.3**	**up**
A_15_P721851	elovl6	NM_199532	**0.016**	**2.3**	**up**
A_15_P366950	dexi	NM_001003764	**0.016**	**2.7**	**up**
A_15_P670161		XM_690985	**0.021**	**2.0**	**up**
A_15_P473965	amd1	NM_001161601	**0.021**	**2.4**	**up**

A_15_P755341	trnau1ap	NM_001020609	**0.014**	**2.1**	**up**
A_15_P673731	atp5f1	NM_001005960	**0.015**	**2.1**	**up**
A_15_P438200		XM_001345774	**0.022**	**2.5**	**up**
A_15_P151713	caspxa	NM_001083862	**0.016**	**3.6**	**up**
A_15_P423700	zgc:153086	NM_001077381	**0.013**	**2.1**	**up**
A_15_P114650	psmc6	NM_001003832	**0.017**	**2.2**	**up**
A_15_P145841	LOC566487	NM_001127514	**0.019**	**2.4**	**up**
A_15_P115320	zgc:123007	NM_001037557	**0.013**	**2.4**	**up**
A_15_P629851	LOC100006275	NM_001126481	**0.017**	**2.9**	**up**
A_15_P727671	ap1s2	NM_205558	**0.014**	**2.3**	**up**
A_15_P198741	si:dkey-181m9.10	NM_001111188	**0.018**	**2.0**	**up**
A_15_P761555	clic1	NM_212682	**0.018**	**2.0**	**up**
A_15_P668456	zgc:162824	NM_001083574	**0.018**	**4.6**	**up**
A_15_P168611		EB890885	**0.019**	**2.5**	**up**
A_15_P118696			**0.015**	**2.5**	**up**
A_15_P357045		XM_691888	**0.015**	**2.0**	**up**
A_15_P568087		EB943821	**0.013**	**4.1**	**up**
A_15_P117010	zgc:55886	NM_200959	**0.014**	**2.4**	**up**
A_15_P748641	Ilvbl	NM_200666	**0.013**	**2.0**	**up**
A_15_P744421	jak1	NM_131073	**0.016**	**2.1**	**up**
A_15_P157386	ash2l	NM_001110105	**0.015**	**2.3**	**up**
A_15_P243697	LOC100002334	NM_001098260	**0.015**	**2.3**	**up**
A_15_P541887	abcg4b	NM_001111212	**0.020**	**2.3**	**up**
A_15_P740561	si:ch211-194d6.3	NM_001128557	**0.013**	**4.1**	**up**
A_15_P130216	zgc:100933	NM_001003629	**0.018**	**2.6**	**up**
A_15_P736716	mboat7	NM_200537	**0.013**	**2.1**	**up**
A_15_P153441	mdkb	NM_131716	**0.016**	**2.6**	**up**
A_15_P217621	zgc:153628	NM_001077294	**0.015**	**2.5**	**up**
A_15_P376995		XM_002666403	**0.015**	**2.3**	**up**
A_15_P740421	atp1a3b	NM_131685	**0.015**	**2.0**	**up**
A_15_P366515	slc48a1b	NM_200006	**0.023**	**2.1**	**up**
A_15_P546447		XM_694390	**0.013**	**2.5**	**up**
A_15_P587952	mobkl3	NM_001003439	**0.015**	**2.2**	**up**
A_15_P724646	tubb2c	NM_198809	**0.014**	**2.1**	**up**

A_15_P615397	mpp1	NM_214692	**0.017**	**2.0**	**up**
A_15_P169596	adcyap1a	NM_152885	**0.013**	**2.5**	**up**
A_15_P687366		XM_003198708	**0.016**	**3.0**	**up**
A_15_P586642		CK019294	**0.020**	**2.7**	**up**
A_15_P120848	zgc:158782	NM_001083009	**0.017**	**2.4**	**up**
A_15_P190291	pcdh2ab1	NM_001009588	**0.013**	**2.2**	**up**
A_15_P750871	calm2b	NM_214736	**0.016**	**2.3**	**up**
A_15_P631136	dia1	NM_200189	**0.018**	**2.1**	**up**
A_15_P515392		EH505116	**0.016**	**2.1**	**up**
A_15_P630326	si:ch211-146f4.3	NM_001045197	**0.013**	**2.2**	**up**
A_15_P592617	wu:fc26h12	EE302152	**0.017**	**2.1**	**up**
A_15_P224141	zgc:158403	NM_001080084	**0.025**	**2.4**	**up**
A_15_P105109	cd81	NM_131518	**0.019**	**2.0**	**up**
A_15_P136246	si:ch1073-351m12.1	NM_001102570	**0.015**	**2.0**	**up**
A_15_P723521	slc25a5	NM_173247	**0.016**	**2.1**	**up**
A_15_P331056	nae1	NM_200499	**0.015**	**2.4**	**up**
A_15_P732966	slc25a5	NM_173247	**0.015**	**2.2**	**up**
A_15_P628026	cxcr7b	NM_001144814	**0.015**	**2.3**	**up**
A_15_P654586	mafl	NM_001082940	**0.013**	**2.5**	**up**
A_15_P404660	wu:fa12e08	EE714630	**0.022**	**2.3**	**up**
A_15_P162116	dhcr24	NM_001008645	**0.017**	**2.3**	**up**
A_15_P728886		EE716300	**0.021**	**2.6**	**up**
A_15_P623851	tbcb	NM_212775	**0.022**	**2.0**	**up**
A_15_P143816		XM_686046	**0.015**	**2.5**	**up**
A_15_P536642	Smo	NM_131027	**0.018**	**2.1**	**up**
A_15_P471785	zgc:77222	NM_207099	**0.015**	**2.2**	**up**
A_15_P121209		CK028674	**0.024**	**2.7**	**up**
A_15_P500832			**0.021**	**2.6**	**up**
A_15_P259671	vcam1	NM_001083835	**0.015**	**2.2**	**up**
A_15_P755701	rtn1a	NM_001029967	**0.015**	**2.5**	**up**
A_15_P768725	LOC795918	BC155243	**0.018**	**2.0**	**up**
A_15_P528552	hpca	NM_200723	**0.016**	**2.1**	**up**
A_15_P174351	mycbp2	NM_001012247	**0.015**	**2.0**	**up**
A_15_P512367	tmem50a	NM_213529	**0.014**	**2.5**	**up**

A_15_P672076	zgc:165409	NM_001098756	**0.017**	**2.6**	**up**
A_15_P659931	timm13	NM_001002465	**0.015**	**2.1**	**up**
A_15_P394450			**0.013**	**2.0**	**up**
A_15_P727631	zgc:153973	NM_001077547	**0.018**	**2.5**	**up**
A_15_P728691	zgc:111961	NM_001024429	**0.015**	**2.0**	**up**
A_15_P113299	zgc:112421	NM_001024414	**0.019**	**2.1**	**up**
A_15_P375271			**0.021**	**2.1**	**up**
A_15_P500032			**0.015**	**2.0**	**up**
A_15_P738899	zgc:92510	NM_001002365	**0.016**	**2.0**	**up**
A_15_P484775			**0.018**	**2.0**	**up**
A_15_P751301	wu:fc14e06	EL647041	**0.023**	**2.1**	**up**
A_15_P366275	galk1	NM_001006002	**0.013**	**2.0**	**up**
A_15_P168201		XR_029160	**0.013**	**2.0**	**up**
A_15_P109236			**0.023**	**2.5**	**up**
A_15_P105440		XM_680251	**0.013**	**2.1**	**up**
A_15_P673166	zgc:175221	NM_001114741	**0.018**	**2.1**	**up**
A_15_P169411	rorca	NM_001082819	**0.015**	**2.1**	**up**
A_15_P547462			**0.018**	**2.4**	**up**
A_15_P468280		XM_002664139	**0.017**	**2.2**	**up**
A_15_P569032	LOC569091	XM_701384	**0.013**	**2.3**	**up**
A_15_P211671	Thrb	NM_131340	**0.017**	**2.0**	**up**
A_15_P529567	prkab1a	NM_001002632	**0.017**	**2.6**	**up**
A_15_P269117		XM_003199798	**0.014**	**2.0**	**up**
A_15_P202481		CF265703	**0.013**	**2.4**	**up**
A_15_P196661	zgc:92467	NM_001003494	**0.016**	**2.2**	**up**
A_15_P502542		DV589762	**0.015**	**2.1**	**up**
A_15_P732156	slc25a5	NM_173247	**0.017**	**2.1**	**up**
A_15_P110650	Tsku	NM_199733	**0.015**	**2.1**	**up**
A_15_P750531	ccng1	NM_199481	**0.021**	**2.1**	**up**
A_15_P343540	hsp90ab1	NM_131310	**0.016**	**2.0**	**up**
A_15_P723501	trmt2b	NM_001122943	**0.023**	**2.3**	**up**
A_15_P733931	clic1	NM_212682	**0.017**	**4.8**	**up**
A_15_P146491	gpr107	NM_001080046	**0.019**	**2.2**	**up**
A_15_P607712	scdb	NM_001020705	**0.015**	**2.0**	**up**

A_15_P752466	obfc1	NM_200389	**0.024**	**2.1**	**up**
A_15_P741106	zgc:85858	NM_212943	**0.017**	**2.4**	**up**
A_15_P268781	si:dkey-188i13.11	EH565016	**0.013**	**2.0**	**up**
A_15_P753381	calm3b	NM_199570	**0.018**	**2.4**	**up**
A_15_P761936			**0.017**	**2.9**	**up**
A_15_P109933		CK024579	**0.013**	**2.1**	**up**
A_15_P108097	Rgra	NM_001017877	**0.016**	**2.1**	**up**
A_15_P446860		XM_003200988	**0.013**	**3.3**	**up**
A_15_P724211	elovl1b	NM_213416	**0.020**	**2.1**	**up**
A_15_P731487	slc25a5	NM_173247	**0.017**	**2.1**	**up**
A_15_P754831	opn3	NM_001111164	**0.016**	**2.3**	**up**
A_15_P740066	Gpia	NM_144763	**0.017**	**2.0**	**up**
A_15_P772286	LOC558654	NM_001099992	**0.014**	**2.2**	**up**
A_15_P111839	Npl	NM_207051	**0.022**	**3.0**	**up**
A_15_P728465	zgc:103652	NM_001007364	**0.014**	**2.7**	**up**
A_15_P504897	zgc:91926	NM_001003453	**0.017**	**2.3**	**up**
A_15_P496192	cln6	NM_001005982	**0.015**	**2.2**	**up**
A_15_P106840	b9d1	NM_001024373	**0.016**	**2.3**	**up**
A_15_P753361	etf1	NM_201460	**0.023**	**2.1**	**up**
A_15_P757121	nprl2	NM_001045847	**0.018**	**2.2**	**up**
A_15_P226676	isoc2	NM_001079953	**0.019**	**2.1**	**up**
A_15_P758721			**0.018**	**4.2**	**up**
A_15_P747471	pygl	NM_001008538	**0.014**	**2.3**	**up**
A_15_P764126			**0.025**	**2.2**	**up**
A_15_P667626	Ntm	NM_001040293	**0.013**	**2.2**	**up**
A_15_P104758	scdb	NM_001020705	**0.013**	**2.1**	**up**
A_15_P626551	dnajc5aa	NM_001002464	**0.015**	**2.7**	**up**
A_15_P142406	zgc:101798	NM_001007396	**0.019**	**2.1**	**up**
A_15_P736007	psma8	NM_213166	**0.015**	**2.1**	**up**
A_15_P613582			**0.014**	**2.3**	**up**
A_15_P516477	zgc:194210	NM_001130609	**0.016**	**2.2**	**up**
A_15_P462225		CK030228	**0.020**	**2.2**	**up**
A_15_P186071		XM_677756	**0.015**	**2.0**	**up**
A_15_P432925		EE694161	**0.018**	**2.0**	**up**

A_15_P625953	cyp2p7	NM_001083049	**0.015**	**4.9**	**up**
A_15_P221356		DT878895	**0.016**	**2.2**	**up**
A_15_P347455	mga	NM_001170739	**0.018**	**2.1**	**up**
A_15_P336785	ywhai	NM_212757	**0.014**	**2.2**	**up**
A_15_P285246	fbln5	NM_001005979	**0.019**	**2.6**	**up**
A_15_P721411	cd9b	NM_213428	**0.014**	**2.2**	**up**
A_15_P446672			**0.014**	**3.3**	**up**
A_15_P724976	fth1b	NM_001004562	**0.016**	**2.0**	**up**
A_15_P656226	pcdh1g9	NM_001024109	**0.013**	**2.0**	**up**
A_15_P130531	abcc8	NM_001172647	**0.015**	**2.2**	**up**
A_15_P163386	tm2d1	NM_001099263	**0.013**	**2.0**	**up**
A_15_P208066	impdh2	NM_201464	**0.015**	**2.3**	**up**
A_15_P107464	zgc:110143	NM_001013518	**0.018**	**2.1**	**up**
A_15_P145251		XM_003201552	**0.019**	**2.0**	**up**
A_15_P144051	hmgb3a	NM_001122836	**0.014**	**2.1**	**up**
A_15_P270031			**0.017**	**2.3**	**up**
A_15_P196331	camk2d2	NM_001002542	**0.020**	**2.0**	**up**
A_15_P766806	wu:fj39h06	CA473253	**0.016**	**2.5**	**up**
A_15_P281991			**0.021**	**2.3**	**up**
A_15_P413845	yipf1	NM_001003991	**0.019**	**2.1**	**up**
A_15_P165896		XM_684948	**0.015**	**3.2**	**up**
A_15_P731811	tram1	NM_153669	**0.015**	**2.2**	**up**
A_15_P203071		BC091841	**0.017**	**2.3**	**up**
A_15_P540817	sgk2a	NM_001113622	**0.019**	**2.2**	**up**
A_15_P284911	psmb1	NM_001003889	**0.014**	**2.1**	**up**
A_15_P721431		BC059691	**0.015**	**2.1**	**up**
A_15_P571715		XM_001336484	**0.014**	**2.2**	**up**
A_15_P113421	ndufaf1	NM_001013308	**0.016**	**2.1**	**up**
A_15_P620491	LOC558654	NM_001099992	**0.014**	**2.4**	**up**
A_15_P255601	lsm14ab	NM_214745	**0.015**	**2.4**	**up**
A_15_P629116	cldnk	NM_001003464	**0.013**	**2.3**	**up**
A_15_P714511	reep5	NM_200058	**0.017**	**2.5**	**up**
A_15_P143506	fgf22	NM_001040095	**0.014**	**2.1**	**up**
A_15_P365060	gsk3a	NM_131390	**0.022**	**2.2**	**up**

A_15_P581262	dda1	NM_213127	**0.016**	**2.1**	**up**
A_15_P161796	lgi2a	NM_001039641	**0.014**	**2.0**	**up**
A_15_P517287		CF997136	**0.018**	**2.0**	**up**
A_15_P737108	calm2b	NM_214736	**0.018**	**2.1**	**up**
A_15_P517467	ptprna	NM_001130588	**0.020**	**2.0**	**up**
A_15_P115781	Lipf	NM_213404	**0.015**	**2.1**	**up**
A_15_P687126	Ppib	NM_213019	**0.015**	**2.1**	**up**
A_15_P108818	mppe1	NM_001017701	**0.015**	**2.1**	**up**
A_15_P737676	pgam1a	NM_198804	**0.015**	**2.0**	**up**
A_15_P142396	rln3a	NM_001037803	**0.013**	**2.1**	**up**
A_15_P193431	Vcp	NM_201481	**0.014**	**2.0**	**up**
A_15_P656956	whsc1	NM_001082551	**0.013**	**2.0**	**up**
A_15_P642691	zgc:162824	NM_001083574	**0.022**	**4.4**	**up**
A_15_P761776	crhbp	NM_001003459	**0.018**	**2.1**	**up**
A_15_P108607		XM_001339501	**0.018**	**2.1**	**up**
A_15_P410880		XM_691028	**0.013**	**2.2**	**up**
A_15_P721231	sc5dl	NM_001004630	**0.015**	**2.8**	**up**
A_15_P405435	ulk1a	NM_001130631	**0.018**	**2.2**	**up**
A_15_P654501	pnrc2	NM_001130192	**0.023**	**2.3**	**up**
A_15_P286772		CK027590	**0.024**	**2.2**	**up**
A_15_P763191	acox1	NM_001005933	**0.013**	**2.8**	**up**
A_15_P529507		XM_001918925	**0.019**	**2.1**	**up**
A_15_P366225	asb5	NM_001017753	**0.018**	**2.1**	**up**
A_15_P721581	cd99l2	NM_194369	**0.015**	**2.2**	**up**
A_15_P194046	riok3	NM_001003614	**0.018**	**2.2**	**up**
A_15_P111605	abcg4b	NM_001111212	**0.013**	**2.7**	**up**
A_15_P272401		XM_688733	**0.016**	**4.2**	**up**
A_15_P761481	flj13639	NM_203511	**0.013**	**2.2**	**up**
A_15_P345240			**0.021**	**2.2**	**up**
A_15_P508674		FJ392638	**0.014**	**2.6**	**up**
A_15_P473645	Apc	NM_001143840	**0.014**	**2.2**	**up**
A_15_P167271		XM_677874	**0.013**	**2.0**	**up**
A_15_P506242		XM_001923782	**0.017**	**2.0**	**up**
A_15_P274916		EG577327	**0.013**	**2.1**	**up**

A_15_P140636	plxna3	NM_001098489	**0.014**	**2.1**	**up**
A_15_P720031	arhgdia	NM_213461	**0.016**	**2.0**	**up**
A_15_P741531	zgc:112300	NM_001030268	**0.013**	**2.0**	**up**
A_15_P740106		CN024664	**0.013**	**4.4**	**up**
A_15_P670096		XM_003198822	**0.016**	**2.4**	**up**
A_15_P164731		XM_683079	**0.024**	**2.4**	**up**
A_15_P202431		XM_002665135	**0.018**	**2.5**	**up**
A_15_P256286	zgc:154067	NM_001076651	**0.015**	**2.4**	**up**
A_15_P666231	rgma	BC165864	**0.014**	**2.0**	**up**
A_15_P182346	jarid2b	NM_001202459	**0.024**	**2.1**	**up**
A_15_P746316	eif5	NM_199591	**0.016**	**2.2**	**up**
A_15_P116273	nsa2	NM_199568	**0.015**	**2.0**	**up**
A_15_P143286	plxna4	NM_001004495	**0.014**	**2.1**	**up**
A_15_P730386	aldh7a1	NM_212724	**0.020**	**2.1**	**up**
A_15_P755155	hmga1a	NM_213168	**0.021**	**2.4**	**up**
A_15_P226536		DN765442	**0.014**	**2.4**	**up**
A_15_P729551	vta1	NM_213140	**0.014**	**2.3**	**up**
A_15_P100705	zgc:101798	NM_001007396	**0.016**	**2.3**	**up**
A_15_P110984	crym	NM_001080687	**0.013**	**4.2**	**up**
A_15_P652406	zgc:172128	NM_001114473	**0.018**	**2.4**	**up**
A_15_P223936	tmed2	NM_199548	**0.015**	**2.0**	**up**
A_15_P732456	spna2	NM_001098488	**0.015**	**2.2**	**up**
A_15_P169911	cdc42	NM_001018120	**0.017**	**2.1**	**up**
A_15_P397885	hapln2	NM_001082816	**0.020**	**2.3**	**up**
A_15_P192056	si:dkey-19f21.3	NM_001128524	**0.014**	**2.1**	**up**
A_15_P413280	zgc:162962	NM_001110482	**0.021**	**2.3**	**up**
A_15_P650841	arl8ba	NM_205566	**0.015**	**3.2**	**up**
A_15_P627191	tpbgl	NM_194392	**0.021**	**2.6**	**up**
A_15_P287396	zgc:73275	NM_200773	**0.013**	**2.1**	**up**
A_15_P772181			**0.017**	**3.3**	**up**
A_15_P553762	ankra2	NM_213129	**0.019**	**2.2**	**up**
A_15_P305221	LOC100149820	NM_001145633	**0.014**	**2.4**	**up**
A_15_P475682	gad2	NM_001017708	**0.016**	**2.2**	**up**
A_15_P201521	tmeff2a	NM_001128365	**0.016**	**2.0**	**up**

A_15_P669551	zgc:194209	NM_001130792	**0.022**	**2.8**	**up**
A_15_P466875		XR_082585	**0.013**	**2.4**	**up**

Table S11: Significantly altered transcripts **in liver** of adult zebrafish males are shown after exposure to 383 µg/L OC revealed by microarray analysis (Agilent 1-color) and GeneSpring GX 12.0. In liver, 136 transcripts are significantly altered over dmso by setting the threshold at FC ≥ 1 and $p \leq 0.05$. Expression levels are expressed as absolute fold-change (FC abs).

ProbeName	GeneSymbol	GenbankAccession	*p*-value	FC (abs)	Regulation
A_15_P684061	rhbdf1	NM_001002228	**5.7E-04**	**1.9**	**down**
A_15_P106013	cx32.3	NM_199612	**1.0E-03**	**1.6**	**down**
A_15_P700402	zgc:110380	NM_001017660	**1.2E-03**	**2.0**	**down**
A_15_P103083	dhcr7	NM_201330	**1.3E-03**	**1.7**	**down**
A_15_P659706	zgc:110380	NM_001017660	**9.4E-04**	**2.0**	**down**
A_15_P681476			**5.8E-04**	**8.1**	**down**
A_15_P282191	papss2b	NM_212562	**1.0E-03**	**1.4**	**down**
A_15_P112648		XM_003199895	**6.3E-04**	**1.5**	**down**
A_15_P740056	zgc:85752	NM_212932	**1.1E-03**	**1.3**	**down**
A_15_P574807	ppdpfa	NM_200079	**1.1E-03**	**2.0**	**down**
A_15_P402510	mg:cb01g09	CN330459	**1.3E-03**	**1.9**	**down**
A_15_P157981	hyou1	NM_212703	**5.7E-04**	**2.0**	**down**
A_15_P434245	antxr2a	NM_001044709	**1.3E-03**	**1.9**	**down**
A_15_P117051	si:dkeyp-86e4.1	NM_001099255	**1.1E-03**	**1.4**	**down**
A_15_P734956		EH542688	**7.9E-04**	**3.0**	**down**
A_15_P151356	h1fx	NM_199276	**1.0E-03**	**1.9**	**down**
A_15_P141931	cebpa	NM_131885	**9.4E-04**	**2.4**	**down**
A_15_P237746		XM_001918989	**1.1E-03**	**1.9**	**down**
A_15_P605587		XM_003199654	**9.5E-04**	**2.0**	**down**
A_15_P737206	apoeb	NM_131098	**1.1E-03**	**1.5**	**down**
A_15_P754126	khdrbs1a	NM_130925	**1.2E-03**	**1.2**	**down**
A_15_P473355		BC115201	**5.7E-04**	**1.8**	**down**
A_15_P751356	col1a1a	NM_199214	**1.3E-03**	**2.5**	**down**
A_15_P143146	si:dkey-177p2.6	NM_001075116	**1.2E-03**	**1.4**	**down**

A_15_P576397	junb	NM_213556	**1.2E-03**	**2.3**	**down**
A_15_P160171		DQ017623	**1.1E-03**	**5.9**	**down**
A_15_P731931	ctsl1a	NM_212584	**5.8E-04**	**1.5**	**down**
A_15_P698971	zgc:113377	BC152494	**1.3E-03**	**1.2**	**down**
A_15_P633546	zgc:173585	NM_001102632	**1.2E-03**	**1.4**	**down**
A_15_P208226	slc16a10	NM_001080028	**3.7E-04**	**2.3**	**down**
A_15_P433820		BC131854	**1.1E-03**	**2.0**	**down**
A_15_P622641	bhlhe41	NM_001039107	**1.0E-03**	**2.3**	**down**
A_15_P115201	tnip1	NM_001079952	**5.8E-04**	**1.6**	**down**
A_15_P767681	ccnb1	NM_131513	**1.2E-03**	**2.7**	**down**
A_15_P202336		XM_682931	**1.0E-03**	**1.4**	**down**
A_15_P661591	pafah1b1b	NM_201346	**5.7E-04**	**1.3**	**down**
A_15_P723581	LOC561719	BC142830	**1.2E-03**	**1.3**	**down**
A_15_P108040	klf2a	NM_131856	**1.3E-03**	**1.4**	**down**
A_15_P103112	slc43a2a	NM_200251	**1.1E-03**	**1.7**	**down**
A_15_P290396	mid1ip1b	NM_200832	**1.2E-03**	**2.6**	**down**
A_15_P629251	col1a1a	NM_199214	**1.3E-03**	**2.3**	**down**
A_15_P641526	zgc:112052	NM_001017665	**1.1E-03**	**1.5**	**down**
A_15_P420580		XM_002665309	**6.3E-04**	**1.3**	**down**
A_15_P731386		BC161671	**1.2E-03**	**1.2**	**down**
A_15_P301271	LOC569089	DT078727	**1.1E-03**	**2.3**	**up**
A_15_P665511	si:ch211-154o6.6	BC153393	**1.2E-03**	**1.3**	**up**
A_15_P402455	si:ch1073-170o4.1	CO355144	**1.4E-04**	**3.1**	**up**
A_15_P768023	rbp4	NM_130920	**3.1E-04**	**2.2**	**up**
A_15_P104141	rbp4	NM_130920	**5.0E-04**	**2.2**	**up**
A_15_P120494	msi2b	NM_201109	**1.3E-03**	**1.5**	**up**
A_15_P699876	zgc:92161	NM_001004556	**8.8E-04**	**2.4**	**up**
A_15_P621106	hif1al	NM_200405	**1.4E-04**	**2.4**	**up**
A_15_P654116	cyp2x12	NM_001079853	**3.7E-04**	**2.3**	**up**
A_15_P111718	zgc:92161	NM_001004556	**6.3E-04**	**2.2**	**up**
A_15_P365960	slc6a13	NM_001004533	**1.1E-03**	**3.0**	**up**
A_15_P771326			**3.1E-04**	**2.9**	**up**
A_15_P380265	zgc:85939	NM_212938	**1.3E-03**	**1.3**	**up**
A_15_P196336	rcl1	NM_001003865	**1.0E-03**	**1.8**	**up**

A_15_P721241	zgc:64095	NM_213142	**1.2E-03**	**1.5**	**up**
A_15_P119608	mettl7a	NM_001080039	**1.0E-03**	**1.6**	**up**
A_15_P101798	terfa	NM_173243	**1.3E-03**	**1.4**	**up**
A_15_P404495	hif1al	NM_200405	**8.8E-04**	**2.1**	**up**
A_15_P101222	rcl1	NM_001003865	**1.3E-03**	**1.7**	**up**
A_15_P496462	dcaf5	NM_001077552	**1.1E-03**	**1.5**	**up**
A_15_P754216	cyp2x12	NM_001079853	**3.1E-04**	**2.3**	**up**
A_15_P115867	rorab	NM_201067	**1.1E-03**	**1.9**	**up**
A_15_P104052	rorab	NM_201067	**1.0E-03**	**1.8**	**up**
A_15_P115269	nr1i2 (pxr)	NM_001098617	**1.2E-03**	**1.8**	**up**
A_15_P141561	synj2bp	NM_199917	**5.7E-04**	**1.3**	**up**
A_15_P669857	rbp4	CF595094	**3.7E-04**	**2.2**	**up**
A_15_P109312	ugdh	NM_001110402	**1.1E-03**	**1.5**	**up**
A_15_P117750	ncor1	NM_200276	**5.7E-04**	**1.9**	**up**
A_15_P663231	slc6a13	NM_001004533	**1.2E-03**	**3.0**	**up**
A_15_P657811		BC152165	**1.3E-03**	**1.2**	**up**
A_15_P206291	zgc:136652	NM_001044325	**1.1E-03**	**1.2**	**up**
A_15_P678091			**7.6E-04**	**1.4**	**up**
A_15_P706116	si:dkey-236e20.2	NM_001082876	**1.4E-04**	**4.9**	**up**
A_15_P547869	LOC798811	NM_001113639	**1.3E-03**	**2.1**	**up**
A_15_P597597	wu:fb39d01	CN500264	**1.1E-03**	**1.4**	**up**
A_15_P671736	zgc:136564	NM_001083558	**6.3E-04**	**1.4**	**up**
A_15_P623886	ypel3	NM_212790	**6.3E-04**	**1.7**	**up**
A_15_P139926	tchp	NM_001040343	**1.1E-03**	**1.4**	**up**
A_15_P403780	mepce	NM_001128529	**1.0E-03**	**1.5**	**up**
A_15_P676226		XM_701300	**1.1E-03**	**2.0**	**up**
A_15_P210836	zgc:92090	NM_001002367	**5.7E-04**	**2.1**	**up**
A_15_P740891	zgc:92090	NM_001002367	**1.0E-03**	**2.0**	**up**
A_15_P108159	tbxas1	NM_205609	**1.0E-03**	**1.8**	**up**
A_15_P195266	si:ch211-219a15.3	NM_001045007	**1.2E-03**	**3.2**	**up**
A_15_P626011	rorab	NM_201067	**1.1E-03**	**1.9**	**up**
A_15_P626941	LOC798811	NM_001113639	**1.2E-03**	**2.0**	**up**
A_15_P144901	pc	BC091826	**1.2E-03**	**2.3**	**up**
A_15_P150786	arg2	NM_199611	**5.8E-04**	**2.5**	**up**

A_15_P206736	si:dkey-236e20.2	NM_001082876	**5.7E-04**	**3.7**	**up**
A_15_P413855	hpx	NM_001111147	**8.8E-04**	**1.3**	**up**
A_15_P101228	manea	NM_001004566	**5.7E-04**	**1.6**	**up**
A_15_P208446	uspl1	NM_001003880	**5.7E-04**	**1.5**	**up**
A_15_P488730			**3.7E-04**	**2.8**	**up**
A_15_P101039	arntl1a	NM_131577	**7.1E-04**	**2.1**	**up**
A_15_P164561	rtn3	NM_201072	**1.3E-03**	**1.5**	**up**
A_15_P162226	zgc:113317	NM_001033725	**1.0E-03**	**5.3**	**up**
A_15_P174806	hhip	NM_001080012	**1.1E-03**	**1.7**	**up**
A_15_P103654	dstyk	NM_205627	**1.2E-03**	**1.4**	**up**
A_15_P121028	smad7	NM_175082	**8.5E-04**	**1.4**	**up**
A_15_P657176	cul1b	NM_213495	**1.3E-03**	**1.1**	**up**
A_15_P215501	ncor1	NM_200276	**6.0E-04**	**1.4**	**up**
A_15_P154051	bbox1	NM_001017717	**1.1E-03**	**3.4**	**up**
A_15_P168101	cry1b	NM_131790	**1.1E-03**	**2.2**	**up**
A_15_P172871	zgc:100869	NM_001003756	**1.3E-03**	**1.3**	**up**
A_15_P103787	spsb4b	NM_001003474	**1.2E-03**	**1.4**	**up**
A_15_P210196		BC092939	**1.1E-03**	**1.3**	**up**
A_15_P527772	cyp3a65	NM_001037438	**1.2E-03**	**1.8**	**up**
A_15_P472900	polr3e	NM_212754	**1.2E-03**	**1.4**	**up**
A_15_P625896	ncor1	NM_200276	**3.1E-04**	**1.6**	**up**
A_15_P591077		XM_001922652	**5.8E-04**	**1.3**	**up**
A_15_P271141	zgc:175096	NM_001114425	**1.2E-03**	**1.3**	**up**
A_15_P106916	sfpq	NM_213278	**3.1E-04**	**1.6**	**up**
A_15_P103005	mao	NM_212827	**1.0E-03**	**1.7**	**up**
A_15_P392430	wu:fj88a02	EH500189	**6.3E-04**	**1.6**	**up**
A_15_P634631	ugt2b1	NM_001177338	**1.1E-03**	**1.8**	**up**
A_15_P201211		XM_001333308	**1.4E-04**	**22.1**	**up**
A_15_P723632	vtg7	NM_001102671	**1.3E-03**	**1.8**	**up**
A_15_P104922	acox1	NM_001005933	**8.8E-04**	**2.0**	**up**
A_15_P660276	lancl1	NM_001009891	**1.3E-03**	**1.4**	**up**
A_15_P171327	ppp1caa	BC070008	**1.3E-03**	**1.4**	**up**
A_15_P621844	cyp2x12	NM_001079853	**1.3E-03**	**2.7**	**up**
A_15_P517187	gata6	NM_131557	**5.8E-04**	**1.2**	**up**

A_15_P149541	Lpl	NM_131127	**1.3E-03**	**1.7**	**up**
A_15_P419910	cyp4v7	NM_001079996	**1.0E-03**	**1.8**	**up**
A_15_P110256	si:dkey-181m9.10	NM_001111188	**1.2E-03**	**1.2**	**up**
A_15_P628261	cry1a	NM_001077297	**1.0E-03**	**1.4**	**up**
A_15_P103896	dctn2	NM_201166	**1.2E-03**	**1.2**	**up**
A_15_P281686	hif1al	NM_200405	**3.1E-04**	**2.7**	**up**
A_15_P576707	cdkn1d	BC124236	**1.1E-03**	**2.0**	**up**
A_15_P171131	zgc:153507	NM_001077736	**8.8E-04**	**1.3**	**up**
A_15_P206546	Apobl	NM_001030062	**1.0E-03**	**1.2**	**up**
A_15_P110663	luc7l	NM_001002116	**8.4E-04**	**1.2**	**up**

Table S12: Ten most significantly altered GO processes by 383 µg/L OC in adult male zebrafish brain and liver as obtained by MetaCore pathway analysis on 628 transcripts in brain (FC ≥ 2, p ≤ 0.05) and 136 transcripts in liver (FC ≥ 1, p ≤ 0.05).

	GO processes	Total [a]	p-value [b]	in data [c]	Genes affected by 383 µg/L OC [d]
BRAIN	developmental processes	5334	2.78E-10	80	JARID2, eIF4A, HSP90, Annexin XIII, PACAP, PU.1, eIF5A, RhoB, RBP2, LIPA, eIF4A1, RhoG, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, FAIM2, PEDF (serpinF1), Cx30, HPCA, R-spondin 3, LOXL2, HSP90 beta, MMP-9, PAX7, IMD2, MEOX1, Reticulocalbin 1, CTGF, Mesogenin 1, BLIMP1 (PRDI-BF1), KIAA1333, Carboxypeptidase M, TAK1(MAP3K7), Lysyl oxidase, Dynamin-3, PDGF-R-alpha, DGAT2, LECT2, COBL, PCDHA3, HMG4, Ionotropic glutamate receptor, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, Asporin, Thyroid hormone receptor, ST6GAL2, FUT7, Histone H3, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, DHC24, BMP9, Histone H3.3, Calpain 2(m), B9D1, Calcineurin B (regulatory), Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, MYCBP2, GSK3 alpha, Galpha(t)-specific GPCRs, COL9A2, SCD, CD166, Foxb2, Galpha(q)-specific PACAP GPCR, Midkine, IGFBP7/8, SOCS3, Plexin A4, Dynamin
	anatomical structure development	4620	2.83E-10	73	JARID2, eIF4A, HSP90, PACAP, PU.1, RhoB, RBP2, LIPA, eIF4A1, RhoG, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, FAIM2, PEDF (serpinF1), Cx30, HPCA, R-spondin 3, HSP90 beta, MMP-9, PAX7, IMD2, MEOX1, Reticulocalbin 1, CTGF, Mesogenin 1, BLIMP1 (PRDI-BF1), KIAA1333, Carboxypeptidase M, TAK1(MAP3K7), Lysyl oxidase, Dynamin-3, PDGF-R-alpha, DGAT2, LECT2, COBL, PCDHA3, Ionotropic glutamate receptor, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, Asporin, Thyroid hormone receptor, FUT7, Histone H3, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, DHC24, BMP9, Histone H3.3, Calpain 2(m), B9D1, Calcineurin B (regulatory), Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, MYCBP2, GSK3 alpha, Galpha(t)-specific GPCRs, COL9A2, CD166, Foxb2, Midkine, IGFBP7/8, SOCS3, Plexin A4, Dynamin
	system development	4195	5.82E-10	68	JARID2, eIF4A, HSP90, PACAP, PU.1, RhoB, RBP2, LIPA, eIF4A1, RhoG, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, FAIM2, PEDF (serpinF1), Cx30, HPCA, R-spondin 3, HSP90 beta, MMP-9, PAX7, IMD2, Reticulocalbin 1, CTGF, BLIMP1 (PRDI-BF1), TAK1(MAP3K7), Lysyl oxidase, Dynamin-3, PDGF-R-alpha, DGAT2, LECT2, COBL, PCDHA3, Ionotropic glutamate receptor, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, Asporin, Thyroid hormone receptor, FUT7, Histone H3, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, DHC24, BMP9, Histone H3.3, Calpain 2(m), Calcineurin B (regulatory), Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, MYCBP2, GSK3 alpha, Galpha(t)-specific GPCRs, COL9A2, CD166, Foxb2, Midkine, IGFBP7/8, SOCS3, Plexin A4, Dynamin
	multicellular organismal process	7153	1.29E-09	95	JARID2, eIF4A, HSP90, CLN6, GluR6, PACAP, HSP47, PU.1, RhoB, RBP2, LIPA, Galpha(q)-specific putative/unclassified GPCRs, CRYM, eIF4A1, RhoG, Myelin P0 protein, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, CXCR3, FAIM2, PEDF (serpinF1), Cx30, HPCA, RGR, R-spondin 3, HSP90 beta, MMP-9, PAX7, IMD2, MEOX1, nAChR gamma, Reticulocalbin 1, IRF7, CTGF, Mesogenin 1, BLIMP1 (PRDI-BF1), ATP1A3, KIAA1333, TAK1(MAP3K7), CaMK II delta, Lysyl oxidase, nAChR gamma/epsilon, Dynamin-3, PDGF-R-alpha, DGAT2, LECT2, COBL, PCDHA3, HMG4, Ionotropic glutamate receptor, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, P2X3, Asporin, Thyroid hormone receptor, ST6GAL2, FUT7, Histone H3, COCH, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, Melatonin receptor 1B, GPR39, DHC24, BMP9, Histone H3.3, Calpain 2(m), Calcineurin B (regulatory), Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, MYCBP2, GSK3 alpha, Galpha(t)-specific GPCRs, COL9A2, Coagulation factor V, CD166, Kv2.1, GAD2, Foxb2, Galpha(q)-specific PACAP GPCR, Midkine, IGFBP7/8, SOCS3, Plexin A4, HBE, Dynamin
	multicellular organismal development	4895	1.58E-09	74	JARID2, eIF4A, HSP90, PACAP, PU.1, RhoB, RBP2, LIPA, eIF4A1, RhoG, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, FAIM2, PEDF (serpinF1), Cx30, HPCA, R-spondin 3, HSP90 beta, MMP-9, PAX7, IMD2, MEOX1, Reticulocalbin 1, CTGF, Mesogenin 1, BLIMP1 (PRDI-BF1), KIAA1333, TAK1(MAP3K7), Lysyl oxidase, Dynamin-3, PDGF-R-alpha, DGAT2, LECT2, COBL, PCDHA3, HMG4, Ionotropic glutamate receptor, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, Asporin, Thyroid hormone receptor, ST6GAL2, FUT7, Histone H3, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, DHC24, BMP9, Histone H3.3, Calpain 2(m), Calcineurin B (regulatory), Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, MYCBP2, GSK3 alpha, Galpha(t)-specific GPCRs, COL9A2, CD166, Foxb2, Galpha(q)-specific PACAP GPCR, Midkine, IGFBP7/8, SOCS3, Plexin A4, Dynamin
	response to steroid hormone stimulus	662	1.39E-08	22	HSP90, PGHD, PACAP, PDGF receptor, PEDF (serpinF1), PPCKM, MMP-9, CTGF, ERK7 (MAPK15), Lysyl oxidase, PDGF-R-alpha, Ionotropic glutamate receptor, Thyroid hormone receptor, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, Kainate receptor, ASH2L, Galpha(q)-specific PACAP GPCR, Midkine, IGFBP7/8, SOCS3
	anatomical structure morphogenesis	2277	1.40E-08	44	eIF4A, HSP90, PU.1, RhoB, LIPA, eIF4A1, RhoG, GSK3 alpha/beta, PDGF receptor, Cx30, R-spondin 3, HSP90 beta, MMP-9, PAX7, MEOX1, CTGF, Mesogenin 1, Carboxypeptidase M, TAK1(MAP3K7), PDGF-R-alpha, COBL, Plexin A3, COL11A1, CDC42, ADAM8, Chondromodulin-I, Cyr61, Thyroid hormone receptor, NUR77, TR-beta1, Galpha(q)-specific peptide GPCRs, BMP9, Calpain 2(m), B9D1, Calcineurin B (regulatory), AP complex 2 small (sigma) chain, Calcineurin B1, MYCBP2, GSK3 alpha, COL9A2, CD166, IGFBP7/8, SOCS3, Plexin A4
	positive regulation of cellular process	3704	7.56E-08	58	JARID2, HSP90, CLN6, PSMC6, GluR6, PACAP, PU.1, eIF5A, RhoB, Calpain 1(mu), Galpha(q)-specific putative/unclassified GPCRs, RhoG, GSK3 alpha/beta, PDGF receptor, PEDF (serpinF1), CD81, HSP90 beta, MMP-9, MEOX1, IRF7, CTGF, BLIMP1 (PRDI-BF1), ERK7 (MAPK15), TAK1(MAP3K7), CaMK II delta, CGI-141, PDGF-R-alpha, Cell death activator CIDE-3, Ionotropic glutamate receptor, Plexin A3, CDC42, ADAM8, Cyr61, P2X3, Thyroid hormone receptor, VIP receptor 2, Villin 1, NUR77, TR-beta1, CCDC98 (Abraxas), Galpha(q)-specific peptide GPCRs, GPR39, BMP9, TM2D1, Calcineurin B (regulatory), Kainate receptor, ASH2L, Calcineurin B1, CIDEC, VCP, GSK3 alpha, SCD, Galpha(q)-specific PACAP GPCR, Midkine, IGFBP7/8, SOCS3, HURP, Dynamin
	organ development	3073	1.07E-07	51	JARID2, eIF4A, HSP90, PACAP, PU.1, RBP2, LIPA, eIF4A1, GSK3 alpha/beta, LOC440093, BCAN, PDGF receptor, PEDF (serpinF1), Cx30, HPCA, R-spondin 3, HSP90 beta, MMP-9, PAX7, IMD2, Reticulocalbin 1, CTGF, BLIMP1 (PRDI-BF1), Lysyl oxidase, PDGF-R-alpha, DGAT2, Plexin A3, COL11A1, CDC42, Chondromodulin-I, Cyr61, Asporin, Thyroid hormone receptor, FUT7, Histone H3, TR-beta1, Galpha(q)-specific peptide GPCRs, DHC24, BMP9, Histone H3.3, Calpain 2(m), Calcineurin B (regulatory), ASH2L, Calcineurin B1, GSK3 alpha, Galpha(t)-specific GPCRs, Foxb2, Midkine, IGFBP7/8, SOCS3, Plexin A4
	cellular component organization	4386	3.91E-07	63	JARID2, HSP90, CLN6, GluR6, HSP47, NDUFAF1, PU.1, RhoB, LIPA, RhoG, Myelin P0 protein, GSK3 alpha/beta, LOC440093, PDGF receptor, PBK, LOXL2, HSP90 beta, MMP-9, Anillin, SET1A, IMD2, Septin 11, CTGF, TAK1(MAP3K7), Lysyl oxidase, Dynamin-3, PDGF-R-alpha, Ionotropic glutamate receptor, Plexin A3, COL11A1, Tim23, CDC42, PREI3, ADAM8, Chondromodulin-I, Cyr61, P2X3, Villin 1, Histone H3, CCDC98 (Abraxas), Galpha(q)-specific peptide GPCRs, DHC24, Histone H3.3, B9D1, REDD1, Calcineurin B (regulatory), MAT1A, Kainate receptor, AP complex 2 small (sigma) chain, ASH2L, Calcineurin B1, VCP, MYCBP2, COL9A2, CD166, Kv2.1, MTO1, IGFBP7/8, Fibulin-5, Plexin A4, Protein MTO1 homolog, mitochondrial precursor, HURP, Dynamin
LIVER	xenobiotic metabolic process	170	1.82E-06	6	MAOB, PXR, MAO, UGDH, THAS, PAPSS2
	cellular response to xenobiotic stimulus	171	1.89E-06	6	MAOB, PXR, MAO, UGDH, THAS, PAPSS2
	urea cycle	12	2.11E-06	3	C/EBP, ARG2, C/EBPalpha
	response to xenobiotic stimulus	175	2.16E-06	6	MAOB, PXR, MAO, UGDH, THAS, PAPSS2
	amide biosynthetic process	14	3.48E-06	3	C/EBP, ARG2, C/EBPalpha
	urea metabolic process	15	4.34E-06	3	C/EBP, ARG2, C/EBPalpha
	nitrogen cycle metabolic process	16	5.34E-06	3	C/EBP, ARG2, C/EBPalpha
	lung development	229	1.01E-05	6	RBP4, C/EBP, ARG2, C/EBPalpha, HIP, DHCR7
	respiratory tube development	232	1.09E-05	6	RBP4, C/EBP, ARG2, C/EBPalpha, HIP, DHCR7
	response to vitamin B2	3	1.39E-05	2	C/EBP, C/EBPalpha

[a]: Total number of genes involved within the particular GO process; [b]: GO processes are listed by significance; [c]: Number of altered genes involved within GO process by 383 µg/L OC; [d]: Name and symbol, respectively, of affected genes by 383 µg/L OC.

Table S13: Affected **pathway maps** (human based) are shown for adult zebrafish exposed to 383 µg/L OC. The list is sorted by significance (*p*-value) and shows the involved number of altered transcripts within each pathway. Results are obtained from MetaCore software after enrichment analysis of differentially expressed genes revealed by GeneSpring software.

	Pathway maps	Total [a]	p-value [b]	in data [c]	Genes affected by 383 µg/L OC [d]
BRAIN	Hypoxia-induced EMT in cancer and fibrosis	9	1.92E-05	3	LOXL2, CTGF, Lysyl oxidase
	Cytoskeleton remodeling_Role of PDGFs in cell migration	24	4.33E-04	3	PDGF receptor, PDGF-R-alpha, CDC42
	Signal transduction_JNK pathway	42	2.27E-03	3	TAK1(MAP3K7), GCKR(MAP4K5), CDC42
	Development_WNT5A signaling	46	2.95E-03	3	TAK1(MAP3K7), CDC42, Calcineurin B (regulatory)
	Some pathways of EMT in cancer cells	51	3.96E-03	3	PDGF receptor, TAK1(MAP3K7), PDGF-R-alpha
	Translation _Regulation of EIF4F activity	53	4.41E-03	3	eIF4A, TAK1(MAP3K7), CDC42
	Transport_Macropinocytosis regulation by growth factors	63	7.16E-03	3	PDGF receptor, PDGF-R-alpha, CDC42
	Reproduction_GnRH signaling	72	1.03E-02	3	PACAP, CDC42, NUR77
	Translation _Regulation of translation initiation	27	1.23E-02	2	eIF4A, eIF1
	Immune response_Antigen presentation by MHC class I	28	1.32E-02	2	HSP90, HSP90 beta
LIVER	Role of Diethylhexyl Phthalate and Tributyltin in fat cell differentiation	29	5.29E-05	3	LPL, C/EBPalpha, N-CoR
	Putative pathways for stimulation of fat cell differentiation by Bisphenol A	32	3.04E-03	2	LPL, C/EBPalpha
	Signal transduction_Activin A signaling regulation	33	3.23E-03	2	SMAD7, N-CoR
	Transcription_Sin3 and NuRD in transcription regulation	38	4.26E-03	2	PSF, N-CoR
	Regulation of metabolism_Triiodothyronine and Thyroxine signaling	48	6.74E-03	2	TAT1, N-CoR
	Polyamine metabolism	68	1.32E-02	2	MAOB, ARG2
	Arginine metabolism/ Rodent version	97	2.58E-02	2	MAOB, ARG2
	Development_Role of nicotinamide in G-CSF-induced granulopoiesis	12	3.07E-02	1	C/EBPalpha
	Cell cycle_Chromosome condensation in prometaphase	21	5.31E-02	1	Histone H1
	Atherosclerosis_Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis	21	5.31E-02	1	LPL

[a]: Total number of genes involved within the particular pathway map; [b]: pathway maps are listed by significance; [c]: Number of altered genes involved within pathway map by 383 µg/L OC; [d]: Name and symbol, respectively, of affected genes by 383 µg/L OC.

Table S14: Affected **process networks** (human based) are shown for adult zebrafish exposed to 383 µg/L OC. The list is sorted by significance (*p*-value) and shows the involved number of altered transcripts within each process. Results are obtained from MetaCore software after enrichment analysis of differentially expressed genes revealed by GeneSpring software.

	Process networks	Total [a]	p-value [b]	in data [c]	Genes affected by 383 µg/L OC [d]
BRAIN	Cell adhesion_Platelet-endothelium-leucocyte interactions	175	4.59E-04	9	PDGF receptor, CD81, MMP-9, CTGF, PDGF-R-alpha, COL11A1, Cyr61, Coagulation factor V, IGFBP7/8
	Translation_Regulation of initiation	127	1.35E-03	7	eIF4A, eIF4A1, GSK3 alpha/beta, eIF3S1, TAK1(MAP3K7), CDC42, GSK3 alpha
	Development_EMT_Regulation of epithelial-to-mesenchymal transition	232	3.31E-03	9	PDGF receptor, LOXL2, MMP-9, CTGF, TAK1(MAP3K7), Lysyl oxidase, PDGF-R-alpha, CDC42, Calcineurin B1
	Protein folding_Folding in normal condition	119	4.65E-03	6	HSP90, HSP47, HSP90 beta, TCP1-eta, Calcineurin B (regulatory), Calcineurin B1
	Development_Cartilage development	66	1.09E-02	4	CTGF, COL11A1, Chondromodulin-I, COL9A2
	Protein folding_Response to unfolded proteins	69	1.27E-02	4	HSP90, HSP47, HSP90 beta, VCP
	Transport_Calcium transport	192	1.30E-02	7	Calpain 1(mu), HPCA, nAChR gamma, Reticulocalbin 1, Ionotropic glutamate receptor, P2X3, Calcineurin B1
	Reproduction_Gonadotropin regulation	199	1.55E-02	7	PACAP, Ionotropic glutamate receptor, CDC42, VIP receptor 2, NUR77, Kainate receptor, Galpha(q)-specific PACAP GPCR
	Protein folding_ER and cytoplasm	45	2.10E-02	3	HSP90, HSP47, VCP
	Reproduction_GnRH signaling pathway	166	2.18E-02	6	PACAP, Ionotropic glutamate receptor, CDC42, VIP receptor 2, Kainate receptor, Galpha(q)-specific PACAP GPCR
LIVER	Inflammation_IL-6 signaling	119	3.59E-03	3	C/EBP, C/EBPalpha, Hemopexin
	Signal transduction_ESR1-nuclear pathway	216	1.85E-02	3	MAOB, MGMT, N-CoR
	Cytoskeleton_Intermediate filaments	81	1.91E-02	2	DCTN2, Trichoplein
	Signal Transduction_BMP and GDF signaling	91	2.38E-02	2	SMAD7, C/EBPalpha
	DNA damage_DBS repair	116	3.73E-02	2	MGMT, PSF
	Signal transduction_Androgen receptor nuclear signaling	126	4.34E-02	2	C/EBPalpha, N-CoR
	Transcription_Chromatin modification	128	4.46E-02	2	Histone H1, N-CoR
	Signal Transduction_TGF-beta, GDF and Activin signaling	154	6.21E-02	2	SMAD7, C/EBPalpha
	Cell cycle_Mitosis	179	8.08E-02	2	DCTN2, Histone H1
	Transcription_Nuclear receptors transcriptional regulation	197	9.52E-02	2	PXR, N-CoR

[a]: Total number of genes involved within the particular process networks; [b]: process networks are listed by significance; [c]: Number of altered genes involved within process network by 383 µg/L OC; [d]: Name and symbol, respectively, of affected genes by 383 µg/L OC.

RT-qPCR analysis.

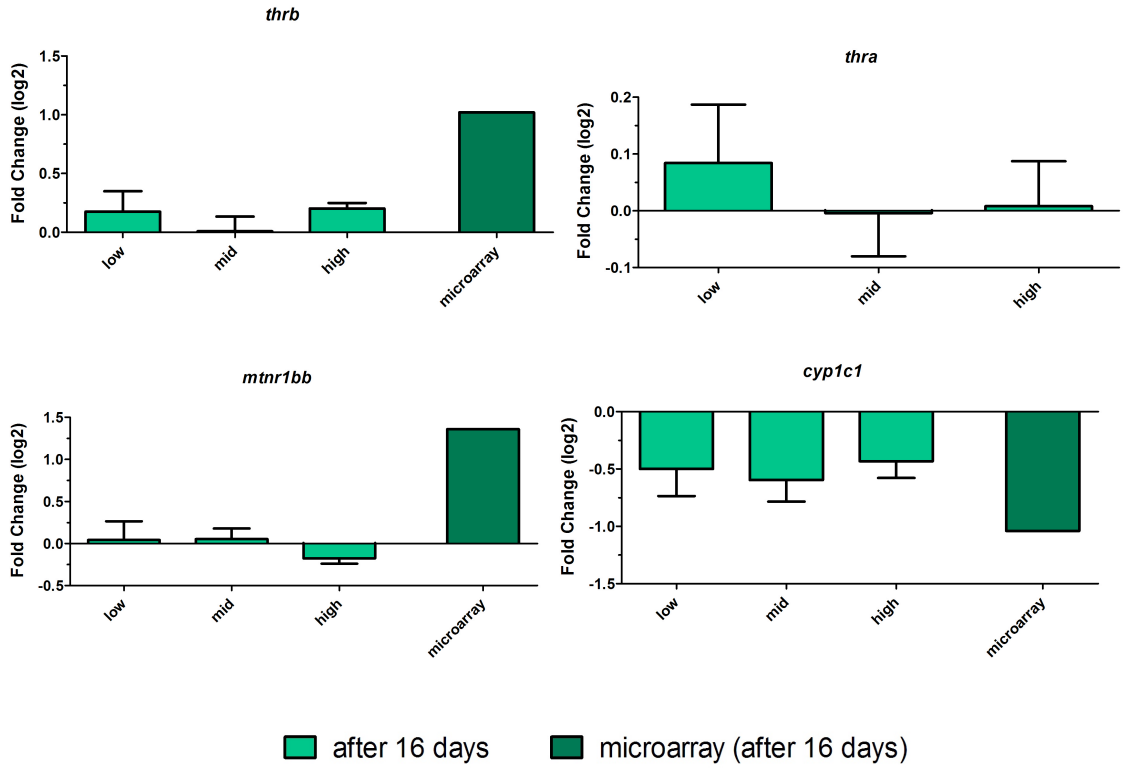

Fig. S1: Relative gene expression in **brain** of adult zebrafish males after 16 days exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Transcripts of thyroid hormone receptor beta/alpha *thrb* and *thra*, melatonin receptor 1B *mtnr1bb* and P450 enzyme *cyp1c1*, including microarray data after 16 days. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates).

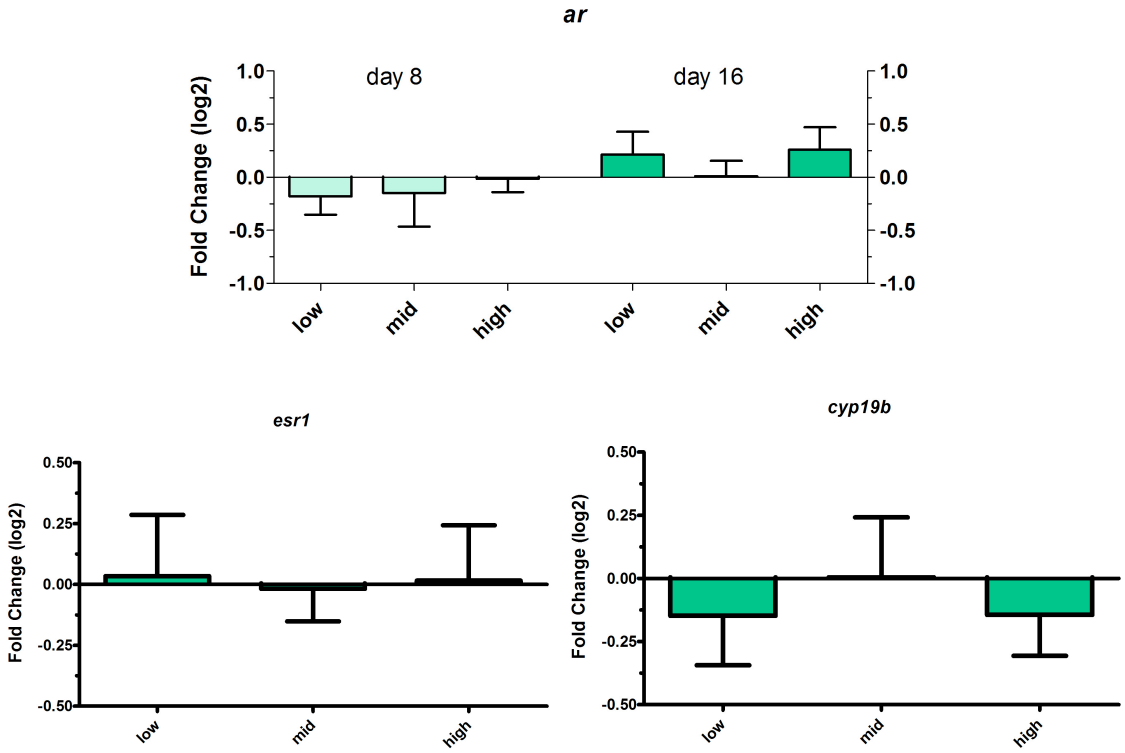

Fig. S2: Relative gene expression in **brain** of adult zebrafish males after (8 and) 16 days exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Transcripts of androgen receptor *ar*, estrogen receptor alpha *esr1* and P450 aromatase B *cyp19b*. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates).

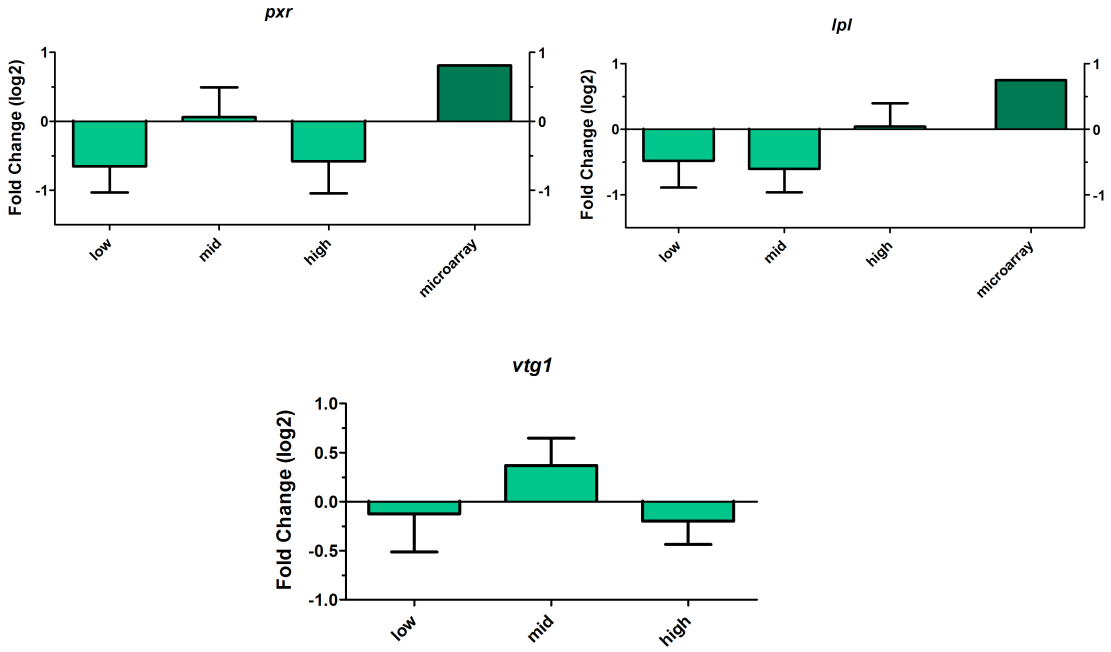

Fig. S3: Relative gene expression in **liver** of adult zebrafish males after 16 days exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Transcripts of pregnane X receptor *pxr*, lipoprotein lipase *lpl*, including microarray data after 16 days, and vitellogenin 1 *vtg1*. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates).

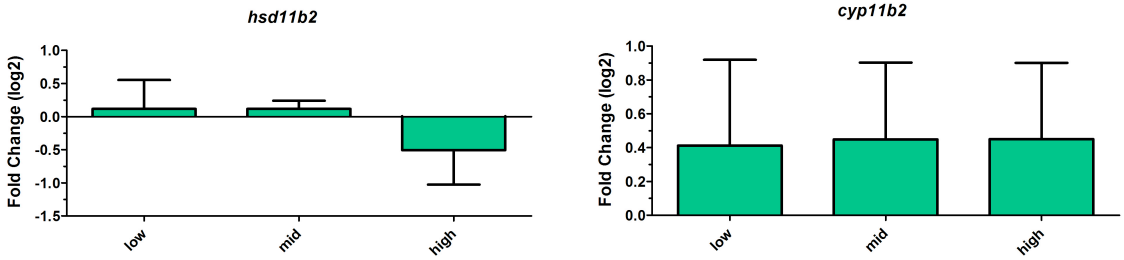

Fig. S4: Relative gene expression in **testes** of adult zebrafish males after 16 days exposure to 22 (low), 209 (mid) and 383 (high) µg/L OC. Transcripts of 11β-hydroxysteroid dehydrogenase type 2 *hsd11b2* and 11β-hydroxylase *cyp11b2*. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=4-5 replicates).

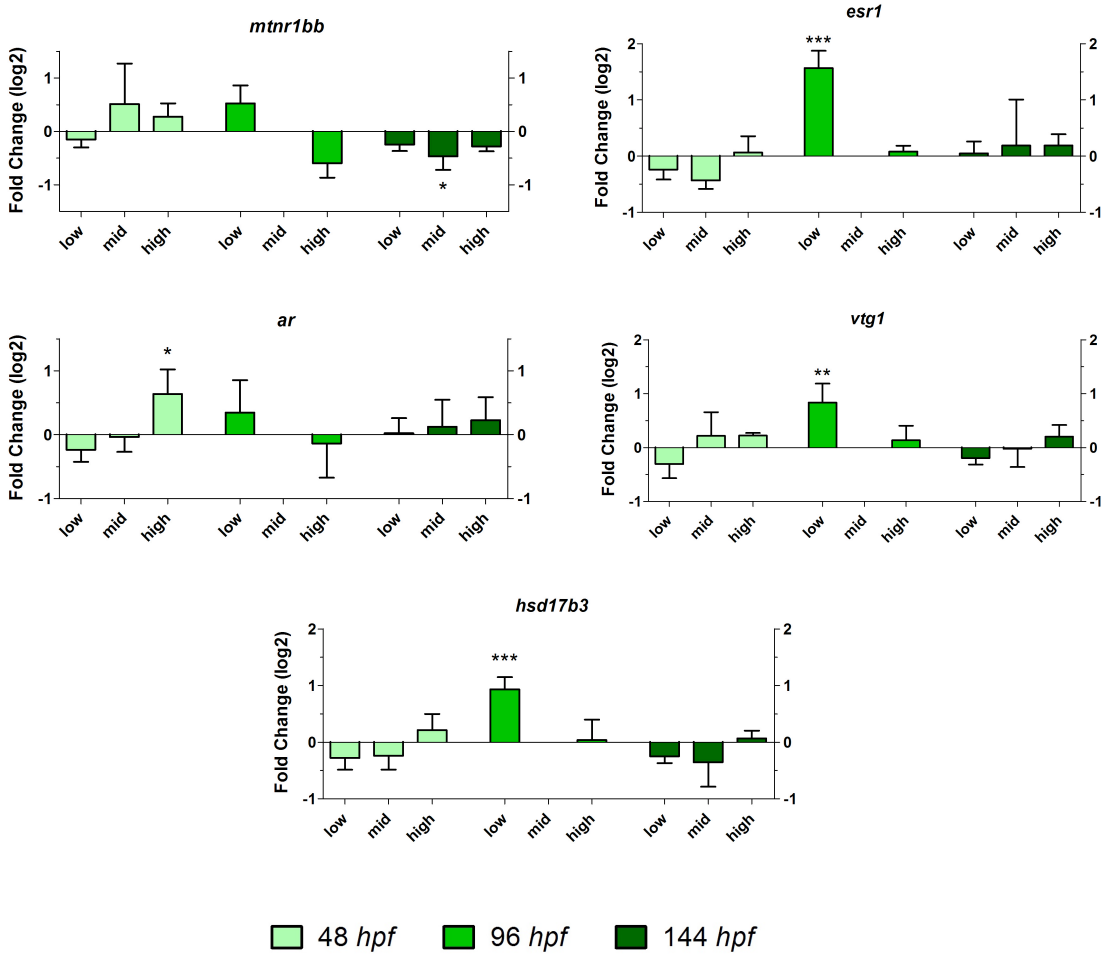

Fig. S5: Relative gene expression in **eleuthero-embryos** after exposure to 69 (low), 293 (mid) and 925 (high) µg/L OC. Transcripts of melatonin receptor 1B *mtnr1bb*, estrogen receptor alpha *esr1*, androgen receptor *ar*, vitellogenin *vtg1* and 17β-hydroxysteroid dehydrogenase type 3 *hsd17b3* after 48, 96 and 144 *hpf*. Relative transcript abundance quantified by RT-qPCR; fold changes (log2) determined using $2^{-\Delta\Delta Ct}$ method; target gene abundance referred to solvent control. Results are given as mean ± S.D. (*n*=3-4 replicates). Asterisks indicate significantly different expression to solvent control (* $p < 0.05$), (** $p < 0.001$), (*** $p < 0.0001$).

A3.1.4. Steroid hormone level.

The main androgen 11-ketotestosterone (11-KT) was measured in 20-40 µL blood plasma of adult male zebrafish using Radioimmunoassay (RIA) and is shown in Fig. S6. Calculated mean concentrations are 8.8 ± 2.5, 8.6 ± 3.2, 8.0 ± 3.2, 8.0 ± 2.3 and 8.4 ± 1.9 ng/mL 11-KT in control, solvent control, 22, 209 and 383 µg/L OC, respectively.

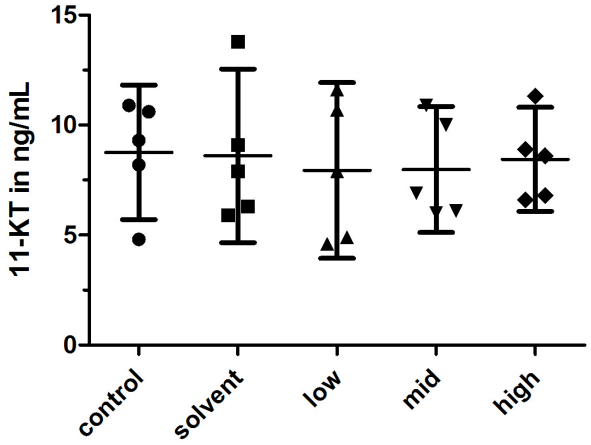

Fig. S6: Steroid hormone level of 11-KT were measured in blood plasma of adult male zebrafish after exposure to control, solvent control (0.005 % DMSO), 22 (low), 209 (mid) and 383 (high) µg/L OC, respectively and are expressed in ng/mL. Individual concentrations and mean values are shown (*n*=5 samples per treatment) including the 95 % confidence interval.

Appendix A4

Supporting Information to Chapter 5

Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (*Danio rerio*): 1. Reproductive and early developmental effects

A4.1. Methods

Analytical chemistry

Liquid Chromatographic - Tandem Mass Spectrometry (HPLC-MS/MS). The HPLC system consisted of a 1200 Series Binary Pump SL and Autosampler (Agilent Technologies, Santa Clara, CA). The MS system was an API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source (AB–Sciex, Thornhill, Ontario, Canada). The chromatographic separation was performed using an XTerra MS C18 3.5 µm, 1x100 mm column acquired from Waters (Waters Corp., Milford, MA) by gradient elution. The aqueous buffer was acetic acid 0.1 % in Milli-Q water (solvent A) and acetonitrile was used as organic phase (solvent B). The flow rate was set at 70 µL/min and the analysis started with 60 % of eluent A, followed by a 10-min linear gradient to 70 % of eluent B, a 1-min linear gradient to 100 % of eluent B, a 3-min isocratic washing step with eluent B and a 1-min linear gradient to the initial conditions that were maintained for 12 min to equilibrate the column. The injection volume was 2 µL and the column was kept at room temperature.

Table S1: LC-MS/MS analyzer conditions for SRM determination of the selected analytes.

	Precursor ion (m/z)	***Quantifier Product ion (m/z) and collision energy (eV)***	***Qualifier Product ion (m/z) and collision energy (eV)***
Progesterone (P4)	315.1	97.1 (27)	109.1 (31)
Mifepristone (MIF)	430.2	134.1 (35)	372.2 (30)
Medroxyprogesterone	345.2	123.1 (30)	-

The MS analysis was done in the positive ion mode and the Turbo Ion Spray source settings were: Ion Spray Voltage (IS) 5500V; Source Temperature 400°C; Curtain Gas 30; Collision Gas (CAD) 7; Ion Source Gas 1 (GS1) 40; Ion Source Gas 2 (GS2) 45. The choice of fragmentation products for each analyte and the optimization of collision energies and other instrument parameters were performed in continuous-flow mode using standard solutions at concentration of 100 pg/µL. The declustering potential (DP) and entrance potential (EP) were 40 and 8.0 V, respectively. Mass spectrometer analyses were done in the selected

reaction monitoring (SRM) mode, and the quantification was done measuring the two most abundant precursor/product ion transitions for each compound (Table S1). Quantification was performed using Analyst software, version 1.5.2 (AB–Sciex, Thornhill, Ontario, Canada).

A4.2 Results

A4.2.1. Analytical chemistry.

Method validation and quality control. The validation of the extraction method provides reproducible recoveries in the range of 48 - 51 % for MIF and 70 - 87 % for P4, which are comparable to previously reported validation results (Zucchi et al., 2012). Details are given in Table S2. The calculated accuracy of QC samples ranged between 38 - 87 % for MIF and 90 - 102 % for P4 and details are given in Table S3. For both the validation and the extraction blank samples, none of the analytes were detected. Although an extracted calibration curve is usually preferred considering potential matrix interferences, it is the other way around for MIF. Thus, all samples were quantified using the external calibration, both for MIF and P4. A linear analytical response was obtained for both analytes (MIF and P4) in the range of 3 - 100 pg/mL and correlation coefficients were ≥ 0.996 (MIF) and ≥ 0.999 (P4), respectively. The instrumental repeatability was assessed by 5-times injection of a standard solution and relative standard deviations (RSD) are 0.16 (MIF) and 2.44 (P4) %, respectively. LODs were 0.12 (MIF) and 0.08 (P4) ng/L and LOQs were 0.23 (MIF) and 0.14 (P4) ng/L. Details for instrumental quality control can be found in Table S4. All standard and exposure solutions (dissolved in DMSO and methanol) were stable at 4 °C (data not shown).

Chemical stability test. The results of the stability tests showed that a concentration dependent loss of MIF, up to 83 %, occurs during storage at -20 °C within 3 months (see Table S5). This loss is more pronounced for higher MIF concentrations. The stability of MIF during storage seems to be influenced by the water quality, as MIF is even less stable in deionized water compared to reconstituted water. Interestingly, we found already lower than expected concentrations at T0 for higher nominal MIF treatments. These results, together with our accuracy tests suggest that the recovery efficiencies at higher MIF concentrations are probably lower and/or some other factors may influence the recovery, although recovery tests did not show any difference. The most reasonable explanation is the

water quality (e.g. organic matter in (exposure) fish water), which may play a more important role at higher MIF concentrations. In contrast, P4 is more stable during storage at -20 °C, losing only up to 7 % (see Table S5). Also the water quality does not affect the stability of P4 to the same extend as it does MIF.

Table S2: Results of the extraction method validation for MIF and P4. Concentrations of 40 and 400 ng/L were validated within the present study using polymeric reversed-phase Strata-X cartridges (Phenomenex). The level of 20 ng/L was previously validated and reported (Zucchi et al., 2012) using C18 silica-bonded phases (Bakerbond).

	Recoveries [%] (mean ± SD)	
Nominal Concentration	MIF	P4
40 ng/L	48 ± 12	87 ± 6
400 ng/L	51 ± 3	70 ± 4
20 ng/L *	46 ± 11	97 ± 4

*: Results obtained within the study of Zucchi et al., 2012

Table S3: Accuracy of QC samples originating from spiked exposure water samples. Three randomly selected exposure water samples were extracted twice, whereas one sample of which was spiked with a mixture of MIF and P4 and served as the QC sample. Accuracies are calculated as measured analyte concentration/expected analyte concentration x 100.

Treatment	Water sample taken for QC	Accuracy [%]		Accuracy % (mean ± sd)	
		MIF	P4	MIF	P4
Low (4 ng/L MIF)	Gr 3 S1 t0	98	89		
	Gr 3 S1 t0 E	71	97	81 ± 15	90 ± 6
	Gr 3 S1 t1 E	73	86		
Mid (40 ng/L MIF)	Gr 4 S3 t2	46	88		
	Gr 4 S1 t2	60	105	87 ± 60	93 ± 11
	Gr 4 S1 t1	155	86		
High (400 ng/L MIF)	Gr 5 S1 t2	47	105		
	Gr 5 S1 t0 E	22	100	38 ± 13	102 ± 3
	Gr 5 S3 t1	44	101		
Blanks (extraction)	4.2.2013	N/A	N/A		
	5.2.2013	N/A	N/A		
	6.2.2013	N/A	N/A		
	7.2.2013	N/A	N/A		

N/A: not analyzed

Table S4: Instrumental quality control parameter for LC-MS/MS analysis of MIF and P4, including LOQ, LOD and instrumental repeatability are shown. LOD and LOQ are determined from sample chromatograms and instrumental repeatability was assessed by 5-times injection of a standard solution.

Instrumental Quality Control	MIF	P4
LOQ (S/N ≥ 5)	0.23 ng/L	0.14 ng/L
LOD (S/N ≥ 3)	0.12 ng/L	0.08 ng/L
Instrumental Repeatability	1.24 ± 0.002 ng/L, RSD = 0.16 %	1.38 ± 0.034 ng/L, RSD = 2.44 %

Table S5: Results of chemical stability test for MIF and P4. Reconstituted water and deionized water, respectively, was spiked with nominal concentrations of 4, 40 and 400 ng/L MIF+P4, and analyzed after T0 and after 3 months storage at -20 °C (T3). Stability and loss of MIF and P4 during storage is expressed in % and referred to measured concentration at T0.

Treatment	Measured Concentrations [ng/L]			
MIFEPRISTONE (MIF)				
Nominal conc.	**Reconstituted water 4 ng/L**	**Reconstituted water 40 ng/L**	**Reconstituted water 400 ng/L**	**Deionized water 40 ng/L**
T0	**4.0**	**39.3**	**158**	**26.4**
T 3 (after 3 months storage at -20°C)				
1	4.3	24.0	17.6	11.2
2	3.0	20.8	26.5	6.4
3	3.8	16.5	35.6	12.9
Mean [ng/L]	**3.7**	**20.4**	**26.6**	**10.2**
SD	**0.6**	**3.8**	**9.0**	**3.4**
RSD (%)	16.9	18.4	33.9	33.2
Stability (%)	**93**	**52**	**17**	**38**
Loss (%)	**7**	**48**	**83**	**62**
PROGESTERONE (P4)				
Nominal conc.	**Reconstituted water 4 ng/L**	**Reconstituted water 40 ng/L**	**Reconstituted water 400 ng/L**	**Deionized water 40 ng/L**
T0	**4.1**	**41.5**	**346**	**42.2**
T 3 (after 3 months storage at -20°C)				
1	4.0	35.9	366	40
2	4.0	40.9	347	39.4
3	3.9	39.3	348	37.6
Mean [ng/L]	**4.0**	**38.7**	**353.7**	**39.0**
SD	**0.0**	**2.6**	**10.7**	**1.2**
RSD (%)	1.2	6.6	3.0	3.2
Stability (%)	**97**	**93**	**102**	**92**
Loss (%)	**3**	**7**	**0**	**8**

Table S6: Measured MIF and P4 concentrations in individual exposure water samples of adult and embryo tanks. The intra-day (T0, T1 and T2 (after 24 h)) and the inter-day (sampling day 1, 2, 3) variability are shown for MIF and P4.

Treatment	Sampling date	Replicate Tank	Time	Sample ID	Measured concentration [ng/L]		Intra-day variability (daily replicates) [ng/L]			Inter-day variability (replicates A, D) [ng/L]			Intra-day variability (daily replicates) [ng/L]			Inter-day variability (replicates A, D) [ng/L]		
					MIF	P4	Mean	SD	RSD [%]	Mean	SD	RSD [%]	Mean	SD	RSD [%]	Mean	SD	RSD [%]
							Mifepristone (MIF)						Progesterone (P4)					
water control	23.8.2013	C	11:30	Gr 1 S2 t0	N/A	N/A	N/A						N/A					
	23.8.2012	C	18:30	Gr 1 S2 t1	N/A	N/A												
	24.8.2012	C	10:00	Gr 1 S2 t2	N/A	N/A												
	23.8.2012	E	11:30	Gr 1 S2 t0 E	N/A	N/A	N/A						N/A					
	24.8.2012	E	10:00	Gr 1 S2 t1 E	N/A	N/A												
dmso (0.01%)	17.8.2012	A	11:00	Gr 2 S1 t0	N/A	0.17	N/A						0.15	0.03	16.82			
	17.8.2013	A	15:00	Gr 2 S1 t1	N/A	0.12												
	18.8.2012	A	11:00	Gr 2 S1 t2	N/A	0.16				N/A						0.30	0.21	70.35
	17.8.2012	E	11:00	Gr 2 S1 t0 E	N/A	0.55	N/A			(adult tanks, A+D)			0.44	0.16	36.16	(adult tanks, A+D)		
	18.8.2012	E	11:00	Gr 2 S1 t1 E	N/A	0.33												
	31.8.2012	D	11:15	Gr 2 S3 t0	N/A	0.26	N/A			N/A			0.44	0.26	59.06	0.35	0.12	34.55
	31.8.2012	D	17:00	Gr 2 S3 t1	N/A	N/A				(embryo tanks, E)						(embryo tanks, E)		
	1.9.2012	D	11:00	Gr 2 S3 t2	N/A	0.63												
	31.8.2012	E	11:15	Gr 2 S3 t0 E	N/A	0.18	N/A						0.27	0.12	44.66			
	1.9.2012	E	11:00	Gr 2 S3 t1 E	N/A	0.35												
low (4 ng/L MIF)	17.8.2012	A	11:00	Gr 3 S1 t0	7.93	1.36	7.26	1.06	14.55				2.68	3.30	123.04			
	17.8.2013	A	15:00	Gr 3 S1 t1	6.04	0.25												
	18.8.2012	A	11:00	Gr 3 S1 t2	7.80	6.44				6.14	1.58	25.70				2.21	0.67	30.23
	17.8.2012	E	11:00	Gr 3 S1 t0 E	4.45	0.60	4.13	0.46	11.02	(adult tanks, A+D)			0.87	0.38	43.78	(adult tanks, A+D)		
	18.8.2012	E	11:00	Gr 3 S1 t1 E	3.81	1.14												
	31.8.2012	D	11:15	Gr 3 S3 t0	5.65	3.49	5.03	0.73	14.57	3.99	0.21	5.16	1.74	1.53	88.02	0.82	0.07	8.85
	31.8.2012	D	17:00	Gr 3 S3 t1	5.20	1.05				(embryo tanks, E)						(embryo tanks, E)		
	1.9.2012	D	11:00	Gr 3 S3 t2	4.22	0.68												
	31.8.2012	E	11:15	Gr 3 S3 t0 E	4.22	0.39	3.84	0.53	13.81				0.77	0.54	69.97			
	1.9.2012	E	11:00	Gr 3 S3 t1 E	3.47	1.15												
										(including re-analysis)						*(including re-analysis)*		
	23.8.2013	C	11:30	Gr 3 S2 t0	1.12	N/A	1.18	0.07	6.00	4.49	3.07	68.45	N/A			1.47	1.36	92.35
	23.8.2012	C	18:30	Gr 3 S2 t1	1.17	N/A				(adult tanks, A+D)						(adult tanks, A+D)		
	24.8.2012	C	10:00	Gr 3 S2 t2	1.26	N/A												
	23.8.2012	E	11:30	Gr 3 S2 t0 E	0.47	N/A	0.50	0.04	7.14	2.82	2.02	71.60	N/A			0.55	0.48	87.11
	24.8.2012	E	10:00	Gr 3 S2 t1 E	0.52	N/A				(embryo tanks, E)						(embryo tanks, E)		
mid (40 ng/L MIF)	17.8.2012	A	11:00	Gr 4 S1 t0	75.85	0.17	51.94	27.19	52.36				0.17	0.03	19.76			
	17.8.2013	A	15:00	Gr 4 S1 t1	57.62	0.13												
	18.8.2012	A	11:00	Gr 4 S1 t2	22.36	0.20				39.44	17.67	44.81				0.26	0.13	50.66
	17.8.2012	E	11:00	Gr 4 S1 t0 E	14.12	0.24	15.77	2.33	14.79	(adult tanks, A+D)			0.29	0.06	22.38	(adult tanks, A+D)		
	18.8.2012	E	11:00	Gr 4 S1 t1 E	17.42	0.33												
	31.8.2012	D	11:15	Gr 4 S3 t0	17.80	N/A	26.95	8.05	29.87	15.33	0.62	4.07	0.35			0.23	0.09	39.08
	31.8.2012	D	17:00	Gr 4 S3 t1	30.09	N/A				(embryo tanks, E)						(embryo tanks, E)		
	1.9.2012	D	11:00	Gr 4 S3 t2	32.95	0.35												
	31.8.2012	E	11:15	Gr 4 S3 t0 E	15.80	0.14	14.89	1.29	8.65				0.16	0.03	16.48			
	1.9.2012	E	11:00	Gr 4 S3 t1 E	13.98	0.18												
high (400 ng/L MIF	17.8.2012	A	11:00	Gr 5 S1 t0	99.07	N/A	89.22	12.63	14.16				4.38	0.21	4.76			
	17.8.2013	A	15:00	Gr 5 S1 t1	93.61	4.53												
	18.8.2012	A	11:00	Gr 5 S1 t2	74.97	4.24				76.89	17.43	22.67				4.38		
	17.8.2012	E	11:00	Gr 5 S1 t0 E	24.22	N/A	22.99	1.75	7.61	(adult tanks, A+D)			5.10			(adult tanks, A+D)		
	18.8.2012	E	11:00	Gr 5 S1 t1 E	21.75	5.10												
	31.8.2012	D	11:15	Gr 5 S3 t0	59.64	N/A	64.57	4.40	6.81	25.80	3.97	15.40	N/A			4.65	0.63	13.46
	31.8.2012	D	17:00	Gr 5 S3 t1	68.11	N/A				(embryo tanks, E)						(embryo tanks, E)		
	1.9.2012	D	11:00	Gr 5 S3 t2	65.95	N/A												
	31.8.2012	E	11:15	Gr 5 S3 t0 E	29.79	4.15	28.61	1.67	5.84				4.21	0.08	1.88			
	1.9.2012	E	11:00	Gr 5 S3 t1 E	27.42	4.27												
P4 (400 ng/L P4)	17.8.2012	A	11:00	Gr 6 S1 t0	N/A	12.15	N/A						13.35	2.03	15.20			
	17.8.2013	A	15:00	Gr 6 S1 t1	N/A	12.22												
	18.8.2012	A	11:00	Gr 6 S1 t2	N/A	15.70				N/A						24.46	15.71	64.22
	17.8.2012	E	11:00	Gr 6 S1 t0 E	N/A	276.91	N/A			(adult tanks, A+D)			275.78	1.59	0.58	(adult tanks, A+D)		
	18.8.2012	E	11:00	Gr 6 S1 t1 E	N/A	274.65												
	31.8.2012	D	11:15	Gr 6 S3 t0	N/A	33.72	N/A			N/A			35.57	7.75	21.80	253.82	31.06	12.24
	31.8.2012	D	17:00	Gr 6 S3 t1	N/A	44.08				(embryo tanks, E)						(embryo tanks, E)		
	1.9.2012	D	11:00	Gr 6 S3 t2	N/A	28.91												
	31.8.2012	E	11:15	Gr 6 S3 t0 E	N/A	252.31	N/A						231.86	28.92	12.47			
	1.9.2012	E	11:00	Gr 6 S3 t1 E	N/A	211.41												

color code: green: re-analysis; blue: adult tanks; red: embryo tanks

A4.2.2. Macroscopic observations.

Table S7. Biological measurement of adult zebrafish males and females after 21 days exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. The corrected gender ratio of females and males present per tank, survival of fish, body wet weight, body length, condition factor CF (CF = body wet weight [mg] / body length [mm] x 100) and gonadosomatic index (GSI = testes weight [mg] / body weight [mg] x 100) are shown. Total number of exposed fish considered for CF calculations were maximum *n*=16 and 24 for females and males, respectively, and for GSI calculations *n*=12 per gender and treatment.

Treatment	survival [%]	gender ratio [a] female/male	body weight [mg]		body length [cm]		condition factor (CF)		gonadosomatic index (GSI)	
			females	males	females	males	females	males	females	males
Water Control	100	4/5, 3/7, 4/6, 4/6	477.5 ± 84.8	481.3 ± 45.5	3.6 ± 0.2	3.8 ± 0.2	1.4 ± 0.1	1.3 ± 0.0	14.9 ± 3.2	1.1 ± 0.2
Solvent Control	100	5/5, 4/6, 4/6, 4/6	479.8 ± 119.5	439.8 ± 49.6	3.6 ± 0.2	3.7 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	14.9 ± 1.8	1.1 ± 0.2
5 ng/L MIF	100	4/6, 4/4, 3/5, 4/6	470.1 ± 58.9	424.1 ± 55.6	3.6 ± 0.2	3.6 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	12.6 ± 2.1	1.4 ± 0.1
39 ng/L MIF	100	4/6, 4/6, 4/4, 4/6	484.6 ± 127.6	506.8 ± 58.2	3.6 ± 0.3	3.9 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	12.8 ± 1.6	1.5 ± 0.1
77 ng/L MIF	100	5/4, 5/4, 4/6, 4/6	452.6 ± 110.5	405.8 ± 37.4	3.6 ± 0.2	3.7 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	13.5 ± 0.5	1.7 ± 0.3
25 ng/L P4	98	4/6, 4/6, 4/6, 7/2	494.7 ± 115.3	476.8 ± 60.3	3.7 ± 0.3	3.7 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	***10.4 ± 2.0	1.3 ± 0.2

[a]: Corrected gender ratio (number of females/males) per replicate tank (*n*=4 tanks per treatment) after sampling. *** Significant different GSI to solvent control ($p<0.001$).

A4.2.3. Reproduction.

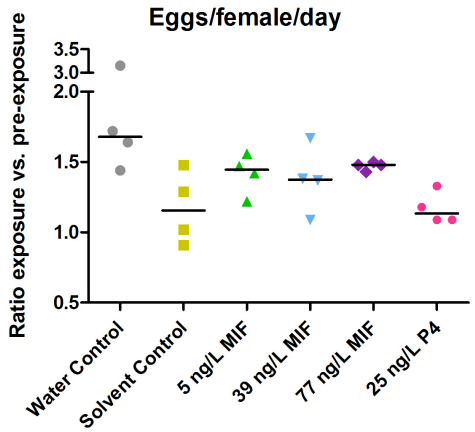

Fig. S1: Average number of eggs per female per day expressed as ratio between exposure and pre-exposure for each treatment. A ratio of 1 indicates no difference in rate of egg production between exposure and pre-exposure within the same treatment group. A ratio > 1 indicates an increased egg production during exposure within one treatment group, and a ratio < 1 indicates decreased egg production during exposure. Individual ratios of the four replicate tanks per treatment are shown as scatter-dot plot, including the mean values per treatment (horizontal lines).

Table S8: Summarized data of average number of eggs per female per week, assuming seven days a week, after two weeks pre-exposure followed by three weeks exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Data are represented as mean values ± S.D. of four replicate tanks per week and treatment. In addition, the overall mean of pre- and exposure is given.

	Number of eggs / female / week (Mean ± S.D.)						
Treatment	pre-week 1	pre-week 2	**pre-exposure mean**	week 1	week 2	week 3	**exposure mean**
Water Control	13.3 ± 5.9	11.4 ± 4.3	**12 ± 1**	23.3 ± 4.8	23.9 ± 5.4	20.9 ± 3.2	**23 ± 2**
Solvent Control	19.3 ± 2.6	19.3 ± 3.0	**19 ± 0**	24.7 ± 3.7	19.8 ± 1.2	22.2 ± 5.7	**22 ± 2**
5 ng/L MIF	25.1 ± 7.5	27.7 ± 4.9	**26 ± 2**	35.6 ± 10.8	34.2 ± 5.7	41.7 ± 8.9	**37 ± 4**
39 ng/L MIF	19.8 ± 1.7	15.7 ± 4.1	**18 ± 3**	23.0 ± 4.7	22.3 ± 4.7	27.1 ± 3.6	**24 ± 3**
77 ng/L MIF	19.1 ± 2.1	23.1 ± 2.7	**21 ± 3**	28.8 ± 4.5	33.5 ± 5.2	30.9 ± 5.5	**31 ± 2**
25 ng/L P4	24.8 ± 2.5	26.5 ± 3.0	**26 ± 1**	28.6 ± 7.3	31.5 ± 4.1	30.2 ± 4.4	**30 ± 1**

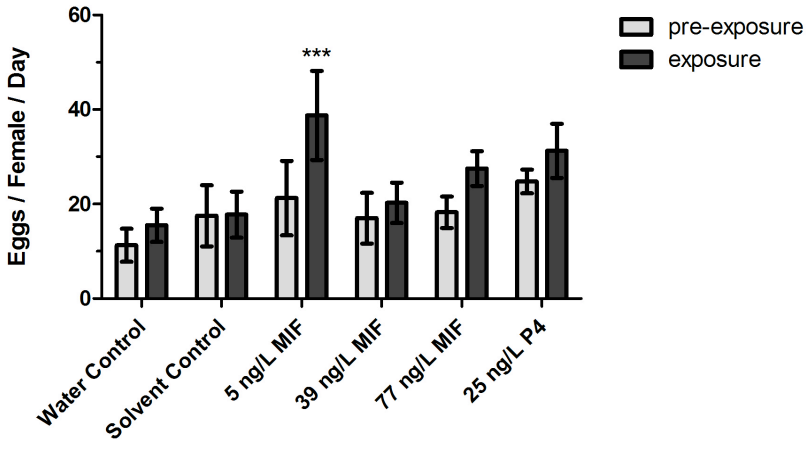

Fig. S2: Average number of eggs per female per day after 14 days pre-exposure followed by 21 days exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Each bar represents the mean value ± S.D. of four replicate tanks per treatment, comparing pre-exposure and exposure. Asterisks indicate significantly different number of eggs per female and day compared to solvent control (*** $p < 0.0001$).

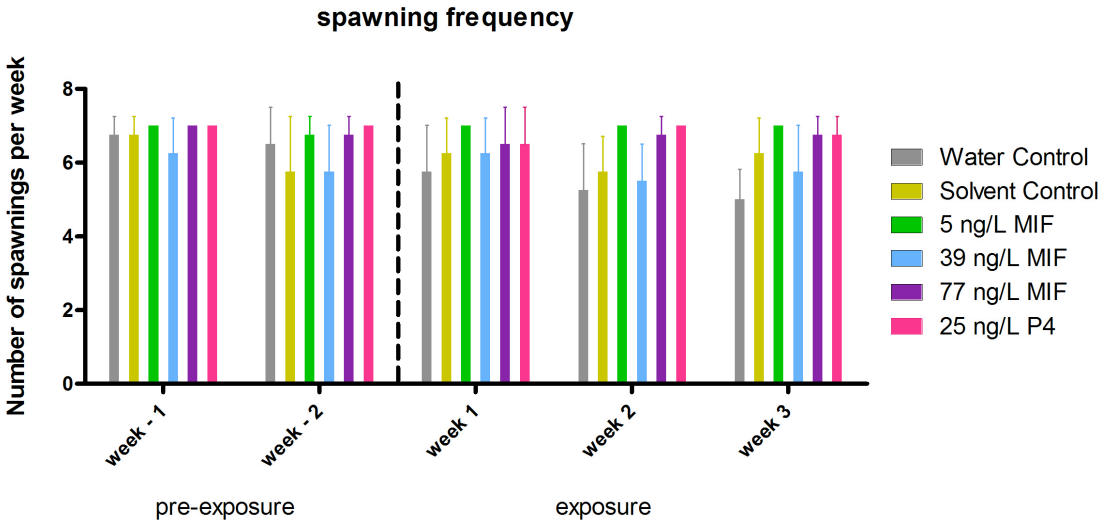

Fig. S3: Spawning frequency of groups of zebrafish, meaning how often the breeding groups spawned per week, assuming seven days a week. Spawning frequencies are shown for the two weeks pre-exposure, followed by three weeks exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Each bar represents the mean value ± S.D. of four replicate tanks per treatment and week.

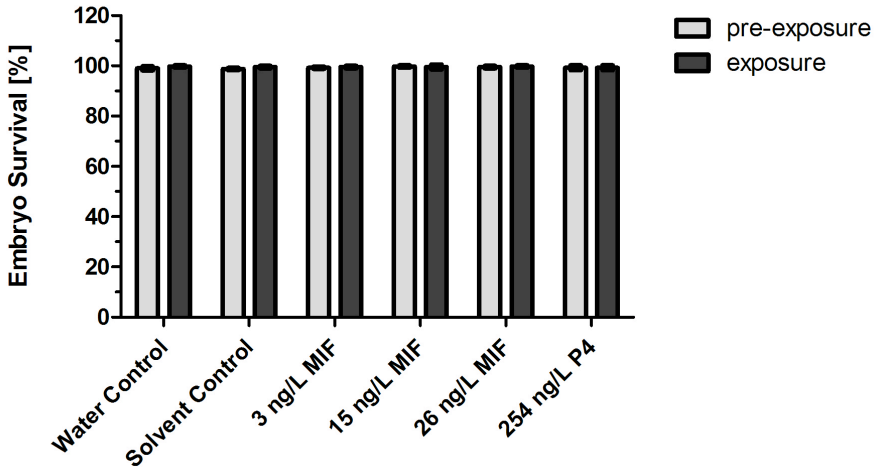

Fig. S4: Embryo survival assessed after 96 *hpf* of exposure to water control, solvent control (0.01 % DMSO), 3, 15, 26 ng/L MIF and 254 ng/L P4. Hatched and non-hatched but alive embryos were considered as alive for calculating the survival rate. The bars represent mean values ± S.D. of four replicates per treatment, expressed in percentage, and comparing pre-exposure and exposure periods.

A4.2.4. Histology.

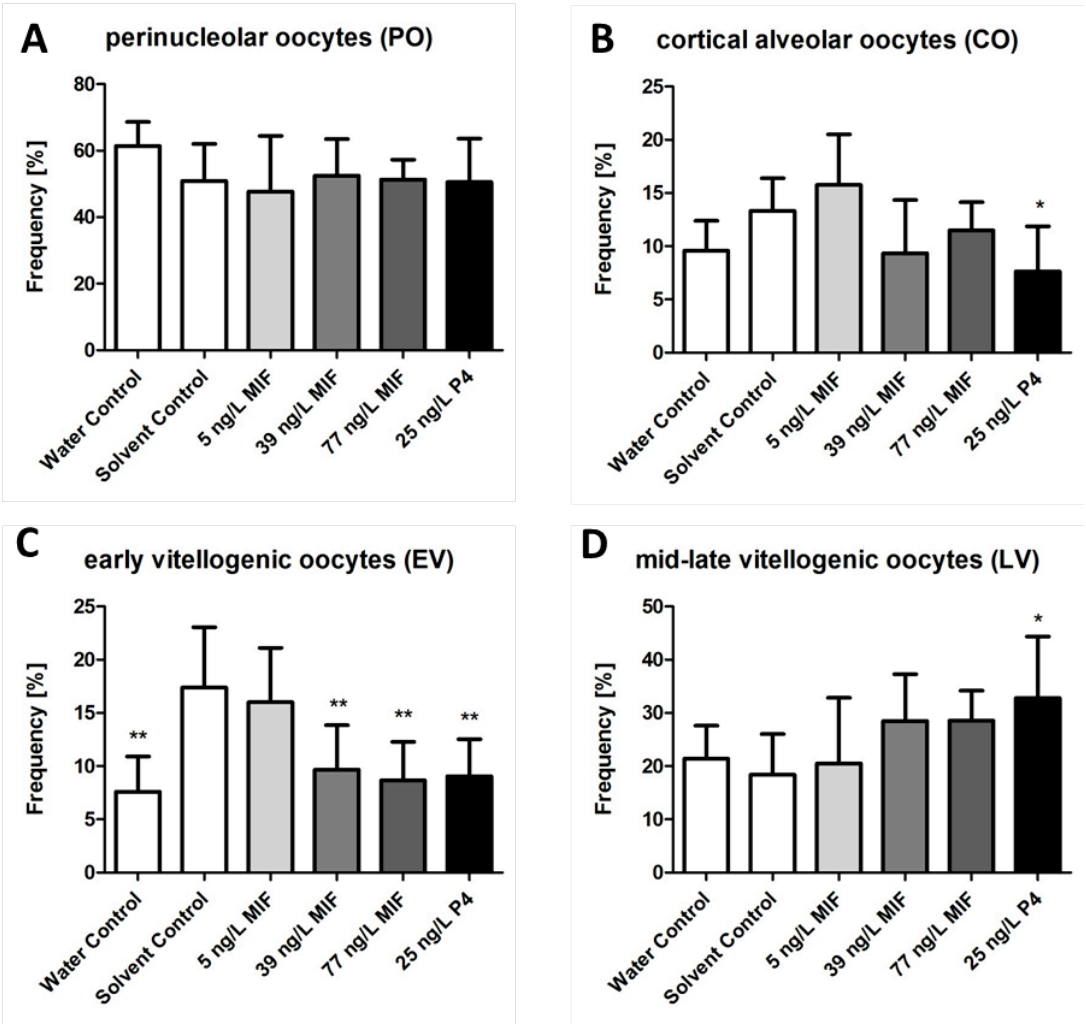

Fig. S5: Relative proportions of **A:** perinucleolar oocytes (PO), **B:** cortical alveolar oocytes (CO), **C:** early vitellogenic (EV) and **D:** mid-late vitellogenic oocytes (LV) in ovaries of adult zebrafish females after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Results are shown as mean values (*n*=6-8 ovaries per treatment) ± S.D. Asterisks indicate significantly different proportions to solvent control (* $p<0.05$), (** $p<0.001$).

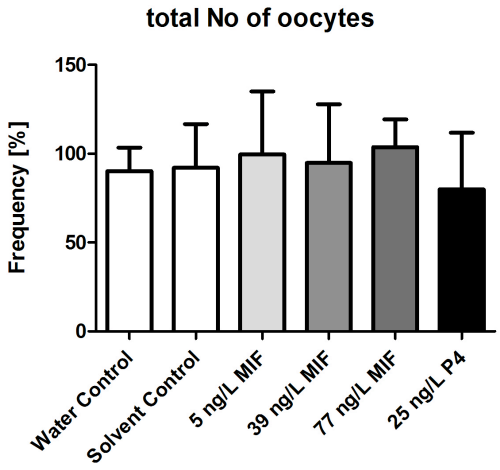

Fig. S6: Total number of oocytes in adult zebrafish females after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Numbers are shown as mean values ± S.D. (*n*=6-8 ovaries per treatment).

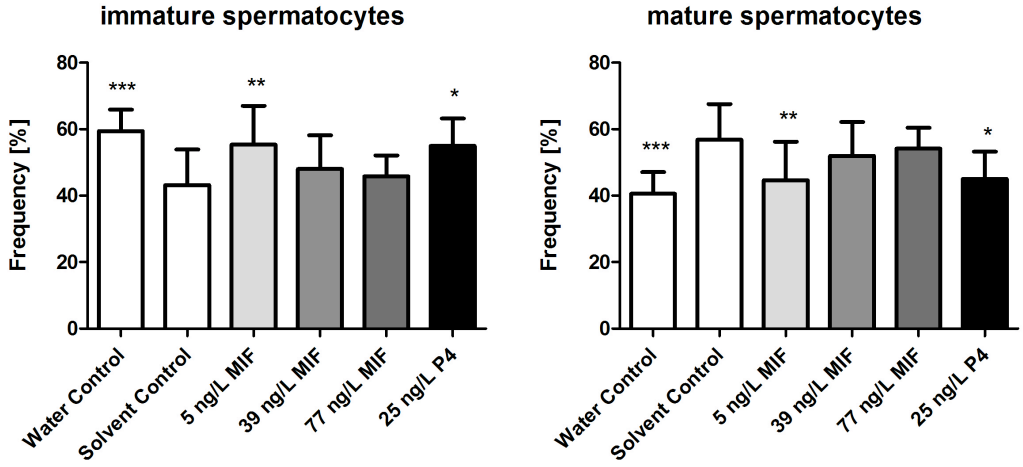

Fig. S7: Relative percentage of immature (spermatogonia and spermatocytes) (left) and mature (spermatids and spermatozoa) (right) spermatocytes in adult zebrafish males after exposure to water control, solvent control (0.01 % DMSO), 5, 39, 77 ng/L MIF and 25 ng/L P4. Results are shown as mean values (*n*=12-16 male gonads per treatment). Asterisks indicate significantly different percentage to solvent control (* $p<0.05$), (** $p<0.001$), (*** $p<0.0001$).

Curriculum Vitae

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