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Novel assessment methods in ecotoxicology for the identification of hormonal active substances: Combining the fish sexual development test with gene expression endpoints

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Novel assessment methods in ecotoxicology for the identification of hormonal active substances: Combining the fish sexual development test with gene expression endpoints

by

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Kurzbeschreibung

Zur Erfassung hormonaktiver Wirkungen von Substanzen auf Fische sind unter Regie der OECD eine Reihe von Testrichtlinien validiert worden, die in das harmonisierte Prüfrichtlinien-Programm für Endokrine Disruptoren (OECD 2010) eingegliedert sind. Die Untersuchung von physiologischen suborganismischen Parametern im Rahmen dieser Tests ist begrenzt auf etablierten Biomarker wie Vitellogenin oder 11-keto Testosteron in Blutplasma oder Lebergewebe. Ein vielversprechender Ansatz zur Verfeinerung bestehender Testrichtlinien ist die Integration molekularer Endpunkte, da diese sehr schnell auf Schadstoffe reagieren. Durch die Aufklärung von Zusammenhängen mit den auslösenden Ereignissen (der initialen Wirkung) der Schadsubstanz können sie indikativ für mögliche adverse Effekte auf Organismen sein. Dies entspricht dem Konzept *Adverse Outcome Pathway* (AOP), das als Weiterentwicklung der „*Toxicity Pathways*" als Interpretationshilfe für die Risikobewertung dienen soll. Hierbei werden die Zusammenhänge zwischen einem initialen molekularen Ereignis (*Molecular Initiating Event*; MIE) und dem daraus resultierenden apikalen Endpunkt auf der Organismus- oder Populationsebene auf eine einfache Kette von Schlüsselereignissen herunter gebrochen. Die so identifizierten Wege können die Basis für effektivere Bewertungsstrategien für toxische Substanzen, einschließlich endokrin wirksamer Substanzen (*Endocrine Disrupting Chemicals*; EDC), bilden und die Entwicklung von passenden Prüfstrategien erleichtern. In der hier präsentierten *Proof-of-Principle*-Studie wurden die Effekte der Exposition mit dem nicht-steroidalen Aromatase-Inhibitor Fadrozol (Testkonzentrationen 10 µg/L, 32 µg/L, 100 µg/L) in einem *Fish Sexual Development Test* (FSDT; OECD TG 234) mit Zebrabärbling (*Danio rerio*) untersucht. Dieser Test wurde durch Genexpressionsanalysen zu unterschiedlichen Zeitpunkten der Entwicklung (48 hpf, 96 hpf, 28 dpf, 63 dpf) ergänzt. Das Ziel der Studie war die Identifikation von molekularen Endpunkten, die durch Aromatase-Inhibition beeinflusst werden und einen adversen Effekt auf der Populationsebene auslösen. Zu den beobachteten Effekten zählte eine vollständige Verschiebung des Geschlechterverhältnisses in Richtung Männchen sowie eine erhöhte Gonadenreife der männlichen Tiere bereits in der niedrigsten Testkonzentration. Diese Effekte wurden auf die spezifische Wirkweise von Fadrozol zurückgeführt. Durch das MIE der Inhibition der Aromatase-Aktivität wird der Umbau von C19-Androgenen zu C18-Östrogenen verhindert und somit das Verhältnis der Steroidhormone, die das Geschlechterverhältnis regulieren, verändert. Eines der direkt folgenden molekularen Schlüsselereignisse (*Molecular Key Event*; KE) war anhand der Studienergebnisse, die Herabregulation der direkt Östrogen-sensitiven Gene *vtg1* und *cyp19a1b* bereits nach 48 hpf, die außerdem in unterschiedlichem Maße geschlechtsdimorphe Expression bei 63 dpf zeigten. Neben diesen beiden Genen wurden drei weitere potenzielle Biomarker–Gene identifiziert, die an unterschiedlichen Schlüsselstellen des Steroidsignalweges eingreifen: *igf1* (*Insulin-like growth factor 1*; Transkriptionsregulation), *lss* (*Lanosterol synthase*; Umbau von Fettsäuren zu Cholesterol) und *star* (*Steroidogenic acute regulatory protein*, Transport von Cholesterol zur inneren Mitochondrienmembran).

Diese Markergene ermöglichen einen umfassenderen Einblick in die zugrunde liegenden Mechanismen der Aromatase-Inhibition während der sexuellen Entwicklung von Fischen. Darüber hinaus konnte der bereits existierende AOP der Aromatasehemmung speziell auf die Geschlechtsentwicklung von Fischen angepasst werden. Auf Basis der vorgestellten Ergebnisse ist die Anwendung von Genexpressionsanalysen im Rahmen von etablierten Testmethoden und Verfahren zu diskutieren. Anwendungsmöglichkeiten bieten der *Fish Embryo Test* (Erweiterung mit Genanalysen hin zu einem Screening Werkzeug für EDCs), die Erweiterung von etablierten chronischen Tests zur Untersuchung von Substanzen mit unbekannter Wirkweise sowie ein Einsatz für das Biomonitoring zum wirkorientierten Nachweis von Schadstoffeinwirkungen in biologischen Matrices.

Abstract

For the evaluation of effects of hormone-active substances to fish, several test guidelines have been validated by the OECD which are included in the *Conceptual Framework for Testing and Assessment of Endocrine Disruptors* (OECD, 2010). In these tests, the assessment of physiological parameters is limited to the established biomarkers like vitellogenin or 11-keto testosterone in blood plasma or liver tissue. A promising approach to refine existing testing strategies is the integration of molecular endpoints, which rapidly respond to exposure. Furthermore, these endpoints can be indicative of potential adverse effects at the organismal level by providing information, which connect to the initiating effect of a substance. This is in line with the concept of Adverse Outcome Pathways (AOP), which evolved from the "toxicity pathway" approach as a mechanistic evaluation tool for the risk assessment. An AOP describes the linkage between a molecular initiating event (MIE) and the adverse outcome at apical level by a simplified sequence of key events at different levels of organization. Identified AOPs can provide the basis for the development of more effective and reliable toxicity testing and evaluation strategies also for endocrine disrupting chemicals (EDC). The present proof-of-principle study investigated the effects of, the non-steroidal aromatase inhibitor fadrozole (test concentrations: 10 µg/L, 32 µg/L, 100 µg/L) on zebrafish (*Danio rerio*) in a Fish Sexual Development Test (FSDT; OECD TG 234). The FSDT was combined with gene expression analyses at different developmental time points (48 hpf, 96 hpf, 28 dpf, 63 dpf). The purpose was to define molecular endpoints which are affected by aromatase inhibition and result in adverse apical effects. Observed apical effects of fadrozole during the sexual development of fish comprised a complete shift of the sex ratio towards males and accelerated maturation of male gonads already at low concentrations. These effects were attributed to the mode of action of fadrozole. The MIE of fadrozole to specifically inhibit the activity of aromatase, led to an inhibition of the conversion of C19-androgens to C18-estrogens, and thus, to an imbalance of the sex steroid hormones controlling the sex ratio. One of the subsequent molecular key events (KE) identified by the study was the down regulation of estrogen-responsive genes like *vtg1* and *cyp19a1b* as early as 48 hpf, and which showed sexdimorphic expression patterns at 63 dpf. In addition to these two genes, three other genes were defined as potential biomarkers, which represent key processes along the steroidogenesis signalling pathway: *igf1* (insulin-like growth factor 1; transcription regulation), *lss* (lanosterol synthase; conversion of fatty acids to cholesterol) and *star* (steroidogenic acute regulatory protein, transport of cholesterol to the inner mitochondrial membrane).

These marker genes allow a more in-depth insight into the underlying mechanisms of aromatase inhibition during sexual development in fish. Moreover, the existing AOP for aromatase inhibition could be refined specifically for the sexual development of fish. Based on the results of this study the integration of gene expression analysis in established test guidelines should be discussed. Possible applications include an implementation in the fish embryo test as a screening tool for EDCs, secondly an extension of chronic fish tests for the assessment of substances with unknown mode of action and finally the use for monitoring purposes to analyse the aftermath of pollution impacts in biological matrices in an effect directed approach.

Content

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Abbreviations*

AOP	Adverse outcome pathway
AR	Androgen receptor
BPA	Bisphenol A
C_{Cal}	Analyte concentration of the calibration samples
CCN proteins	Derived from the names of the first three identified family members: Cyr61, CTGF and Nov
cDNA	Complementary deoxyribonucleic acid
CF	Conceptual framework
Ct	Threshold cycle (derived from PCR amplification curve)
ΔΔCt	Delta delta Ct
(DD)-RT-PCR	Differential display RT-PCR
dpf	Days post fertilization
E2	Estradiol
EATS	Estrogen, androgen, thyroid and steroidogenesis signalling
EDC	Endocrine disrupting chemical
EDTA	Endocrine disrupters testing and assessment
EE2	17α-Ethinyl estradiol
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	Estrogen receptor
ERE	Estrogen receptor-responsive element
F_1, F_2	Filial 1 generation, Filial 2 generation
FET	Fish embryo test
FFLT	Fish full life-cycle test
FHM	Fathead minnow
FLCTT	Fish life-cycle toxicity test
FSA	Fish screening assay
FSDT	Fish sexual development test
FSTRA	Fish short-term reproduction assay
GD	Guidance document
GLP	Good Laboratory Practice
hpf	Hours post fertilization
HPG	Hypothalamus-pituitary-gonadal axis
KE	Key event (of an AOP)
$LC(_{50})$	Lethal concentration (50% of organisms display lethality)

LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
mFET	Medaka FET
MIE	Molecular initiation event (of an AOP)
MMGT	Medaka multigeneration test
MOA	Mode of action
mRNA	Messenger ribonucleic acid
NB	Nicht bestimmbar
ND	Not determinable
n.d.	not detected
NOEC	No observed effect concentration
NP	4-tert-nonylphenol
NRC	National Research Council
NSB	Non-specific binding
OECD	Organization for Economic Cooperation and Development
P	Parental generation
PAR	Peak area ratio (IS conc. × peak area analyte / peak area IS)
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
QSAR	Quantitative structure-activity relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
RT	Reverse transcription
SEM	Standard error of the mean
SSH	Suppression subtractive hybridisation
TG	Test guideline
TGT	Two generation test
TIU	Trypsin-inhibitor unit
VTG	Vitellogenin
WHO	World Health Organisation
zFET	Zebrafish FET

*Abbreviated zebrafish gene names are not included. They can be found in section 4, Table 4.

1 Zusammenfassung

Einleitung

Seit den neunziger Jahren des vergangenen Jahrhunderts werden Umweltschadstoffe, die mit dem endokrinen System von Organismen interagieren und dadurch Schädigungen hervorrufen und die Reproduktion beeinträchtigen können, auch über Generationen hinweg, als sogenannte endokrine Disruptoren bezeichnet. Der Anstieg von Erkrankungen, die die menschliche Fortpflanzungsfähigkeit betreffen sowie Beobachtungen in der Tierwelt, etwa von Veränderungen bei den Fortpflanzungsorganen von Vertebraten und Invertebraten, sowie der Rückgang von Individuenzahlen bei Fischen und Amphibien, haben die Thematik in die Öffentlichkeit gerückt. Diese Indizien wurden wissenschaftlich eindeutig als Risiko bewertet und öffentlich dementsprechend kommuniziert. Die Problematik der endokrinen Disruptoren wurde auf einem Symposium im britischen Weybridge von Vertretern aus Wissenschaft und Behörden 1996 erstmals international diskutiert. Eines der Hauptergebnisse dieses Workshops war die Einigung auf eine Definition für endokrine Disruptoren, welche später auch von der WHO in einer leicht abgewandelten Form übernommen wurde. Diese besagt: „*An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations*" (WHO/IPCS 2002). Seitdem hat das Thema „Endokrine Disruptoren" den Weg in diverse Verordnungen und Zulassungsrichtlinien gefunden, z.B. in REACH, die Pestizid- und Biozid-Verordnungen sowie in die Wasserrahmenrichtlinie (2000/60/EG). Zusätzlich wurde von der „*Endocrine Disruptors Testing and Assessment*" (EDTA) Arbeitsgruppe ein gemeinsames Rahmenrichtlinien-Konzept entwickelt, welches schließlich auf dem 6. EDTA-Workshop 2002 in Tokio verabschiedet wurde (ENV/JM/TG/EDTA (2002)4). Auf dieser Basis wurde die Entwicklung und Validierung von OECD Testrichtlinien zur Detektion von endokrin wirksamen Substanzen auf den Weg gebracht.

Eine hormonaktive Wirkung zeigt sich in Organismen oder einer Population meist erst nach längerer Exposition im subakuten oder chronischen Konzentrationsbereich. Schon deshalb ist die Identifizierung von endokrinen Substanzen mit einem hohen Zeit- und Kostenaufwand verbunden. Für diese umfangreichen Experimente wird zudem eine große Anzahl an Tieren benötigt. Die Entwicklung von Alternativen zu diesen Testverfahren, welche die Prinzipien des 3R-Konzepts (Replacement, Reduction and Refinement) nach Russell and Burch (1959) berücksichtigen, wird seit Langem vorangetrieben. Kurzzeittests zur Bestimmung apikaler Endpunkte können diesen Zweck nicht erfüllen. Sie dienen als Screening Tests, die komplexere Studien wie etwa den *Fish life-cycle test* triggern, aber nicht ersetzen können. Standard Tests, wie etwa der *Fish early life stage toxicity test*, führen zu nicht zufriedenstellenden Aussagen, da sie wichtige Expositionsfenster, wie etwa die Sexualentwicklung, ausklammern. Bei der Untersuchung potentiell hormonaktiver Substanzen ist es zudem wichtig, neben den populationsrelevanten auch schnell-reagierende indikative Endpunkte zu untersuchen. Sie können wichtige Informationen zu den zugrunde liegenden Wirkmechanismen liefern.

Einen vielversprechenden Ansatz bietet die Untersuchung molekularer Endpunkte, da diese sehr schnell auf Schadstoffe reagieren. Sie können zudem auch Hinweise auf mögliche adverse Effekte auf Organismus-Ebene liefern (Piersma, 2006). Verschiedene Studien konnten mit Hilfe von genomischen Analysen einen entscheidenden Beitrag zur Aufklärung molekularer Mechanismen leisten, die schließlich in adversen Effekte auf apikaler Ebene ihre Ausprägung fanden (Ankley et al., 2006; Sawle et al., 2010). Umfassende Transkriptomanalysen erlauben aussagekräftige Untersuchungen von adversen Effekten in Zellen, Organen und dem Gesamtorganismus. Durch ihre hohe Sensitivität können Wirkungen bereits in einem Konzentrationsbereich erkannt werden, der unterhalb des Schwellenwerts für den sensitivsten Endpunkt eines „konventionellen" ökotoxikologischen Tests liegt. Diese höhere Empfindlichkeit von molekularen Analysen ist eine Voraussetzung, um Effekte, die aus systemischer Toxizität resultieren, von den endokrinen Effekten abzugrenzen (Scholz and Mayer, 2008; Wang et al., 2010).

Der Fortschritt bei molekularen und Computer-basierten (*in silico*) Methoden können als Basis für den Paradigmenwechsel in der Ökotoxikologie (NRC 2007) angesehen werden. Neue Entwicklungen und Denkansätze zu alternativen Teststrategien und Interpretationshilfen für die Umweltrisikobewertung wurden auf den

Weg gebracht, wie z.B. die „*Adverse Outcome Pathways*“ (AOP; Ankley et al., 2010; Villeneuve and Garcia-Reyero, 2011) oder die „*Toxicity Pathways*“ (Bradbury et al., 2004). Beim AOP Konzept wird der direkte Zusammenhang zwischen einem initialen molekularen Ereignis („*Molecular Initiating Event*“; MIE) und dem daraus resultierendem apikalen Endpunkt auf der Organismus- oder Populationsebene untersucht (Kramer et al., 2011; Segner, 2011, 2009). Die so identifizierten AOPs können als Basis für die Entwicklung von effektiveren und sicheren Modellen und Teststrategien zur Identifikation endokrin wirksamer Substanzen fungieren.

Das hier präsentierte Forschungsprojekt beabsichtigte eine Abschätzung, ob die Analyse von Genexpressionsveränderungen im Rahmen eines von der OECD validierten Fischtests zur Untersuchung endokriner Wirkungen die Identifizierung von endokrinen Disruptoren erleichtern würde und darüber hinaus, eine Aussage über den zu erwartenden Effekt auf apikaler Ebene abgeleitet werden könnte. Im Rahmen dieser Studie wurde folglich ein *Fish sexual development test* (FSDT) durchgeführt, der als eine Modifikation der OECD Testrichtlinie 210, dem *Fish Early Life Stage Toxicity Test* (OECD, 2013a), angesehen werden kann. Ursprünglich wurde der FSDT als „*Fish Partial Life Cycle Test*“ bezeichnet. Er wurde entwickelt, um fokussiert auf die Sexualentwicklung spezifisch endokrine Disruptoren zu detektieren, ohne dabei eine komplette Fischgeneration exponieren zu müssen.

Die grundlegende Hypothese, durch zusätzliche Daten und neue Endpunkte die Beweislast für oder gegen eine endokrine Wirkung einer Substanz zu erhöhen, begründete das Projekt. Zusätzliche Endpunkte, die endokrine Mechanismen und apikale Wirkungen verknüpfen können, verbessern das Verständnis und stärken die Beweislage für die Abschätzung einer möglichen Gefährdung, die im regulatorischen Zusammenhang nach den „*weight-of-evidence*“ erfolgt. Zur Zeit werden mechanistische Daten nur auf der Stufe 2 und 3 (Stufe 2: *in vitro*-Assays zur Erhebung von Daten über endokrine Mechanismen; Stufe 3: *in vivo*-Assays zur Erhebung von Daten zu einzelnen endokrinen Mechanismen und Effekten) gemäß des *Conceptual Framework* der OECD *for Testing and Assessment of Endocrine Disruptors* (OECD, 2010) erhoben, während der FSDT auf Stufe 4 (*in vivo*-Assays zur Erhebung von Daten zu multiplen endokrinen Mechanismen und Effekten) selbst nur grundsätzliche Informationen zum endokrinen Signalweg, der durch einen Stoff beeinflusst wird, liefern kann. Zudem fokussiert sich das *Conceptual Framework* bislang auf die Untersuchung von Substanzen, welche die Estrogen-, Androgen- und Thyroid-Signalwege und die Steroidbiosynthese (EATS) beeinflussen. Andere endokrine Signalwege (z.B. Kortikosteroid-, Gestagen- oder Retinol-Signalwege), die ebenfalls durch exogene Stoffe gestört werden können, werden noch nicht berücksichtigt. Der Bedarf an umfassenderen Methoden wurde bereits erkannt und in einem Review Paper der OECD über „*Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors*“ (Kortenkamp et al., 2011; OECD, 2012a) hervorgehoben.

Ein Hauptziel dieses Projektes war die Herausstellung der Vorzüge einer Integration von (Bio-)Marker-Messungen auf Genexpressionsebene für die Interpretation der Ergebnisse eines FSDT. Im experimentellen Teil der Studie wurde hierzu ein FSDT mit Zebrabärbling in Anlehnung an die OECD-Testrichtlinie 234 durchgeführt. Zudem wurden die Studie, wo möglich, unter Berücksichtigung der Prinzipien der Guten Labor-Praxis (GLP) durchgeführt. Zusätzlich zu den Standard-Endpunkten, die im FSDT untersucht werden, wie Schlupferfolg, juveniles Wachstum und das Geschlechterverhältnis sowie die indikativen Endpunke Plasma-Vitellogeninkonzentration und Histopathologie wurde die Expression von endokrinen Markergenen in den frühen Entwicklungsstadien sowie in juvenilen und präadulten Fischen während des Tests und am Testende gemessen.

Die Grundlagen für die experimentelle Phase des Projekts lieferte eine umfassende Literaturrecherche die dazu diente, einen detaillierten Überblick über die bereits existierenden Daten von Genexpressions-Untersuchungen zur Bewertung von endokriner Disruption bei Fischen zu erhalten. Basierend auf dieser Recherche wurde eine geeignete Testsubstanz ausgewählt. Die Ergebnisse dieser Literaturrecherche wurden in einem UBA-internen, nicht-publizierten Interimsreport zusammengestellt. In diesem Report wird an den entsprechenden Stellen auf den Interimsreport verwiesen.

Material und Methoden

Testsubstanz und -konzentrationen

Als Testsubstanz wurde nach einer ausgiebigen Literaturrecherche (Fenske and Teigeler, 2013; first interims report of project FKZ 3712 63 418, Umweltbundesamt) und Diskussion der Ergebnisse Fadrozol ausgewählt. Fadrozol ist ein potenter, selektiver, nicht-steroidaler Aromatase-Inhibitor. Seine genaue Enzyminteraktion ist noch nicht vollständig geklärt. Eine Koordination mit dem Eisen des Porphyrin-Kerns der Aromatase ist sehr wahrscheinlich, da dies eine charakteristische Funktion von Typ-II-Inhibitoren ist (Browne et al. 1991).

Fadrozol ist als Referenzsubstanz für den Wirkmechanismus der Aromatase-Inhibition etabliert. Die Wirkungen einer Fadrozolexposition auf Fische sind in der Literatur mehrfach beschrieben. Die Inhibition der Aromatase bedingt auf apikaler Ebene eine Verschiebung des Geschlechterverhältnisses in Richtung Männchen und zusätzlich eine Unterdrückung der Vitellogeninproduktion (Ankley et al., 2002; Fenske and Segner, 2004). Ferner gibt es Genexpressiondaten aus Kurzzeittests (Villeneuve et al., 2009; Wang et al., 2012), was Fadrozol als Testsubstanz prädestiniert.

Für den FSDT wurde 100 µg Fadrozol/L als höchste Testkonzentration aufgrund von vorhandenen Literaturdaten, die eine Wirksamkeit von Fadrozol in dieser Konzentration auf apikale Endpunkte zeigen (Andersen et al., 2003; Ankley et al., 2002; Panter et al., 2012) festgelegt. In dieser Konzentration wurde Fadrozol außerdem als Positivkontrolle in den Validierungsstudien für den OECD-21-Tage-*Screening-Assay* (OECD report no. 61, 2006) erfolgreich eingesetzt.

Zusätzlich wurden zwei geringere Testkonzentrationen mit einem Abstandsfaktors von 3.16 festgesetzt, woraus sich die nominalen Testkonzentrationen von 10 µg/L, 32 µg/L und 100 µg/L Fadrozol ergaben. Parallel wurde als Negativkontrolle reines Testmedium unter Verwendung von Verdünnungswasser gewählt.

Die Analyse der dosierten Konzentrationen von Fadrozol im Testwasser wurde mittels Hochleistungs-Flüssigchromatographie/ Tandem Massenspektrometrie mit negativer Ionisierung durchgeführt. Wasserproben wurden regelmäßig aus dem mittleren Teil des Wasserkörpers der Testbecken entnommen und durch die Zugabe von Acetonitril (1:1; v/v), welches 0.2 % Ameisensäure beinhaltete, vor der Lagerung stabilisiert.

Durchführung Fish Sexual Development Test (FSDT)

Der Zebrabärbling (*Danio rerio*) wird durch die OECD als Modellorganismus für endokrine Disruption empfohlen. Er wird außerdem explizit in der OECD TG 234 als Testfisch für den FSDT genannt. Zudem ist der Zebrabärbling seit vielen Jahren eine etablierte Fischart in unserer Prüfeinrichtung. Transkriptomik-Daten zu endokriner Disruption im Zebrabärbling waren aus der Literatur (Fenske and Teigeler 2013. First interims report of project FKZ 3712 63 418, Umweltbundesamt) und aus eigenen Experimenten vorhanden. Zu anderen Fischarten (z.B. Medaka (*Oryzias latipes*)) sind weniger Daten publiziert, was den Zebrabärbling zur besser geeigneten Fischart macht.

Der Test wurde in Übereinstimmung mit der OECD Testrichtlinie 234 (*Fish Sexual Development Test*) mit geringen Modifikationen durchgeführt, die eine parallele Durchführung des FSDT mit der Genexpressionsanalyse zu unterschiedlichen Zeitpunkten ermöglichten. In Anlehnung an die Richtlinie wurde der Test mit 3 Testkonzentrationen und einer Kontrolle mit jeweils 4 Replikaten angesetzt. Zum Start des Tests wurden 340 befruchtete Eier pro Konzentration, d.h. 85 Eier pro Beckenreplikat, eingesetzt. Die Eier wurden auf zwei Brutkäfige verteilt. Ein Brutkäfig diente der Aufzucht der Embryos und Larven für die Genexpressionsanalysen, während der zweite Brutkäfig gemäß den Vorgaben der Richtlinie behandelt und dementsprechend weitgehend ungestört belassen wurde. Die biologischen Standardendpunkte des FSDT umfassen den Schlupferfolg, die Überlebensrate nach Schlupf sowie die Körperlänge, das Feuchtgewicht und die Überlebensrate bei Testauflösung. Um Informationen zum Überleben und dem Wachstum explizit der frühen Lebensphase zur erhalten, wurde am Tag 28 nach Befruchtung die Anzahl der überlebenden Larven bestimmt und die Fischlängen gemessen. Am Ende der Studie wurde eine Gonaden-histopathologische Untersuchung der Fische vorgenommen, um das Geschlechterverhältnis zu bestimmen. Außerdem wurde Vitellogenin (VTG) im Blutplasma der Fische mittels ELISA Kit (Biosense, Norwegen) gemessen. Neben der Untersu-

chung der biologischen Standardendpunkte im FSDT wurde eine quantitative Echtzeit PCR (qPCR) als Methode der Wahl zur Genexpressionsanalyse bei Zebrabärblingsembryonen (Alter 48 Stunden nach Befruchtung (hours post fertilisation, hpf)), Eleutheroembryonen (96 hpf), juvenilen Zebrabärblinge (28 Tagen nach Befruchtung (days post fertilisation, dpf)) und präadulten Zebrabärblingen (63 dpf) durchgeführt. Als Messsystem wurde das *iQ5 real-time PCR* Detektionssystem (BioRad®, Hercules, USA) und der SYBR®GreenER qPCR SuperMix (Invitrogen, Carlsbad, USA) verwendet. Die qPCR-Analysen wurden am Fraunhofer IME Standort Aachen durchgeführt. Es wurden insgesamt 30 Gene ausgesucht, basierend auf den Ergebnissen vorhergehenden eigener Genexpressionsstudien mit 48 hpf Zebrabärblingsembryonen und der Literatur (z.B. Villeneuve et al., 2009), die jeweils eine Regulation dieser Gene durch Fadrozol oder einer Steroidhormon-ähnlichen Substanz zeigten. Aufgrund der Ergebnisse aus einem 96 h Fischembryo-Vortest mit Fadrozol wurden drei der Gene, die dort keine Antwort zeigten, durch zwei neue ersetzt und so letztendlich 29 Gene gemessen.

Ergebnisse und Diskussion

Testbedingungen

Die physikalischen Testparameter Temperatur, Sauerstoffsättigung und pH wurden während der Studie aufgezeichnet. Die mittlere Temperatur in den Testgefäßen war in dem Bereich von 24.5°C und 25.1°C. Die mittlere Sauerstoffsättigung lag bei 99.0 % und 103.4 %, und der mittlere pH –Wert lag zwischen 8.08 und 8.17, mit geringen Abweichungen von den jeweiligen Mittelwerten. Die gemessenen Parameter lagen somit in den von der Richtlinie zugelassenen Schwankungsbereichen.

Die mittleren gemessenen Konzentrationen der Applikationslösungen von Fadrozol lagen im Testzeitraum von Woche 1 bis Woche 9 zwischen 94.4 % und 114.8 %. Die Testkonzentrationen überlappten während des Versuchs nicht und lagen im angestrebten Bereich von 80 % bis 120 % der Nominalkonzentrationen. Die Ausnahme bildeten zwei Testbecken der mittleren Testkonzentration, die von der gleichen Pumpe bedient wurden. Für diese wurden in der zweiten Hälfte der Studie erhöhte Werte > 120 % der Nominalkonzentration gemessen. Nichtsdestotrotz konnte während der gesamten Testdauer eine gute Übereinstimmung mit den nominalen Testkonzentrationen und damit stabile Dosierbedingungen festgestellt werden. Die Auswertung der Effektdaten erfolgte daher auf Basis der nominalen Testkonzentrationen.

Biologische Ergebnisse des FSDT

Die Studie wurde mit dem Einsetzen der 85 befruchteten Eier in die Brutschalen der einzelnen Testgefäße gestartet. Der Schlupf der Embryos wurde ab Tag 3 nach Befruchtung beobachtet. An Tag 6 waren >95 % aller verbliebenen Embryos (nach der Entnahme der Embryos nach 48 hpf und 96 hpf aus einem der Brutschalen für die PCR-Analyse) geschlüpft. Die Überlebensrate nach Schlupf wurde an Tag 28 nach Befruchtung bestimmt, wobei die Anzahl der geschlüpften Larven an Tag 6 jeweils für alle Behandlungen als 100 % festgesetzt wurde. Die durchschnittliche Überlebensrate der Larven bis zum Tag 28 pf unter Kontrollbedingungen betrug 81 % und lag damit im validen Bereich von mindestens 70 %. Die Überlebensrate in den Fadrozol-Behandlungen nahm konzentrationsabhängig ab und erreichte einen Wert von 71 % bei 32 µg /L und von 68 % bei 100 µg/L-. Die statistische Analyse ergab eine signifikante Abnahme der Überlebensraten im Vergleich zur Kontrolle bei 32 und 100 µg/L Fadrozol (William's t-test; $p<0.05$; einseitig getestet gegen Abnahme).

Während der frühen Lebensphase sind Zebrabärblings-Larven besonders empfindlich gegenüber chemischen und physikalischen Stimuli (Belanger et al., 2010; Diekmann and Nagel, 2004; Korwin-Kossakowski, 2008; Woltering, 1984), da der Stoffwechsel von intrinsischer Ernährung durch den Dottersack auf externe Ernährung umstellt, was oft zu erhöhter Mortalität auch unter Kontrollbedingungen führt. Andere Tests mit Fadrozol (eigene Studien; Fenske und Segner 2004), die mit anderen Lebensstadien durchgeführt wurden, waren weniger sensitiv. Zebrabärblings-Embryonen zeigten in IME-internen Untersuchungen keine Mortalität bis zu einer Fadrozol-Konzentration bis 1 mg/L (Macherey, 2013), adulte Dickkopfelritzen zeigten in einem 21 Tage Test keine Mortalität bis 50 µg/L (Ankley et al., 2002). Bei der Planung einer Studie ist der

Ausschluss von Testkonzentrationen empfohlen, die zu systemischer Toxizität führen, da diese auch endokrine Endpunkte in einer nicht-endokrin abhängigen Art und Weise beeinflussen können. Dies wiederum führt zu falsch-positiven Ergebnissen bei Substanzen mit einer unbekannten Wirkweise (Ankley and Jensen, 2014; Wheeler et al., 2013). Da die Wirkweise des Fadrozols sehr spezifisch ist, kann man allerdings davon ausgehen, dass die in dieser Studie beobachteten Effekte bei den überlebenden Fischen am Testende tatsächlich auf die hormonaktive Wirkung der Substanz zurückzuführen waren. Auch die regulierten Gene (Kapitel 5.4) lassen dies schlussfolgern. Mit Ausnahme der frühen Lebensphase wurde in keinem weiteren Zeitfenster Mortalität beobachtet. Es kann postuliert werden, dass die Larven in der sensiblen Phase der Futterumstellung durch die substanzinduzierte Dauerinduktion rezeptorgesteuerter Vorgänge einem zusätzlichen Stressor ausgesetzt waren, der dann final zu einer erhöhten Mortalität in den höheren Konzentrationsstufen geführt hat. Die juvenilen und präadulten Fische konnten dies offensichtlich teilweise kompensieren.

Des Weiteren wurde ein erhöhtes Gewicht der Fische in den belasteten Gruppen im beobachtet, welches im Vergleich zur Kontrolle allerdings nicht signifikant unterschiedlich war. Ein erhöhtes Gewicht aufgrund geringerer Fischdichten lässt sich ausschließen, da alle Beckenreplikate am Tag 28 einheitlich auf 25 Tiere pro Becken reduziert wurden. Die histopathologische Untersuchung der Zebrabärblinge zeigte jedoch einen erhöhten Reifegrad der Gonadenzellen der Tiere in den belasteten Gruppen (Kapitel 5.3.4). Ein leicht erhöhtes Körpergewicht ließe sich mit einem Vorsprung bei der Gonadenreife erklären.

Histopathologische Untersuchungen

Die Geschlechtsbestimmung der Fische erfolgte im Rahmen der histopathologischen Untersuchung. Bei zehn Individuen konnte das Geschlecht nicht bestimmt werden, da das Gonadengewebe während der Präparation beschädigt oder verloren gegangen war. Die fehlenden Proben waren gleichmäßig über alle Konzentrationsstufen verteilt (zwei in den Kontrollen, vier in 10 µg Fadrozol/L, drei in 32 µg Fadrozol/L, eine in 100 µg Fadrozol/L) und die Datenqualität dadurch nicht negativ beeinflusst.

In den Kontrollbecken lag das Geschlechterverhältnis im Mittel bei 50% Männchen zu 50% Weibchen und damit in einem Bereich, der für Zebrabärblingspopulationen unter unbelasteten Bedingungen zu erwarten war (Maack and Segner, 2003). In den Testkonzentrationen 32 µg/L und 100 µg/L waren dagegen keine Weibchen vorhanden, und bei 10 µg/L Fadrozol war lediglich ein Weibchen in einem Replikatbecken zu finden. Für die Bewertung der aus der Fadrozol-Behandlung resultierenden Effekte wurden folglich nur phänotypische Männchen berücksichtigt werden.

Eine Bewertung der Gonadenreife der Tiere erfolgte auf Basis des Gonaden-Maturitäts-Index, der von Baumann et al (2013) beschrieben wurde. In dieser Publikation wird für Zebrabärblings-Weibchen im Alter von 60 dpf ein durchschnittlicher Maturitäts-Index von 3 angegeben. Die Weibchen aus der hier vorliegenden Studie zeigten dagegen einen Maturitäts-Index von durchschnittlich 1.8, damit eine deutliche niedrigere Reife. Das einzelne Weibchen aus der 10 µg/l Behandlung, Replikat A zeigte ebenfalls einen niedrigen Maturitäts-Index. Pathologische Veränderungen wurden bei keinen der untersuchten Ovarien gefunden.

Der normale durchschnittliche Maturitäts-Index von Männchen im Alter von 60 dpf liegt zwischen 2.5 und 3 (Baumann et al., 2013). Die Männchen aus der Kontrollgruppe dieser Studie hatten einen durchschnittlichen Maturitäts-Index von 2.1. Mit steigender Fadrozol-Konzentration stieg der durchschnittliche Gonaden-Maturitäts-Index der Männchen an (bis 2.7 in der 100 µg/L Fadrozol Behandlung).

Durch die Inhibition der Aromatase war mit einem Anstieg der androgenen Sexualhormone zu rechnen. Dies führte offensichtlich zu einer Begünstigung des testikulären Reifungsprozesses und zu einem gesteigerten Wachstum der Testes. Seki et al. (2006) berichteten über erhöhte gonadosomatische Indizes bei Zebrabärblingen nach Exposition an 17ß-Trenbolon, welches als ein starkes Androgen gilt. Der gleiche stimulierende Effekt von 17ß-Trenbolon auf die Testis-Reifung beim Zebrabärbling wurde auch bei Morthorst et al. (2010) beschrieben. Ein weiteres Indiz für den niedrigen Entwicklungsgrad der Gonaden bei den Kontroll-Fischen ist der erhöhte Anteil an Männchen mit „Testis-Ova“ (36.8 % in den Kontrollen im Vergleich zu 17.9 % bei 100 µg Fadrozol/L). Diese Beobachtung lässt darauf schließen, dass sich die Gonaden der behandelten Fische schneller entwickelten als die der Kontroll-Fische. Der Differenzierung der protogynen Gonade

in Testes, wie er für (männliche) Zebrabärblinge beschrieben wurde (Maack and Segner, 2003; Takahashi, 1977), war weder in den Kontrollen noch in den Behandlungen vollständig abgeschlossen.

Wie bereits erörtert, führte die Fadrozol-Exposition zu einer vollständigen Verschiebung des Geschlechterverhältnisses zu den Männchen. Bis auf ein Weibchen wurden alle behandelten Fische als phänotypische Männchen identifiziert. Diese Verschiebung wurde durch die Wirkung des Fadrozol als spezifischer Aromatase-Inhibitor hervorgerufen. Die Cytochrom P450-Aromatase (CYP19) katalysiert die Konversion von C19-Androgenen zu C18-Estrogenen bei allen Wirbeltieren einschließlich Fisch (Callard et al., 1978). Zebrabärblinge erweisen sich aufgrund ihrer nicht-funktionellen protogynen Gonadenentwicklung besonders sensitiv gegenüber Aromatase-Modulationen.

Eine Reduktion der VTG-Konzentration im Blutplasma durch Fadrozol-Behandlung konnte in den Männchen nicht nachgewiesen werden. Aufgrund von Erfahrung und Hinweisen aus der Literatur war dies zu erwarten. Unter Kontrollbedingungen ist der VTG-Plasmagehalt männlicher Zebrabärblinge bereits auf einem, je nach Meßmethode, kaum oder gar nicht messbaren Niveau. Eine verlässliche Messung der Abnahme von Plasma-VTG mittels ELISA kann aus diesem Grund nur bei Weibchen erfolgen. Da lediglich ein einzelnes Weibchen in den Behandlungen gefunden wurde, war es in dieser Studie nicht möglich eine Abnahme der VTG- Konzentration durch die Fadrozol-Exposition zu belegen.

Table 1: Zusammenfassung der Effekte der Standard-biologischen Endpunkte von Fadrozol während des FSDTs

Lebensphase/ Testphase	Endpunkt	NOEC	Beobachtung
Frühe Lebensphase	Schlupf	› 100 µg/L	
	Überleben nach Schlupf	10 µg/L	
	Mittlere Länge	› 100 µg/L	
Juvenile Wachstumsphase	Überleben am Testende	› 100 µg/L	
	Mittlere Länge	› 100 µg/L	
	Mittleres Gewicht	› 100 µg/L	
Histopathologie	Geschlechterverhältnis	‹ 10 µg/L	Vollständige Verschiebung des Geschlechterverhältnisses zu Männchen.
	Maturitäts-Index (Männchen)	‹ 10 µg/L	Erhöhter Maturitäts-Index im Vergleich zur Kontrolle indiziert beschleunigte Gonadenreifung.
	Maturitäts-Index (Weibchen)	NB	
	Testis-Ova	‹ 10 µg/L	Anzahl der Männchen mit Testis-Ova sinkt mit steigender Fadrozol Konzentration an und indiziert ebenfalls beschleunigte Gonadenreifung.
Biomarker	Vitellogenin (Männchen)	› 100 µg/L	
	Vitellogenin (Weibchen)	NB	Lediglich ein Weibchen in den Behandlungen vorhanden

Zur NOEC-Bestimmung wurde der William's t-test, *one-sided smaller*, mit einem Signifikanzniveau von $p<0.05$ durchgeführt; NB = nicht bestimmbar.

Ergebnisse Genexpressionsanalyse

Die Expression von 29 Genen wurde mittels quantitativer RT-PCR bei 48 hpf und 96 hpf alten Embryonen und Larven, 28 dpf alten juvenilen und 63 dpf alten (präadulten) Fischen gemessen. Die Gene wurden ausgewählt, weil sie bereits bei 48 hpf und 96 hpf alten Zebrabärblingsembryonen eine Regulation nach Exposition an Steroidhormon-ähnlichen Substanzen gezeigt hatten (Schiller et al., 2014, 2013b und M. Macherey 2013 (unveröffentlichte Masterarbeit)) oder bei ausgewachsenen Fischen durch Kurzzeitexposition an Fadrozol beeinflusst waren (Villeneuve et al., 2009). Das Ziel dieser Untersuchung war es, die Expression und eine mögliche Regulation dieser Gene durch Fadrozol zu analysieren, um so die mögliche Anwendbarkeit dieser Gene als frühe Biomarker endokriner Disruption verursacht durch Aromatase-Hemmung zu prüfen. Die ausgewählten Gene sind an wichtigen Regulationswegen entlang der HPG-Achse beteiligt.

Die qPCR-Ergebnisse zeigten, dass sich das Muster der Genregulation bei den embryonalen (48 hpf) und larvalen (96 hpf) Stadien von den späteren Entwicklungsstadien unterschieden. Insgesamt wurden 17 Gene gefunden, die in mindestens einer der Fadrozol-Expositionsgruppen an einem der vier Zeitpunkte reguliert waren. Sieben der 17 Gene zeigten sich in mehr als einer Expositionsgruppen zu einem gegebenen Zeitpunkt reguliert. Kein Gen wurde an allen Zeitpunkten signifikant reguliert, und lediglich *vtg1, star, igf1* und *zgc:64022* waren an mehr als einen Zeitpunkt signifikant reguliert. Die höchste Anzahl an regulierten Genen fand sich bei 96 hpf, wobei vier Gene (*vtg1, esr2a, gnrhr2, gnrhr3*) herunter- und drei Gene (*igf1, sox9b, zgc:64022*) herauf reguliert waren. Eine konzentrationsabhängige andauernde Hochregulierung zeigte sich für *igf1*. Eine signifikante Hoch- bzw. Herunterregulation sowohl bei 48 hpf und 96 hpf wurde für *vtg1*, und *igf1* nachgewiesen. Für *mvd* zeigte sich nach 48 hpf eine signifikante Hochregulation.

Zum Zeitpunkt 28 dpf zeigten Genregulation wie auch die Expressionsniveaus keine Übereinstimmung mit den früheren Zeitpunkten und nur wenig Übereinstimmung mit dem 63 dpf Zeitpunkt. Keine Gene waren signifikant reguliert. Als ein möglicher Grund für das Fehlen von Effekten auf Genexpression zu diesem Zeitpunkt wird das Poolen mehrerer Fische diskutiert. Zeitpunkt 28 dpf fällt in die frühe Phase der Geschlechts- und Gonadendifferenzierung beim Zebrabärbling (Maack und Segner 2004, Baumann et al. 2013), weshalb von einer bereits beginnenden geschlechtlichen Differenzierung bei den Fischen dieser Studie auszugehen ist (auch wenn dies weder histologisch noch genetisch untersucht wurde). Diese geschlechtliche Differenzierung wirkte sich wahrscheinlich bereits auf die Expression von geschlechtsdimorphen Genen aus, wie es sich zum Zeitpunkt 63 dpf in dieser Studie manifestierte (Kapitel 5.4.4). Daraus kann als Hypothese abgeleitet werden, dass die Streuung der Genexpressionsniveaus bei vielen der regulierten Gene in den Kontrollen und den Fadrozol-Behandlungen höher war als zu den früheren Zeitpunkten und somit keine statistisch signifikanten Ergebnisse erzielt werden konnten. Die Höhe der Fehlerbalken in Figure 10 stützt diese Hypothese. Eine individuelle Bestimmung der Genexpression hätte hier mehr Aufschluss über die Streuung geben können. Durch das Poolen von vier Fischen wurden individuelle Effekte vermutlich verschleiert und aufgrund der geringen n-Zahl die Streuung im Mittel nicht genügend reduziert.

Bei 63 dpf es wurde es möglich die Expression der Zielgene in Rumpfproben (ohne Kopf) individueller Fische zu messen anstatt in gepoolten Proben, wie zu den früheren Zeitpunkten. Während der Analyse der 63 dpf-Daten verdeutlichte sich bei den Kontrollproben eine auffällige Divergenz in den *vtg1* mRNA Mengen, die unterschiedliche Expressionsniveaus bei weiblichen (*high vtg1*) und männlichen Fischen (low *vtg1*) vermuten ließ. Folglich wurden die Kontrollfische in eine Gruppe mit niedrigen und eine Gruppe mit hohen *vtg1* Expressionsniveaus unterteilt und Expressionswerte der anderen Gene entsprechend dieser Gruppierung unterteilt. Die so gebildeten Low-und High-Level *vtg1* Kontrollgruppen erwiesen sich als sehr unterschiedlich in der Regulation der meisten Gene (Figure 12), mit der Ausnahme der Steroidbiosynthese-Gene, *star* und *igf1*, wobei *igf1* eine Induktion nur vermuten ließ. In der Low *vtg1* Kontrollgruppe waren nur *lss, star* und *prox1* hochreguliert, und zwar in mindestens bei einer der Fadrozol-Konzentrationen. In der High *vtg1* Gruppe waren *esr2a*, *cyp19a1b* und *zgc:64022* bei allen Fadrozol-Konzentrationen herunter reguliert, und *star, lss, igf1* und *igfbp5a* in mindestens einer Fadrozol-Konzentration hochreguliert.

Die Fadrozol-Exposition beeinflusste während der frühen Lebensphase lediglich die Gene *vtg1* und *igf1*, wobei *vtg1* bei 48 hpf herunter- und der Wachstumsfaktor *igf1* bei 96 hpf herauf reguliert wurde. Ungleich

reagierten die Steroidsynthese-Gene und des Aromatase-Gens *cyp19a1b* nur bei 63 dpf, was auf eine Stimulation der Steroidhormon-Biosynthese durch Hochregulation von *star* und *lss* schließen lässt und die Hemmung der gehirnspezifischen Aromatase *cyp19a1b* (herunter reguliert) bei den Low *vtg1* Fischen zeigt. Stark herunter reguliert war auch der östrogene Biomarker *vtg1* am 63 dpf Zeitpunkt.

Über die Zeit zeigten sich die deutlichsten Änderungen für die östrogenregulierten Gene *lss, vtg1, star, igf1* und *cyp19a1b* bei mehr als einer Fadrozolkonzentration und für mindestens einen Zeitpunkt. Diese Gene repräsentieren verschiedene regulative Signalwege wie Steroidsynthese, Lipidtransport, Zellwachstum und Aromatase-Aktivität, die bekanntermaßen von endokriner Disruption in unterschiedlicher Weise betroffen sind. Diese Gene wurden als die wahrscheinlichsten potenziellen (Bio-) Markergene für den Nachweis und die Identifizierung von Aromatase-Hemmung im Rahmen des FSDT identifiziert.

Der Wachstumsfaktor Igf1 spielt eine zentrale Rolle bei unterschiedlichen physiologischen Prozessen, wie Wachstum, Differenzierung und Immunreaktionen (z.B. Perez-Sanchez et al., 2000). Die Funktion bei endokrinen Prozessen und Empfindlichkeit gegenüber endokriner Disruption ist für Fischen beschrieben, jedoch sind die Angaben zur Hoch-und Herunterregulierung des Gens oder zum endokrinen Wirkmechanismen uneinheitlich für unterschiedliche Fischarten (z.B. Filby et al., 2007; Shved et al., 2008). Unsere Ergebnisse lassen eine Beteiligung in der Aromatasehemmung vermuten, denn wir fanden eine dauerhafte Hochregulierung durch Fadrozol, wenn auch nicht immer in signifikantem Maße. Igf1 aktiviert IGF-Rezeptoren, was zu einer Induktion von Transkriptionsfaktoren führt und letztlich die Expression von *star* beeinflusst.

Star ist für den Transport von Cholesterin zur inneren Mitochondrienmembran verantwortlich, was als geschwindigkeitsbestimmender Schritt bei der Steroidhormonsynthese gilt. Eine differenzielle Genexpression bei Fischen nach Exposition ist für *star* für verschiedene EDCs beschrieben (Johns et al. 2011; Sharpe et al., 2007). Wir fanden eine Hochregulierung von *star* im Vergleich zu den Kontrollen bei den präadulten 63 dpf Fischen in der höchsten Fadrozol-Exposition.

Gene der Terpenoid-/Isoprenoid-Synthese, wie *lss, sc4mol* oder *mvd*, sind an der Produktion von Cholesterin aus Fettsäuren beteiligt. Eine Regulation dieser Gene konnte für mindestens einen (*mvd*) bzw. zwei Zeitpunkte (*lss*) während der Studie demonstriert werden, was mit der von Schiller et al. (2014) beschriebenen Regulierung dieser Gene oder der entsprechenden Signalwege durch östrogene und anti-androgene Substanzen übereinstimmt. Eine Regulation von *lss* war während der gesamten Exposition, wie bei *star*, nur im Präadultstadium signifikant verändert. Dies könnte möglicherweise eine Feedbackreaktion auf die Reduktion von Östrogenen durch die Aromatasehemmung widerspiegeln.

Das kodierende Gen für das Enzym Aromatase kommt bei den meisten Fischen in zwei Isoformen vor, die unterschiedlicher Regulation unterliegen (Fenske and Segner, 2004; Kishida and Callard, 2001; Menuet et al., 2005). Die im Gehirn vorherrschende Isoform *cyp19a1b* wird über ein Estrogen-Response-Element (ERE), geknüpft an eine Östrogenrezeptor-Aktivierung gesteuert, und ist somit Östrogen abhängig reguliert. Die in den Gonaden vorherrschende Form *cyp19a1a* dagegen ist Östrogen-unempfindlich. In der Tat konnten wir zeigen, dass nur *cyp19a1b* in den erwachsenen Fischen durch Fadrozol signifikant herunter reguliert war. Die andere Isoform *cyp19a1a* war entgegengesetzt reguliert, wenn auch nur in nicht-signifikanten Maße. Interessanterweise war die Regulation beider Gene entgegengesetzt bei 63 dpf im Vergleich zu der High *vtg1* Kontrollfischen und im stärkeren Maß, auch im Vergleich zu den Low *vtg1* Kontrollen. Die Expressionsänderungen von *cyp19a1a* waren jedoch nicht signifikant waren.

Schlussfolgerung und Ausblick

Trotz hoher Komplexität der Ergebnisse und großer Datenmengen war es möglich, ein spezifisches Genexpressionsmuster herauszuarbeiten. Letztendlich gelang es mit der Studie, den bereits definierten *Adverse Outcome Pathway* (AOP) für Aromatase-Inhibition (AOP-WiKi - https://aopkb.org/aopwiki/index.php/Main_Page) für die FSDT Situation anzupassen. Im Rahmen der Studie gelang es uns, vielversprechende Markergene zu identifizieren, die zusätzlich zu den etablierten indikativen Parametern Vitellogenin und Histopathologie der Gonaden, als potenzielle Biomarker für

Aromatasehemmung und/oder Steroidhormonsynthese-Störungen im Rahmen von ED-Tests, wie dem FSDT, genutzt werden könnten.

Trotz eines erfolgreichen Abschluss der Studie ergaben sich basierend auf den Ergebnissen und den gewonnenen Erfahrungen Problem- und Fragestellungen für weiterführende Untersuchungen. Zum einen war die Prüfsubstanz Fadrozol bekanntermaßen eine sehr spezifisch wirkende Substanz, was sich anhand der eindeutigen Effekte auf die apikalen Endpunkte Gonadendifferenzierung und Geschlechterverhältnis präsentierte und die Ergebnisse zur Genexpression prägte. Für die Verifizierung der Ergebnisse dieser Studie wäre als nächster Schritt die Exposition an eine weniger spezifisch wirkende Substanz, die bekanntermaßen ähnliche endokrin relevante adverse Effekte hervorruft, erforderlich. Empfehlenswert wäre ein EDC mit nicht-ausschließlichem Aromatase-Inhibitor Wirkmechanismus oder eine Substanz mit einer anderen ED Modalität, aber gleicher apikaler Wirkung, um in einer Folgestudie Genantworten für die identifizierten Markergene zu prüfen. Abschließend wäre für eine Validierung des Ansatzes die Testung von unbekannten EDCs nötig, wobei bevorzugt endokrine Mechanismen in Betracht gezogen werden sollten, die nicht direkt mit der Wirkung der Sexualhormone in Verbindung stehen. Frühe endokrine Regulationsprozesse, induziert durch Progestine und Gestagene, wären hier mögliche Targets.

Aus der Studie ergab sich ferner die Frage nach der praktischen Anwendung solcher Wirkmechanismen oder indikative molekularen Biomarker im regulatorischen Kontext. Basierend auf den Ergebnissen wurden die folgenden Ansätze diskutiert:

- Ein durch Gen-Biomarker erweiterter Fischembryotest als EDC-Screening-Tool.
  Solch ein erweiterter Fischembryotest könnte die Basis einer gestuften Teststrategie darstellen. Gegenüber den derzeit propagierten zellbasierten Screening-Assays (OECD Conceptual Framework 2010) hat der Fischembryotest den Vorteil, die Untersuchung von endokrin-spezifischen Biomarkern in einem Gesamtorganismus zu ermöglichen. Die Aussagekraft von Daten aus *in vivo*-Assays ist deutlich höher als bei *in-vitro* einzustufen, da Zellsystemen die Komplexität eines Organismus und Zell-Zell-Interaktionen fehlen. Eine dadurch erzielte höhere Verlässlichkeit von Screening-Test durch eine reduzierte falsch-negative Fehlerrate könnte ein Beitrag zur Vermeidung von unnötigen Fischtests (damit Tierversuchen) sein.
- Die Erweiterung der bestehenden Prüfrichtlinien durch Genexpressionsanalysen

  Die steigende Zahl an Zulassungverfahren für Substanzen mit unbekannter Wirkweise in nicht-Zielorganismen wird den Bedarf und die Relevanz von EDC-Testmethoden weiter steigen lassen. Eine Integration von Gen-Biomarkern in bestehende Testrichtlinien könnte ein Beitrag zur verbesserten Identifikation von potenziellen EDC, auch im Rahmen von nicht-endokrinen Tests sein. Dies wäre beispielsweise interessant für pharmazeutische Substanzen, die häufig gezielt auf das Hormonsystem von Mensch oder Zuchtvieh wirken.
- Verwendung von Genanalysen für Biomonitoring Fragestellungen

  Die Untersuchung von biologischen Matrices aus Monitoringprojekten bietet ein breites Einsatzgebiet für Genexpressionsanalysen. Bei der Untersuchung von Biotaproben aus belasteten Lebensräumen wäre neben der klassischen chemischen Analyse auf Schadstoffrückstände (z.B. Pharmazeutika und Pestizide) die Untersuchung der Folgen einer Belastung anhand von Genexpressionsmustern im Rahmen einer wirkungsorientierten Analytik (EDA) denkbar.

2 Summary

Introduction

Environmental pollutants which interact with the endocrine system of organisms, and which exert a negative impact on development and reproduction, became known to the public in the 1990ies as endocrine disruptors. Especially the increase in reproductive disorders in humans, and observations of gonadal alterations including feminisation in aquatic populations (i.e. fish, but also amphibians and gastropods), moved the topic to the public eye. Such evidence was scientifically interpreted as indication of high risk and communicated and published as such. For the first time this risk potential was discussed on an international symposium in Weybridge (UK) in 1996 by representatives from science and authorities. One mayor goal of this workshop was the decision on a definition for endocrine disruptor, which was adopted later on by the WHO in a slightly modified version. It states: "An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO/IPCS 2002). Further, it was defined that "A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or progeny, or (sub)populations".

Meanwhile, the topic "Endocrine disruptors" has found its way into diverse legislations, e.g. into REACH and the water framework directive (2000/60/EG), as well as into the pesticide- and biocide ordinances. Additionally, a joint framework concept was developed by the Endocrine Disrupters Testing and Assessment (EDTA) task force for the analysis of endocrine active risk potential, which was approved on the 6th EDTA workshop in Tokyo 2002 (ENV/JM/TG/EDTA (2002)4). On this basis, the development and validation of OECD test guidelines for the testing of endocrine active substances was implemented.

However, endocrine disruption manifests in an organism or a population only after sub-acute or chronic exposure, and identification of hormone-active substances is therefore time- and cost-intensive. Further, these elaborative experiments require a huge amount of animals, and alternatives, which consider the principles of the 3R-concept (Replacement, Reduction and Refinement) after Russell and Burch (1959), are highly appreciated. However, short-term tests for the determination of apical endpoints are not recommended. They were designed as screening tool to trigger complex studies like the fish life cycle test, which they cannot replace. Moreover, tests focussing only on the early developmental stages (e.g. the fish early life stage toxicity test) might not lead to satisfactory results, as they do not include relevant exposure windows, e.g. the sexual development. Since apical endpoints respond slowly to exposure, it is however advisable to include quick responding parameters which can also be indicative of the underlying MOA.

In this context, inclusion of molecular endpoints may be a promising approach as they react promptly on pollutants. They may further be indicative of adverse effects at organism level (Piersma, 2006). Powerful genomic approaches have contributed essentially to the determination of molecular mechanism resulting in apical adverse endpoints (Ankley et al., 2006; Sawle et al., 2010). Global transcriptomics allow meaningful analyses of adverse effects in cells, organs, and the whole organism at concentrations potentially below thresholds of the most sensitive endpoints of the "conventional" studies. This higher sensitivity of molecular analyses is crucial to prevent the overlay of endocrine effects by other effects, esp. systemic toxicity (Scholz and Mayer, 2008; Wang et al., 2010).

The progress in molecular and computer-based (*in silico*) methods paved the way for the shift of paradigms in ecotoxicology (NRC 2007), and for the development of alternative concepts for environmental risk assessment, e.g. the Adverse Outcome Pathways (AOPs; Ankley et al., 2010; Villeneuve and Garcia-Reyero, 2011), or the Toxicity Pathways (Bradbury et al., 2004). In the sense of AOPs, the direct link between exposure, the molecular initiating events (MIEs), and the resulting apical endpoints on organism/population level should be explored (Kramer et al., 2011; Segner, 2011, 2009) also for endocrine disruption. This will provide the basis for the development of more effective and reliable models for risk assessment.

The here presented research project intended to appraise whether the analysis of changes in gene expression in the context of a validated OECD fish test guideline (TG) could facilitate the identification of endocrine disruptors and perhaps even the prediction of endocrine mediated effects at the apical level. The FSDT can be described as a modification of the OECD guideline 210, the Fish Early-Life Stage Toxicity Test (revised version of 2013). The Fish Sexual Development Test (FSDT) was originally considered as "Fish Partial Life-Cycle Test" and was aimed at providing an alternative test that specifically detects endocrine disrupters in fish without the requirement to expose for a whole generation.

The rationale of these investigations was to generate supplementary data to the FSDT that could help increasing evidence on whether or not a substance acts as an EDC. The use of other, new endpoints may help in understanding the link between endocrine-related mechanisms and apical effects in the weight-of-evidence-approach that is currently pursued in the regulatory context to assess an endocrine disruption hazard (OECD 2012b). Currently, the conceptual framework of the OECD for Testing and Assessment of Endocrine Disruptors (OECD 2010) provides mechanistic data only at level 2 and 3 (level 2: *in vitro* assays providing data on endocrine mechanisms; level 3: *in vivo* assays providing data about single endocrine mechanisms and effects), whereas the FSDT at level 4 (*in vivo* assays providing data about multiple endocrine mechanisms and effects) can deliver only basic information on the endocrine signalling pathway(s) being affected by a chemical. Besides, the conceptual framework so far focuses only on the assessment of chemicals disrupting estrogen, androgen, thyroid signalling processes and steroidogenesis (EATS), but there are several other endocrine pathways (e.g. corticosteroid, gestagenic or retinoid signalling pathways), which may be disrupted by chemical exposure. The need for more comprehensive methods has been acknowledged and has also been highlighted in the Review Paper of the OECD on Novel *In Vitro* and *In Vivo* Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors (Kortenkamp et al., 2011; OECD, 2012b).

The central goal of this project was to demonstrate the benefits of an integration of (bio-) marker measurements at gene expression level for the interpretation of FSDT results. To this end, a FSDT was carried out with zebrafish according to the OECD guideline and in compliance with general principles of GLP.

In addition to the default endpoints (hatch, juvenile growth, sex ratio, plasma vitellogenin, histopathology), the expression of endocrine related marker genes was measured in early life stages as well as in trunks of pre-adult fish during and at the end of the test.

The basis for the experimental phase of this project was provided by an extensive literature review in order to obtain an in-depth overview of the existing data base of gene expression level investigation for the evaluation of endocrine disrupting effects in fish. Based on this review, a suitable test substance was selected. The results of the literature review were presented in an UBA-internal, unpublished interims report. Relating sections of the present report are indicated by the corresponding citation of the report (Fenske and Teigeler 2013; first interims report of project FKZ 3712 63 418, Umweltbundesamt).

Material and Methods

Test item and test concentrations

Based on the results of the extensive literature study (Fenske and Teigeler 2013; first interims report of project FKZ 3712 63 418, Umweltbundesamt) and following internal discussions, fadrozole was chosen as test item. It is a potent, selective, non-steroidal aromatase inhibitor. Its exact enzyme interaction is not fully understood. However, coordination with the iron of the porphyrine core of aromatase is most likely, as this describes a characteristic function of type-II-inhibitors (Browne et al., 1991).

Fadrozole is an established reference substance representing aromatase inhibitors. Effects on fish to fadrozole exposure are well described in the literature. Effects related to aromatase inhibition on the apical level are a shift of the sex ratio towards males and moreover the repression of vitellogenin production (Ankley et al., 2002; Fenske and Segner, 2004). Additionally, gene expression data are present, derived from short-term exposition tests (Villeneuve et al., 2009; Wang et al., 2012), predestining fadrozole as test item.

The maximum concentration of 100 µg fadrozole/L was based on available data from literature, which indicated potency of fadrozole at this concentration on apical endpoints (Ankley et al., 2002; Panter et al., 2004; Andersen et al., 2004). Further, this concentration was successfully applied as positive control for validation studies for the OECD 21-day screening assay (OECD report no. 61, 2006).

Two additional lower test concentrations with a spacing factor of 3.16 were applied, resulting in nominal test concentrations of 10 µg/L, 32 µg/L, and 100 µg/L fadrozole. A negative control (dilution water only) was applied in parallel. Fadrozole substance analysis was performed by high performance liquid chromatography tandem mass spectrometry with negative ionization. Water samples were taken from the vessels from the mid water body on a regular basis and stabilised by addition of acetonitrile (1:1; v+v) containing 0.2 % formic acid prior to storage.

Performance of the Fish Sexual Development Test (FSDT)

Zebrafish (*Danio rerio*) is recommended by the OECD as model organism for testing endocrine disruption. It is explicitly mentioned in the OECD TG 234 as test fish species for Fish Sexual Development Tests (FSDTs). Besides, the zebrafish has been a well-established organism in the test facility for many years. Transcriptomics data on endocrine disruption in zebrafish are available in the literature as well as from data obtained from experiments performed in the test facility. Data on other test species (e.g. medaka), are less frequently published, making this fish species less suitable for the presented project.

The test was performed in accordance to the OECD TG 234 (Fish Sexual Development Test) with only minor modifications allowing parallel performance of the FSDT and analysis of gene expression at different time points during juvenile growth and sexual development. Thus, as requested by the TG 234, the study was performed with 3 test concentrations and a control, in 4 replicates each. The test was started with 340 zebrafish eggs per concentration, i.e. 85 eggs/replicate, allocated to two fry cages. The first fry cage served to provide samples for gene expression analysis, while the second fry cage was handled in accordance to the guideline and was kept mainly undisturbed. Standard biological test endpoints of the FSDT include hatching success, post-hatch survival, body length, wet weight, and survival rate at test termination. To receive additional information on the early life stages, survival and total body length were additionally examined on day 28 post fertilization. At test end, gonad histopathology was performed to assess the sex ratio of the exposed fish groups, and vitellogenin content in blood plasma was measured.

In addition to the standard biological endpoints of the FSDT, quantitative Real-time PCR (qPCR) was used as the method of choice for gene expression analysis in zebrafish embryos (at 48 hpf) and eleutheroembryos (96 hpf) as well as in the 28 dpf and 63 dpf zebrafish. The iQ5 real-time PCR detection system (BioRad®, Hercules, USA) and the SYBR®GreenER qPCR SuperMix (Invitrogen, Carlsbad, USA) were used for the qPCR assays at the Fraunhofer IME in Aachen. 30 genes were selected, which showed regulation after exposure to fadrozole or a steroid hormone like compound in own zebrafish embryo gene expression studies or in the literature (e.g., Villeneuve et al. 2009). Based on the results of a 96 h fish embryo pre-test with fadrozole, three of the 30 genes were dropped due to the lack of response and replaced by two different ones, resulting in 29 genes which were eventually measured.

Results and Discussion

Test conditions

The physical test parameters temperature, oxygen saturation, and pH were recorded during the time course of the study. The mean temperature in the test vessels was in the range between 24.5°C and 25.1°C, the mean oxygen saturation was in the range between 99.0 % and 103.4 %; and the mean pH was between 8.08 and 8.17, with minor deviations. Thus, these parameters were in the range defined by the test guideline.

The mean measured concentrations of the application solutions of fadrozole between week 1 and week 9 of the study ranged between 94.4 % and 114.8 % per treatment. Test concentrations during the time course of the study did not overlap and were in the 20 % range for most of the samples. However, two samples of the middle test concentration, which were served by one pump, displayed higher concentrations in the second half of the study. Overall, measured concentrations were in good accordance with the nominal concentrations over the whole test duration. Thus, the calculation of effect data was based on nominal test concentrations.

Biological results of the FSDT

The study was initiated by introducing 85 eggs into the fry cages of each test vessel. Hatching was observed starting from day 3 post fertilisation (3 dpf), and a > 95% hatching rate of remaining eggs after sampling for gene expression analysis in all concentrations was observed at day 6 pf. The post hatch survival during early life stage was determined at 28 dpf. For that purpose, the number of hatched larvae at day 6 pf was defined as 100 % for all treatment levels. Of the controls, 81 % of larvae survived until day 28 pf in compliance with the valid range of at least 70 %. The survival rate of the treatments decreased concentration-dependent to a value of 71 % at a fadrozole concentration of 32 µg/L and to 68 % at the highest fadrozole concentration of 100 µg/L. This decrease resulted in values statistically significantly different from the control (William's t-test; $p<0.05$; one-sided smaller). During the early life stage, zebrafish are especially sensitive to chemical and physical stimuli (compare Belanger et al., 2010; Diekmann and Nagel, 2004; Korwin-Kossakowski, 2008; Woltering, 1984), as the metabolism changes from intrinsic feeding of the yolk sac to external feeding, which often results in an increased mortality also at control conditions. Tests performed with fadrozole on other life stages are less sensitive, i.e. the zebrafish embryo (no mortality up to a fadrozole concentration of 1 mg/L, unpublished results) or adult fish (fathead minnow; 50 µg/L; Ankley et al., 2002). It is recommended to avoid test concentrations that result in systemic/overt toxicity, as this might influence endocrine endpoints in a non-endocrine manner, and thus would lead to false-positive results for substances with unknown mode of action (Ankley et al., 2014; Wheeler et al., 2013). However, as the mode of action of fadrozole is known to be very specific, the observed effects were assumed to be endocrine related. Since no further mortality was observed during the juvenile growth phase of treated fish, it was postulated that the additional stress of continuous aromatase inhibition during the sensitive phase of food adaptation caused the increased mortality of the exposed larvae. Juvenile and pre-adult fish were able to compensate.

Another biological effect not primarily considered as an adverse endocrine effect was a gain in weight in treatment groups compared to the controls (although statistically insignificant). The mean weight slightly increased from 0.183 g in the controls to 0.196 g in the highest fadrozole exposure concentration. This observation was likely not based on differences in fish density, as replicates were randomly reduced to 25 fish after 28 days of exposure. The histopathological examination of the fadrozole treated fish revealed a higher maturity level of exposed compared to control fish, which could explain the observed weight gain.

Histopathological examinations

The classification of the gonadal sex was part of the histopathological examinations. For ten individuals the fish sex could only be classified as unknown due to technical reasons, as their inner organs were lost during the sampling process. However, these fish were equally distributed over all treatment levels (i.e. 2 in controls; 4 in 10 µg fadrozole/L; 3 in 32 µg fadrozole/L; 1 in 100 µg fadrozole/L) and thus, the impact on the data quality can be considered as low.

The sex ratio in the control vessels was found to be quite close to 50 % males and 50 % females, which can be expected for unexposed populations of zebrafish (Maack and Segner 2003). In contrast, no females were found in the 32 µg/L and 100 µg/L fadrozole exposures, and just one female at 10 µg/L.

The evaluation of gonad maturity was performed as described by Baumann et al. (2013). In this publication, the maturity index of zebrafish females at an age of 60 dpf was specified with 3. However, the females of our study expressed a maturity index of 1.8, which is considerably lower. The single female of group 1 also showed rather immature gonads. Pathological alterations could not be found in any of the ovaries.

Males from the control group had an average gonad maturity index of 2.1. Average maturity indices of males at the age of 60 dpf were found to be in the range of 2.5 -3.0 (Baumann et al., 2013). In this study, the average gonad maturity of males increased with increasing exposure concentrations (up to 2.7 in a treatment with 100 µg fadrozole/L). The increased gonad maturity underlines this impression of a strong masculinizing effect. Elevated androgen levels (either caused directly by exogenous androgens or indirectly by aromatase inhibition) are known to trigger male gonad maturation (Baumann et al., 2013). Seki et al., (2006) reported elevated gonadosomatic indices in zebrafish after exposure to 17ß-trenbolone (strong androgen). The same stimulating effect on testis maturation was also observed by Morthorst et al., (2010), who exposed zebrafish to 17ß-trenbolone for 60 days. A further indication of allow maturity stage of the gonads in control fish is the increased amount of “testis-ova” in males (36.8% in controls, compared to 17.9% in a treatment with 100 µg fadrozole/L). This observation implicates that the gonads of exposed fish were further developed than those of the control fish and therefore had lower occurrence of testis-ova, which are typical for the transition phase of the protogynic gonad as described for zebrafish Takahashi (1977), Maack and Segner (2003), and others.

In summary, the most prominent effect of fadrozole was a skewed sex ratio towards males caused by the specific aromatase inhibitor properties of fadrozole. Apart from one female at the lowest exposure concentration, all exposed fish were identified as gonad phenotypic males. The cytochrome P450 aromatase (CYP19) regulates the conversion of C19-androgens to C18-estrogens in vertebrates including fish (Callard et al., 1978). As zebrafish undergo a developmental gonadal stage described as non-functional protogynous gonad, which transition to either functional ovary or testes is sex steroid hormone regulated, zebrafish can be assumed particularly sensitive to aromatase modulations.

A reduction in VTG concentration was not observed upon fadrozole treatment in males. Based on experience and evidence from the literature, this result was anticipated. The VTG plasma content of male zebrafish at control conditions is barely, if at all, measurable, depending on the method. A decrease in plasma VTG is therefore be measured reliably only in females. Since only one single female remained in the fadrozole treatments, a fadrozole exposure induced decrease of plasma VTG could not be shown in this study.

Summary of effects on standard biological endpoints of fadrozole during the time course of the FSDT

Table 2: Summary of effects on standard biological endpoints of fadrozole during the time course of the FSDT

Life stage/ test phase	Endpoint	NOEC	Observation
Early life stage	Hatch	› 100 µg/L	
	Post hatch survival	10 µg/L	
	Mean length	› 100 µg/L	
Juvenile growth phase	Survival at test termination	› 100 µg/L	
	Mean length	› 100 µg/L	
	Mean weight	› 100 µg/L	
Histopathology	Sex ratio	‹ 10 µg/L[1)]	The sex ratio was shifted to males
	Maturity index (males)	‹ 10 µg/L[2)]	The maturity index was found to be increased in treatments compared to the control, indicating a progress in maturation due to fadrozole treatment.
	Maturity index (females)	ND	as no females were found in fadrozole treatments
	Testis-ova	‹ 10 µg/L[3)]	The number of males with testis-ova was decreased in treatments, indicating a progress in maturation due to fadrozole treatment.
Biomarker	Vitellogenin (males)	› 100 µg/L	
	Vitellogenin (females)	ND	as no females were found in fadrozole treatments

For NOEC determination, William's t-test, one sided smaller, with a significance level of $p<0.05$ was performed; ND = not determinable.

Gene expression analysis results

Expression of 29 genes was measured in 48 hpf and 96 hpf embryos and larvae, in 28 dpf juvenile and 63 dpf pre-adult fish using quantitative RT-PCR. Unless stated otherwise in Table 4, the genes were chosen because they had previously indicated regulation in 48 hpf and 96 hpf zebrafish embryos after exposure to steroid hormone like compounds (Schiller et al., 2014, 2013b; Macherey, 2013) and/or in adult fish after short-term exposure to fadrozole (Villeneuve et al., 2009), The aim of this investigation was to analyse the expression and possible regulation of these genes by fadrozole in order to evaluate their potential application as early biomarkers of endocrine disruption caused by aromatase inhibition. The selected genes are involved in key regulatory pathways along the HPG axis.

The qPCR results obtained for the different time points revealed that the pattern of gene regulation in the embryonic (48 hpf) and larval (96 hpf) stages differed from the later developmental stages. Altogether, 17 genes were regulated in at least one fadrozole exposure group at any of the four time points, and seven of these 17 genes were found regulated in more than one exposure group at a given time-point. No gene was significantly regulated at every time points, and only *vtg1, star, igf1* and *zgc:64022* were significantly regulated at more than one time-point. The highest number of genes regulated was found at 96 hpf, with four genes (*vtg1, esr2a, gnrhr2, gnrhr3*) being down and three genes (*igf1, sox9b, zgc:64022*) being upregulated. An exposure concentration dependent persistent up regulation was demonstrated for *igf1*. Significant down

respectively up regulation both at 48 hpf and 96 hpf was found for *vtg1* and *igf1*. For *mvd*, a significant regulation was observed at 48 hpf.

At 28 dpf, gene regulation and expression levels showed no coherence with the earlier time points and little agreement with the 63 dpf time point. No significantly regulated gene was found. One possible reason for the lack of effects on gene expression at this time point discussed was the pooling of several fish. The 28 dpf time point coincides with the early phase of sexual and gonadal differentiation of zebrafish (Maack and Segner 2004, Baumann et al. 2013) and it can thus be assumed that these fish had already entered this phase (although this was not studied histopathologically or genetically in this study). Consequently, the onset of sexual differentiation in these fish might have also impacted the expression of sexual dimorphic genes. This effect became then more evident at the 63 dpf time point (chapter 5.4.4). Based on this assumption it was hypothesised that the variation in the gene expression levels in the controls and the fadrozole treatments was higher at the 28 dpf than the earlier time points, which led to a statistically non-significant result. The height of the error bars of Figure 9 substantiates this hypothesis. The measurement of gene expression in individual fish would have resolved the high variation, but the pooling of four fish instead most likely obscured individual effect on gene expression. The n-number, on the other hand, was too low to reduce the variation based on an average value.

At 63 dpf it was possible to measure the expression of the target genes in trunk samples of individual fish and not in pooled samples, like for the earlier time points. During the analysis of the 63 dpf data, a divergence in the *vtg1* mRNA amounts of the control samples became apparent and suggested different expression rates linked to female (high *vtg1* expression) and male fish (low *vtg1* expression). Thus, control fish were divided into a low vtg1 and a high l *vtg1* group and regulation of all other genes evaluated according to these two *vtg1* expression categories. Gene regulation at 63 dpf was different for several genes in low and low *vtg1* control fish. The steroidogenesis related genes were in agreement as well as *star* and *igf1*, which were upregulated (insignificantly in the case of *igf1*). In the low *vtg1*control group, *lss, star* and *prox1* were found significantly upregulated in at least one exposure group, whereas in the high *vtg1*control group *lss, star, igfbp5a, and igf1* were significantly upregulated at least in one of the exposure groups and *vtg1, esr2a, cyp19a1b*, and *zgc:64022* down regulated.

Over time, the most significant changes in gene expression in more than one fadrozole exposure group for at least one time point were determined for estrogen regulated genes *lss, vtg1, star, igf1* and *cyp19a1b*. These genes represent different biological functions related to steroidogenesis, lipid transport, cell growth and aromatase activity, and which are regulative pathways known to be differently affected in by endocrine disruptors. These genes were identified as the most likely potential (bio-) marker genes suitable for the detection and identification of aromatase inhibition in the context of the FSDT. Fadrozole exposure during early life affected only *vtg1* and *igf1*, with *vtg1* responding by up regulation at 48 hpf and with the growth factor *igf1* up regulation at 96 hpf. Different was the response of the steroidogenic genes and the aromatase gene *cyp19a1b*, since their response only occurred at 63 dpf, indicating a stimulation of steroid hormone biosynthesis due to up regulation of *star* and *lss*, and inhibition of the brain-specific aromatase in low *vtg1* expressing fish, where *cyp19a1b* was down regulated. Strongly down regulated was also the estrogenic biomarker gene *vtg1* at the 63 dpf time point.

The growth factor *igf1* plays a central role in diverse physiological processes, including growth, differentiation and immune responses (e.g. Perez-Sanchez et al., 2000). Its function in endocrine processes and sensitivity to endocrine disruption in fish has been de scribed but demonstrated inconsistent gene up- or down regulation among different fish species or MOAs of endocrine disruption (e.g. Filby et al., 2007; Shved et al., 2008). Our results suggest an involvement in aromatase inhibition, as we found a constant up-regulation upon fadrozole treatment, though not always significant. Igf1 activates IGF receptors, leading to an induction of transcription factors, which ultimately affect expression of *star*.

Star is responsible for the transport of cholesterol to the inner mitochondrial membrane, which is considered as rate-limiting step during steroidogenesis, and its differential gene expression upon exposure of fish has

been described for several EDCs (Johns et al., 2011; Sharpe et al., 2007). We found an up-regulation of *star* in comparison to the controls in the pre-adult 63 dpf fish at the highest fadrozole concentration.

Genes related to terpenoid/isoprenoid synthesis like *lss, sc4mol* or *mvd* are involved in the production of cholesterol from fatty acids. We demonstrated regulation of these genes at least for one (*mvd*) respectively two (*lss*) sampling time points throughout the study, what was in agreement with the regulation of these genes respectively regulative pathway by estrogenic and anti-androgenic substances described by Schiller et al. (2014). The regulation of *lss* throughout exposure was, like star, significantly changed only at the pre-adult stage, what may reflect a feedback reaction to aromatase inhibition induced estrogen reduction.

The gene encoding the enzyme aromatase appears in fish in two isoforms, which are known to show divergent regulation (Fenske and Segner, 2004; Kishida and Callard, 2001; Menuet et al., 2005). The brain predominating isoform *cyp19a1b* is regulated by an estrogen response element (ERE), linked to estrogen receptor activation and is thus estrogen dependent, whereas the gonad predominating form *cyp19a1a* is estrogen-independent. And in fact, we demonstrated significant down regulation by fadrozole in the pre-adult fish only for *cyp19a1b*. The other isoform *cyp19a1* showed contrary regulation, although at a non-significant level. Interestingly, the regulation of both genes was reversed in comparison to the control fish at 63dpf with low *vtg1* expression and more pronounced, to the low *vtg1* group. Although, the expression changes of *cyp19a1a* were not significant.

Conclusion and outlook

Although the complexity of results and the amount of data was high, a distinctive picture of the gene expression was obtained. Finally, the study enabled us to adapt the previously defined *Adverse Outcome Pathway* (AOP) for aromatase inhibition (AOP-WiKi -https://aopkb.org/aopwiki/index.php/Main_Page) to fit the FSDT situation. In addition to the established indicative parameters like vitellogenin and gonad histopathology, we were able to identify a couple of promising marker genes, which may potentially be used as biomarkers of aromatase inhibition and /or steroidogenesis disruption in the context of an endocrine disruption assay like the FSDT.

Despite the successful completion of the study, issues and questions arose from the results and the experiences gained, which should be addressed by further investigations. The test chemical fadrozole was known to act rather specific, which was confirmed by the clear effects on the apical endpoint gonad differentiation and sex ratio and reflected by the results of the gene expression. However, in order to verify these results, a less specific acting compound should be tested as well. A not exclusively as aromatase inhibitor acting compound or an EDC of another endocrine modality, which knowingly cause similar endocrine relevant apical effects would be recommended to test whether a similar response of the marker genes can be obtained. Finally, for the validation of the approach, an unknown substance suspected to act as endocrine disruptor, preferably by a mechanisms not strictly related to the sex hormone activity, has to be tested. Early endocrine regulatory processes induced by progestins and gestagens would be possible targets.

Another important question that arose from the study was how such mechanism-indicative molecular biomarkers could be used for regulatory purposes. Based on the results of this study, the following approaches were being discussed:

- A gene biomarker enhanced fish embryo test as EDC screening tool.

  Such an extended fish embryo test could provide the basis for a tiered testing strategy. The advantage of the fish embryo test over the currently promoted cell-based screening assays (Conceptual Framework of the OECD 2010) is the possible evaluation of endocrine-specific biomarker genes in a whole organism. The significance of data obtained *in vivo* can certainly be considered higher than *in-vitro*, since cell-based systems lack the complexity of whole organisms and cell-cell interactions. Consequently, the achievement of a higher reliability of screening assays through the reduction of false negative results could be a factor also promoting the avoidance of unnecessary fish tests (i.e. animal tests).

- Extension of existing test guidelines by gene expression analysis

  Due to an increasing number of applications for authorization of compound of uncertain mode of action in non-target organisms, the demand for and relevance of EDC test methods will continue to increase. The integration of gene biomarkers into existing test guidelines could improve the identification of EDC also in the context of non-EDC tests. This may be of particular interest for pharmaceutical compounds which often target specifically the hormone system of humans or of livestock.

- Use of gene expression analysis for biomonitoring purposes

  A broad field of application for gene expression analysis is provided by biomonitoring projects and the examination of biological matrices. The analysis of biota samples taken from contaminated habitats, which is routinely performed by chemical analysis of key contaminants (like pharmaceuticals or pesticides), could be supplemented by MOA-based gene expression analysis in the context of an effect-directed analysis (EDA).

3 Introduction

3.1 State of the art in endocrine disruption testing

Environmental pollutants which interact with the endocrine system of organisms, and which exert a negative impact on development and reproduction, became known to the public in the 1990ies as endocrine disruptors. Especially the increase in reproductive disorders in humans, and observations of gonadal alterations including feminisation in aquatic populations (i.e. fish, but also amphibians and gastropods), moved the topic to the public eye. Such evidence was scientifically interpreted as indication of high risk and communicated and published as such. For the first time this risk potential was discussed on an international symposium in Weybridge (UK) in 1996 by representatives from science and authorities. One mayor goal of this workshop was the decision on a definition for endocrine disruptor, which was adopted later on by the WHO in a slightly modified version. It states: “An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (WHO/IPCS 2002). Further, it was defined that “A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or progeny, or (sub)populations”.

Meanwhile, the topic “Endocrine disruptors” has found its way into diverse legislations, e.g. into REACH and the water framework directive (2000/60/EG), as well as into the pesticide- and biocide ordinances. Additionally, a joint framework concept was developed by the Endocrine Disrupters Testing and Assessment (EDTA) task force for the analysis of endocrine active risk potential, which was approved on the 6th EDTA workshop in Tokyo 2002 (ENV/JM/TG/EDTA (2002)4). On this basis, the development and validation of OECD test guidelines for the testing of endocrine active substances was implemented. These TGs include the OECD 229 (Fish Short Term Reproduction Assay), OECD 230 (21-Day Fish Assay), OECD 231 (Amphibian Metamorphosis Assay), OECD 234 (Fish Sexual Development Test), OECD 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents), OECD 440 (Uterotrophic Bioassay in Rodents), OECD 441 (Hershberger Bioassay in Rats), OECD 455 (Stably Transfected Human Estrogen Receptor-α Transcriptional Activation Assay for Detection of Estrogenic Agonist-Activity of Chemicals), and OECD 456 (H295R Steroidogenesis Assay).

3.1.1 Integrating novel approaches in existing test strategies

Endocrine disruption generally manifests in an organism or a population only after sub-acute longer-term or chronic exposure, making the identification of hormone-active substances time- and cost-intensive. Further, elaborative chronic experiments required a huge amount of animals, and alternatives, which consider the principles of the 3R-concept (Replacement, Reduction and Refinement) after Russell and Burch, (1959) highly appreciated. However, short-term tests for the determination of apical endpoints are not recommended. They were designed as screening tool to trigger complex studies like the fish life cycle test, which they cannot replace. Also tests with early developmental stages (e.g., the fish early life stage toxicity test) do usually not lead to satisfactory results as they do not include relevant exposure windows, e.g. the sexual development. Since apical endpoints respond slowly to exposure, it is advisable to include quick responding parameters, which can also be indicative of the underlying MOA.

In this context, inclusion of molecular endpoints seems a promising approach as they react promptly on pollutants. They may further be indicative of adverse effects at organism level (Piersma, 2006). Powerful genomic approaches have contributed essentially to the determination of molecular mechanism resulting in apical adverse endpoints (Ankley et al., 2006; Sawle et al., 2010). Global transcriptomics allow meaningful analyses of adverse effects in cells, organs, and the whole organism at concentrations potentially below thresholds of the most sensitive endpoints of the “conventional” studies. This higher sensitivity of molecular analyses is crucial to prevent the overlay of endocrine effects by other effects, esp. systemic toxicity (Scholz and Mayer, 2008; Wang et al., 2010).

The progress in molecular and computer-based (*in silico*) methods paved the way for the shift of paradigms in ecotoxicology (NRC 2007), and for the development of alternative concepts for environmental risk assessment, e.g. the Adverse Outcome Pathways (AOPs; Ankley et al., 2010; Villeneuve and Garcia-Reyero, 2011), or the Toxicity Pathways (Bradbury et al., 2004). In the sense of AOPs, the direct link between exposure, the molecular initiating events (MIEs), and the resulting apical endpoints on organism/population level should be explored (Kramer et al., 2011; Segner, 2011, 2009) also for endocrine disruption. This will provide the basis for the development of more effective and reliable models for risk assessment.

3.1.2 The Fish Sexual Development Test (FSDT)

The Fish Sexual Development Test (FSDT) can be described as a modification of the OECD guideline 210, the Fish Early-Life Stage Toxicity Test, which was adopted in 1992 and revised in 2013. The FSDT was originally considered as "Fish Partial Life-Cycle Test" and was aimed at providing an alternative test that specifically detects endocrine disrupters in fish without the requirement to expose for a whole generation. The rationale behind this assay was to establish an *in vivo* fish test for endocrine disrupting chemicals (EDCs) that was less complex, cheaper and more rapid than a full-life cycle tests. In support of this effort, there was sufficient evidence to show that exposure of fish to EDCs during the sensitive window for sexual development was sufficient to alter relevant endpoints of endocrine disruption, like the plasma vitellogenin concentration, the phenotypic sex ratio, or secondary sexual characteristics (Holbech et al., 2006; Kinnberg et al., 2007; Örn et al., 2003). For zebrafish, the window of sexual differentiation starts at the end of the early life stage phase and undergoes the transition of the protogynous gonad (e.g., Maack and Segner, 2003; Takahashi, 1977). The FSDT was developed only for zebrafish (*Danio rerio*) at first but in 2003 it was decided that other OECD candidate species such as Japanese medaka (*Oryzias latipes*) and three-pined stickleback (*Gasterosteus aculeatus*), should be acceptable as well (OECD, 2012b). On behalf of the Nordic countries, Denmark initiated the validation of the FSDT in 2003, which ended in 2010 and was finalised by a peer review report published by the OECD in 2011 (OECD, 2012a). In this report the FSDT is described as follows: "The Fish Sexual Development Test (FSDT) covers a life-stage where sexual development is particularly sensitive to perturbation caused by endocrine active chemicals. The chemical exposure lasts for about 60 days, at the end of which endpoints of ecological relevance, like the sex ratio of the exposed fish, are assessed and the biomarker endpoint vitellogenin is measured in individual animals".

The test was finally adopted as an OECD approved guideline 234 end of July 2011(OECD, 2011) and has since been included at level 4 into the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupters published in August 2012 (OECD, 2012c).

However, the endocrine relevant endpoints of the FSDT are principally very limited in terms of the interpretation what modality of endocrine disruption is exerted by a test chemical. The diagnostic properties of the phenotypic endpoints sex-ratio, vitellogenin levels and gonad histopathology are confined to the identification of endocrine disruption of either an estrogenic or androgenic MOA. However, without any other existing endocrine-relevant data, e.g., from *in vitro* assays, no reliable conclusion on an endocrine disruption modality could be drawn from the FSDT alone. The additional early life stage parameters hatch, survival and growth, can potentially be sensitive, but have no diagnostic value in terms of effects on estrogen, androgen and thyroid signalling (EATS) at all. Thus, more sensitive endpoints identifying the actual endocrine MOA of any substance, e.g. on the molecular level, in addition to the FSDT is much appreciated.

3.2 Aim of the study

This research project aimed at evaluating whether changes in gene expression would enable early identification or perhaps even the prediction of endocrine mediated effects in the context of currently recommended fish tests for endocrine disruption.

The rationale behind the investigation was that the current OECD fish testing framework for endocrine disruption (OECD, 2012a, 2012b, 2012d, 2010) focuses on reproductive effects with only a very limited scope to discern endocrine mechanism of action from the parameters assessed within the tests. Most mechanistic information currently stem from receptor binding assays and other in-vitro screening assays (testing level 2).

The inclusion of additional biomarkers into the current testing strategy may be a promising approach to generate supplementary information on endocrine mechanisms of action to increase the weight-of-evidence of endocrine disruption hazard (OECD, 2012b). Biomarker endpoints like vitellogenin have shown their value to help understanding the link between endocrine-related mechanisms and apical adverse effects. The FSDT at the Conceptual Framework Level 4 (OECD, 2012b) can deliver only limited information on e.g., the EATS signalling pathway(s) being affected and adverse effects concerning gonad development and sex-ratio of fish. However, the outcome of a level 4-test may decide whether a more comprehensive assessment of adverse effects on endocrine disruption (regulatory) relevant endpoints at Level 5 will be necessary. Hence, a reduction of the error rate at Level 4 is desirable and could be achieved by the inclusion of bio-marker endpoints suitable to indicate the mode or even the mechanism of ED action. If this approach would prove successful, then regulatory authorities may consider a positive result in the FSDT sufficient to confirm a chemical as ED and use the data in future risk assessments. In turn, this would contribute to the reduction of fish life cycle or even multi-generation fish tests. The need for more mechanism-based methods has already been acknowledged and highlighted in the context of a Review Paper of the OECD (No. 178) on Novel In Vitro and In Vivo Screening and Testing Methods and Endpoints for Evaluating Endocrine Disruptors (Kortenkamp et al., 2011; OECD, 2012a), which focused on other endocrine pathways but EATS.

The central goal of the project was to demonstrate the benefits of integrating the expression of genes as potential (bio-) markers of exposure and for the identification of endocrine mechanisms of action to facilitate the interpretation of FSDT results. These gene biomarkers should enable the determination of ED pathways affected by the chemical tested and thus, facilitate the link to the apical ED effects To achieve this goal, the first major task was to identify a suitable test compound by an extensive literature review and subsequently conduct a FSDT with zebrafish according to the OECD guideline and in general compliance with the principles of Good Laboratory Practice (GLP).

In addition to the default endpoints (hatch, juvenile growth, sex ratio, plasma vitellogenin, histopathology), the expression of endocrine related marker genes was measured in early life stages as well as in trunks of pre-adult fish during and at the end of the FSDT.

3.3 Development of the test approach

3.3.1 Information extraction strategy of the literature review

The aim of the literature review was to get an in-depth overview of the existing data base of gene expression level investigations for the evaluation of endocrine disrupting effects in fish. Based on this overview, a suitable test substance was selected.

The review primarily used public databases of peer reviewed journal articles, reports and book chapters but also referred to non-peer reviewed sources for the research. In terms of the public database search, most information and references were taken from the science- specific web search engine Scirus (http://www.scirus.com), the databases of the most relevant publishers of scientific journals, like ScienceDirect (http://www.sciencedirect.com), SpringerLink (http://link.springer.com), Wiley Online Library (http://onlinelibrary.wiley.com), BioMedCentral (http://www.biomedcentral.com/) and citations and full-text archives like PubMed/PubMed Central (http://www.ncbi.nlm.nih.gov/pubmed) or Toxline (TOXLINE http://toxnet.nlm.nih.gov/newtoxnet/toxline.htm) of the NLM (National Library of Medicine) of the NIH in the USA.

The search was structured according to search criteria and the key information to be extracted from the publication. The primary search criteria, which deemed a publication relevant to the review, have been as follows:

- Toxicity tests performed with fish
- Effects of chemicals with known or suspected endocrine disruptive activity
- Investigations included endocrine test endpoints related to gene expression in fish, using methods like (semi-) quantitative PCR (Polymerase Chain Reaction), microarray analysis, or direct sequencing.

From publications that met the mentioned criteria, the following information was extracted:

- Basic data like title, author, year of publication, source
- What type of fish test was conducted and was the conduction in compliance with effective guidelines of the OECD, DIN/ISO or of the Environmental Protection Agency EPA of the USA?
- What fish species was used - trout, zebrafish, Japanese medaka, fathead minnow, or others?
- What test substance/chemical(s) was (were) used for the exposure and was (were) it endocrine active? What primary mode of endocrine action was investigated - estrogenic or anti-estrogenic, androgenic or anti-androgenic, thyroidal or other?
- Which criteria were applied to choose the particular endocrine disruptor?
- What was the experimental design, i.e., how many fish were exposed with what chemical and for how long? How many replicate test vessels were used per treatment and what concentration levels were used? What age or developmental stages of fish were used and have body weight and length been reported?
- What gene expression endpoints were assessed and which genes were evaluated?
- What method was applied for the gene expression analysis?
- What have been the main results in terms of gene expression?

The above mentioned information extracted from publications was summarised in an Excel spreadsheet. The results of the literature review were presented in an UBA-internal, unpublished interims report in February 2013 and are summarised in the following section 3.3.2. Subsequent relating sections of the present report are indicated by the corresponding citation of the report (Fenske and Teigeler, 2013; first interims report of project FKZ 3712 63 418, Umweltbundesamt).

3.3.2 Results of the literature review

In peer-reviewed journals, a total of 104 references were found, including four review articles, reporting of gene expression analyses following exposure of fish to chemicals and mixtures with endocrine disrupting properties. The list of these references was provided by the Excel spreadsheet included in the interims report. The articles were published between 2002 and 2012 and report of studies on 64 different chemical substances, among which the primarily estrogenic acting chemicals 17α- ethinylestradiol (EE2), 17β-estradiol (E2), 4-tert-nonylphenol (NP) and bisphenol A (BPA) were the most commonly, applied substances. In 40 of the 103 references, the method of gene expression analysis was microarrays of different types (cDNA or oligo arrays; custom-made or commercial), 46 studies used real-time RT-PCR and 12 references cited either semi-quantitative RT-PCR as method of analysis or other methods like SSH (Suppression subtractive hybridization), Northern Blot or differential display (DD-) RT-PCR.

In terms of preferences of fish species, the review showed that the majority of studies were conducted in zebrafish (30 of 104). The Japanese medaka (*Oryzias latipes*) was the second most common species and was mentioned in 14 of the 104 references, the fathead minnow (*Pimephales promelas*) came third with 13 entries and rainbow trout (*Oncorhynchus mykiss*) was used in 11 studies. Other species used in more than one of the studies were mostly marine species like the Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), marine medaka (*Oryzias javanicus*) or the self-fertilising hermaphroditic mangrove killifish (*Kryptolebias marmoratus*).

However, in the review most attention was given to the references of zebrafish and Japanese medaka and the EDCs tested with these two species. The decision to use the zebrafish as the model species of choice for this project was confirmed and agreed upon on the kick-off meeting in September 2012, and as such the focus on the literature references of this species is substantiated. Nevertheless, information concerning the Japanese medaka was considered important and supplementary to the zebrafish data, as it represents the second most relevant small fish species in ecotoxicology, and was also used during validation of the FSDT test guideline. It has been acknowledged as beneficial for the evaluation of endocrine disruptors, as is possesses a sex chromosome-linked directly sexual differentiation and determination. Among the fish screens and tests contained within the “OECD Conceptual Framework for the Screening and Testing of Endocrine Disrupting

Chemicals" (CF) and described in the Guidance Document (GD) on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (OECD, 2012c), the Japanese medaka is together with zebrafish, the only species accepted by all TGs. Even more importance will probably be attached to the medaka in the future in case the currently discussed Fish (Medaka) Multi- Generation Test (MMGT) gets approved and included in the CF. In terms of gene expression endpoints, the Japanese medaka literature was also of particular relevance because the IME in-house investigations on EDC-induced changes in the gene expression response of fish embryos, were in many cases conducted in zebrafish and medaka in parallel. Taken these points into consideration, data on endocrine disruption in medaka allow comparison to endocrine disruption in zebrafish, and are regarded useful in the development of a test approach, i.e. in the choice of substance and of candidate genes for expression analysis.

Focusing on the zebrafish and medaka references from the literature review, the number of chemicals studied in zebrafish was 25; in medaka it was 14, of which 7 were duplicates to zebrafish. Together, 32 different EDCs were therefore investigated in zebrafish and medaka (review paper excluded), of which 10 were primarily estrogenic. The remaining 22 chemicals comprise five thyroid function modulator (3,5,3′-triiodo-l-thyroxine, methimazole, microcystin– leucine–arginine, PBDE-47 (2,2′,4,4′-tetrabromodiphenyl ether) and triclosan), three androgens (17α-methyltestosterone), 17α- methyldihydrotestosterone, 17β-trenbolone),three steroid biosynthesis modulators (atrazine, retene and trilostane), three aromatase inhibitor (fadrozole, letrozole and prochloraz), one anti- estrogen (tamoxifen) and one anti-androgen (flutamide). Seven chemicals affect either other signalling pathways of the endocrine system than those disrupting estrogen, androgen, and thyroid signalling processes and steroidogenesis (EATS) primarily covered by the CF (OECD, 2012c), or affect several endocrine pathways at once (fluoxetine, 2,3,7,8-tetrachloro-di-benzodioxin (TCDD), methylmercury (MeHg), mercury (HgCl2), 4-azapyrene, LXR agonist T0901317, benzo[b]fluorene).

Additional to the publications included in the Excel spreadsheet, validation documents for the FSDT (OECD, 2012b, 2012d, 2011b), and their test chemicals, were considered: In phase 1 of the validation studies for the development of the OECD approved test guideline of the FSDT, the EDTA Task Force advised to use weak endocrine-active substances and thus, prochloraz and 4-tert-pentylphenol were selected. They represent different modes of action, weak aromatase inhibition and estrogen receptor agonism, respectively. 17β-estradiol was used as a positive control. For the second phase, another estrogen, 4-tert-octylphenol, and an androgen, dihydrotestosterone, were used as test substances.

As a follow-up to the publication of the OECD Guidance Document (GD) on "Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (OECD, 2012c), the Endocrine Disruption Testing and Assessment Advisory Group (EDTA AG) decided to carry out several case studies to evaluate the conclusions and recommendations of in the GD in light of comprehensive datasets. The comprehensive results of these three case studies for prochloraz, 4-tert-octylphenol, and perchlorate are now published (OECD, 2012b) and contain important information and conclusions drawn also from the FSDTs conducted with these chemicals. This information was included in the decision finding process regarding the suitable test compound(s) for this project.

3.3.3 Results of preliminary work conducted at the Fraunhofer IME

Additional to the information extracted from the literature, the Fraunhofer IME contributed actively to the generation of data on endocrine disruption in fish, either by chronic fish studies with EDCs of different modes of action (MOA) or by fish embryo tests with zebrafish and medaka including gene expression analyses like microarrays and quantitative RT-PCR.

3.3.3.1 Chronic fish studies with EDCs

The screening studies with 4-tert pentylphenol, octylphenol, flutamide and prochloraz were part of the interlaboratory validation for the Fish Short-term Reproduction Assay (FSTRA; OECD 229). Per definition, reproduction was recorded for about 21 days. The Fish Screening Assay (FSA; OECD 230) were performed with a shortened exposure time (14 days instead of 21 days). The Full Life CycleToxicity Tests (FLCTT; US EPA OPPTS 850.1500) with tamoxifen-citrate, trenbolone and flutamide were performed as two generation

tests (TGT). This included exposure of a parental-generation and two filial generations (F_1 complete generation, F_2 only early life stages). In-house, an FSDT study was only performed for the estrogen receptor (ER)-agonist octylphenol. However, Fish Full Life-Cycle (FFLC) studies were performed with several EDCs, encompassing the MOAs ER-agonism, ER-antagonism, androgen receptor (AR)-agonism, AR-antagonism, and aromatase inhibition. As the test design of an FLCT includes, amongst other, the same life stages as the FSDT, these data provide substantial information of EDCs with different MOAs on the sexual development of fish.

In all screening studies, vitellogenin and 11-keto testosterone were measured. With exception of the androgen-linked modes of action, VTG was found to be the most sensitive biomarker. For the anti-androgen flutamide, the 11-keto testosterone level was found to be the sensitive physiological parameter. For the androgen trenbolone, vitellogenin responded, but was clearly less sensitive than the population relevant endpoints (Knacker et al., 2010; Teigeler et al., 2007).

3.3.3.2 Gene expression analysis of EDCs with the fish embryo test

For zebrafish, the endocrine disruptive effects on gene expression were assessed mainly by microarray analysis. Test compounds include EDCs compromising a broad range of MOAs, i.e. ER-agonism, ER-antagonism, AR-agonism, AR-antagonism, and aromatase inhibition. Quantitative RT-PCR analysis was employed only secondary, and had the main purpose to verify the microarray results. Effects on gene expression changes at the mRNA level, analysed by qPCR, were mainly performed with estrogenic and anti-androgenic compounds. Further test compounds include substances with endocrine activity, however with uncertain MOA. The purpose of this analysis was to explore the differences in gene response of zebrafish before and after hatch. The tested genes were related to the hypothalamic-gonadal-axis and to early steroidogenesis. These genes were the androgen receptor (*ar*), 11-beta-hydroxylase (*cyp11b*), aromatase b (*cyp19a1b*), vitellogenin 1 (*vtg1*), estrogen receptor 1 (*esr1*), and the estrogen receptor 2a (*esr2a*) (Schiller et al., 2014, 2013b).

For the quantitative RT-PCR analysis in 7 day old medaka, the following genes were measured to identify either an estrogenic or an androgenic response: aromatase b (*cyp19a1b*), vitellogenin 1 (*vtg1*), estrogen receptor 2a (*esr2a*), androgen receptor (*ar*), 11-beta- hydroxylase (*cyp11b*), gonadotropin-releasing hormone (type 2) receptor 2 (*gnrhr2*), 3beta-hydroxysteroid-dehydrogenase D5-D4 isomerase (*3βhsd*); steroidogenic genes lanosterol synthase (*lss*), sterol- C4-methyl oxidase-like (*sc4mol*) and mevalonate (diphospho) decarboxylase (*mvd*) (Schiller et al., 2014).

For genistein, aromatase a (*cyp19a1a*), paired box gene 2a (*pax2a*), deiodinase 2 (*dio2*), NK2 homeobox 1 (*nkx2.1*), homeobox a9 (*hoxa9a*), tumour suppressor protein 53 (*tp53*), homeobox a10 (*hoxa10b*) and homeobox a11 (*hoxa11b*) were measured additionally in both species (Schiller et al., 2013a).

3.3.4 Conclusions drawn from the literature review

Overall, the literature review showed that a broad range of ED substances have already been tested in conjunction with gene expression as an additional endpoint. Also, the available spectrum of data for substances of different endocrine disruptive properties was quite diverse. More than half (i.e., 57) of the studies compiled by the review were conducted either with zebrafish, Japanese medaka or fathead minnow and therefore provide information that was directly employed in the planning of the FSDT.

3.4 Choice of test substance

The following considerations were included in the decision process which test substance should be used in the study. In the first instance, the selection of an appropriate candidate substance depended on whether this substance was known or very likely to impair the population relevant endpoints of the FSDT. This was very important for the evaluation of the marker genes, because otherwise the critical link between significant changes of potential marker genes and apical adverse effects could not be established. It was therefore advisable to choose a candidate substance with the potential to shift the sex ratio. Potent oestrogens are known to cause a shift to an increased number of females. However, it was agreed on the kick-off meeting to exclude

the estrogenic chemicals for this project, since quite a profound knowledge-base is already available, also for gene expression markers. Alternatively, a potent androgen like methyltestosterone was considered as candidate substance, as it skews the sex-ratio towards males. Data for another androgen, dihydrotestosterone, a metabolite of testosterone, was available for the FSDT from the inter laboratory validation phase 2 (OECD, 2012d). Further, aromatase-inhibitors were considered which cause a shift towards an increased number of males and repress VTG in the females. Candidate test substances included the pharmaceutical fadrozole, which is specifically designed to inhibit the aromatase enzyme and would therefore represent a suitable positive reference. Other aromatase inhibitors identified by the literature review were the imidazole-related fungicides. However, as their molecular mechanisms of action are broader, affecting different endocrine signalling pathways at once, results would be difficult to interpret and transcriptomic changes are due to several molecular initiating events (MIEs). Aromatase inhibitors were favoured due to the good data base available, e.g. for another candidate, prochloraz. Prochloraz was used in phase 1 of the validation of the FSDT (OECD, 2012b) and has also been tested in-house in zFETs and mFETs, ensuring the availability of extensive data on FSDT and transcriptomics endpoints.

It was decided to choose a candidate substance from the list of those chemicals already studied at transcriptome level in in-house FETs and of which there is knowledge in terms of regulatory fish tests. This would be of benefit for the selection of potential (bio-) marker genes as well as the design of the FSDT and facilitate the accompanying chemical analysis. Meeting all these requirements, fadrozole was consequently identified as test substance.

Fadrozole is an established reference substance for aromatase inhibition and thus, suppression of estrogen synthesis. Fadrozole is a potent, selective, non-steroid aromatase inhibitor. Its exact enzyme interaction is not fully understood. However, coordination with the iron of the porphyrine core of the aromatase enzyme is most likely, as this describes a characteristic function of type-II-inhibitors (Browne et al., 1991). Fadrozole therefore exhibits a clearly defined, specific MOA with very few known off-target effects (may also lower cortisol and aldosterone; Demers, 1994). Besides, the water solubility of fadrozole is sufficiently high (see 4.1) to allow waterborne exposure at effective concentrations without the addition of a carrier solvent. Effects of fadrozole exposure on fish are well described in the literature. Effects related to aromatase inhibition on the apical level are a shift of the sex ratio towards males and moreover the repression of vitellogenin production (Ankley et al., 2002; Fenske and Segner, 2004). An important additional reason for choosing fadrozole as test compound was the availability of gene expression data for zebrafish, firstly derived from short-term exposure tests with adults (Villeneuve et al., 2009; Wang et al., 2012), and secondly obtained from in-house transcriptomics studies with embryos. These data provided a basis for the selection of target genes to be analysed during this study. However, long-term gene expression data, which may provide information upon the potential relationship between altered gene expression and adverse effects were missing and identified as a major goal of this study.

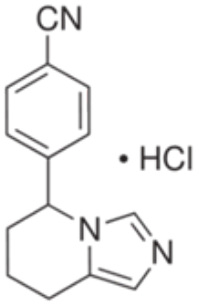
3.5 Choice of candidate genes for gene expression analysis

Several genes were selected for the gene expression analysis primarily based on the results of previous microarray studies with zebrafish 48 hpf and 96 hpf embryos carried out at the Fraunhofer IME (unpublished results; master thesis of Melanie Macherey, and PhD thesis of Viktoria Schiller, both RWTH Aachen University, 2013). From the results, genes or genes representing relevant pathways were extracted, which showed significant regulation after exposure to fadrozole (400 µg/L) or to other sex steroid modulators prone to affect the estrogen-androgen homeostasis. Additional genes were selected from publications. A study by Villeneuve et al. (2009), for instance, presented extensive microarray and qPCR derived data from a fadrozole exposure study on adult zebrafish. This study demonstrated significant regulation of the genes *cyp19a1a*, *fshr* (follicle-stimulating hormone receptor), *npyryb* (neuropeptide Y receptor YB; now called *npy8br*), *sox9b* (sry box-containing gene 9b), *prox1* (prospero-related homeobox gene 1) *star* (steroidogenic acute regulatory protein), and z*gc:64022* (ras-like estrogen-regulated growth inhibitor) in ovaries and/or brains of 100 µg/L fadrozole exposed zebrafish. The genes which were chosen to be included in the current

study and the particular reasons for their inclusion, are described in the following section 4 Material and Methods, in Table 4.

4 Material and Methods

4.1 Test item

Test item name:	Fadrozole-Hydrochloride
Synonyms:	Afema, CGS 16949A, 4-(5,6,7,8-Tetrahydroimidazo[1,5-a]pyridine-5-yl)-benzonitrile
Chemical structure:	
CAS Number:	102676-31-3
Molecular weight:	259.73 g/mol
Water solubility:	32.95 mg/L (@ 25°C: estimate from Log octanol-water partition coefficient)
Log K_{ow} (estimate):	3.20
Water solubility:	14.775 mg/L (from fragments)
Solubility in DMSO:	> 20 mg/mL
Origin of test item:	Adooq Bioscience LLC., Irvine, Canada
Purity:	> 98%
Appearance:	White powder

4.2 Test organism

4.2.1 Justification for the use of the test organism

Zebrafish (*Danio rerio*) is recommended by the OECD as model organism for testing endocrine disruption. It is explicitly mentioned in the OECD TG 234 as test fish for Fish Sexual Development Tests (FSDTs). Besides, the zebrafish has been an established fish species in the test facility for many years. Transcriptomics data on endocrine disruption in zebrafish are available in the literature as well as from experiments performed in-house. Data on other test species (e.g. medaka) are less frequently published, making these fish species less suitable for the present project.

4.2.2 Specification

Species:	*Danio rerio* (Teleostei, Cyprinidae)
Source:	Test facility bred
Origin of the used strain of zebrafish:	West Aquarium GmbH
	37431 Bad Lauterberg, Germany

Fertilised eggs for the study were obtained from individuals reared in the laboratory of the Fraunhofer Institute, Schmallenberg, Germany.

4.2.3 Holding of parental fish and breeding conditions

Parental fish (maximum age: 2 years) were kept in glass aquaria of a total volume of 150 L. Holding water was of the same quality as used for the test. Holding temperature was 25°C ± 2°C, the photoperiod 12 h light: 12 h dark, light intensity approximately 1000 lux, measured 5 cm above water surface in the middle of each vessel. Flow through rate was adjusted to reach a 1-fold exchange of water per day at minimum. Animals were fed twice daily, once ad libitum with TetraMin® Hauptfutter (Tetra Werke, Melle, Germany) and once brine shrimp nauplii (*Artemia salina*). Fish larvae were fed with breeding food (Tetra Werke, Melle, Germany). The broodstock was visually checked every day for mortality, illness, parasites or abnormal behaviour. No prophylactic treatment of fish took place. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilised eggs.

4.2.4 Obtaining of fertilised eggs for the study

Eggs were collected in a glass spawning-tray which was placed at the bottom of the holding vessels. The tray was covered with a stainless steel lattice to prevent pre-adult fish from devouring the eggs. An artificial substrate was attached to the lattice to stimulate spawning above the tray.

The turn on of lighting (one neon lamp per vessel, light intensity approximately 1000 lux, measured 5 cm above the water surface in the middle of the test vessel) induced mating and spawning of fish.

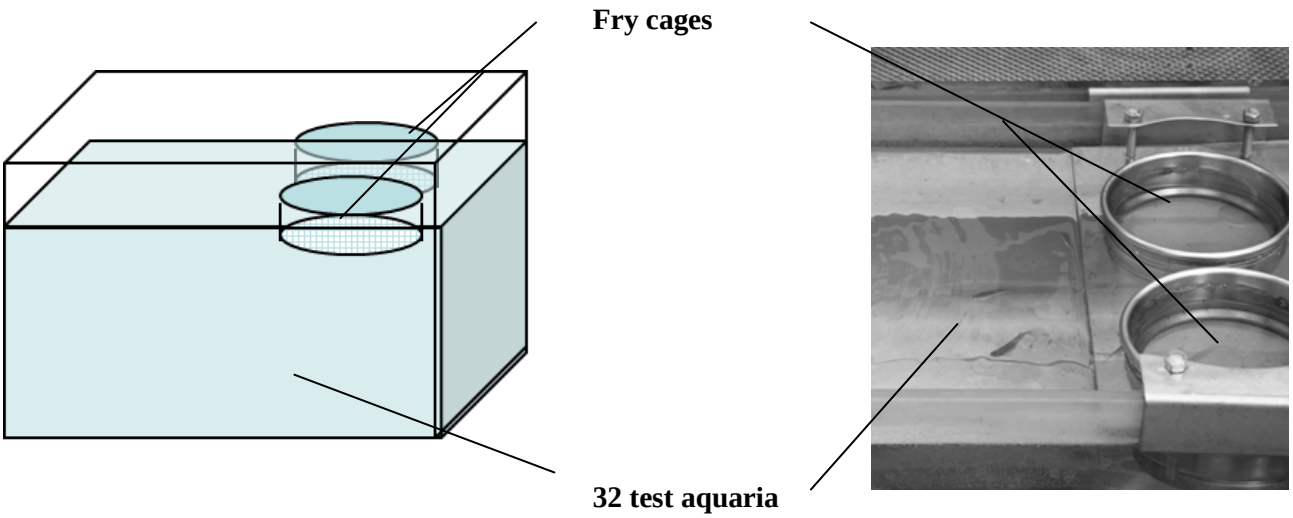
The collected eggs were transferred from the spawning-tray into a sieve (tea strainer), rinsed with clean water in order to remove any debris and then put into glass dishes. Fertilised eggs (microscopic determination of >four cell stage, i.e. early blastula stage) were subsequently transferred into the test chambers using a pipette with widened and smoothed-edged pipette tips. The time span from spawning until transferring into the test solutions was kept as short as possible.

4.3 Test conditions

4.3.1 Test aquaria

The test vessels were glass aquaria with a total volume of 12 L. At test start, each test vessel was equipped with two fry cages. These cages were analytical sieves of stainless steel with a diameter of 10 cm and a brim height of 4.5 cm. Due to a limited number of fry cages of this size, the two smaller fry cages were replaced in replicate C of all treatment levels by one fry cage with an approx. two-fold size. The mesh width of the sieve net at the bottom was 355 µm. Each replicate group was kept in an individual fry cage. Each test chamber was labelled with the vessel number and test concentration. At day 28 pf, the larvae were introduced into the main aquaria.

Figure 1: Test aquaria holding the fry cages; (A) schematic drawing; (B) image



4.3.2 Physical test parameters

The light regime was 12 hours light / 12 hours dark. Water temperature in the test vessels was adjusted to 25°C ± 2°C. Oxygen saturation of the test solution was assured to be not lower than 60%. Gentle aeration by an adjustable, centrally controlled air pump was established in all test vessels during the equilibration phase and throughout the study. The water temperature was measured on each workday (five days/week) in all test vessels. Oxygen concentration and pH of the water were measured in each vessel before adding the fish and afterwards at least twice per week. Mean values for physical test parameters during the time course of the study can be found in the results part in Section 5, Table 5; detailed values can be found in Annex 8, Table 16 to Table 18.

4.3.3 Flow-through system

An individual dosage system for continuous supply of the test substance throughout the test was used for two replicates each, i.e., two dosage systems for each concentration with four replicates were used. The dosage system was setup as follows. The dilution water was delivered into a mixing chamber placed on a magnetic stirrer by means of a membrane pump (Prominent, Heidelberg, Germany). A corresponding amount of the application solution was dosed into the mixing chamber using another membrane pump with a stainless steel head (Prominent, Heidelberg, Germany). The prepared test solutions were dispensed into the test vessels via flexible Teflon tubes and dosed to the two vessels by an electronically regulated distributor (driven by compressed air), alternating between the tanks. Controls were fed by dilution water only. Control and all test concentrations were run in 4 replicate aquaria each. For every test vessel pair fed by one pump, a water flow rate of 2.5 L/h was adjusted, resulting in a daily turnover of 5 water volumes per vessel. At test start, the flow

through system was served by test solutions at least for 21 days before introducing the eggs to equilibrate the system. The pumping devices as well as the consumption of the stock solution was checked daily.

4.4 Test performance

4.4.1 Test item concentrations

Three test concentrations were applied in the study. The maximum concentration of 100 µg fadrozole/L was based on available data from literature, which indicate potency of fadrozole in this concentration on apical endpoints (Andersen et al., 2005; Ankley et al., 2002; Panter et al., 2004). Further, this concentration was successfully applied as positive control for validation studies for the OECD 21-day screening assay.

Two additional lower test concentrations with a spacing factor of 3.16 were applied, resulting in nominal test concentrations of 10 µg/L, 32 µg/L, and 100 µg/L fadrozole.

A negative control (dilution water only) was applied in parallel.

4.4.2 Test procedure

4.4.2.1 Group introduction and handling

For the purpose of comparability to similar studies, this study was conducted in accordance to the OECD test guideline 234 with only minor modifications allowing parallel performance of the FSDT and analysis of gene expression at different time points during juvenile growth and sexual development. Thus, as requested by the TG 234, the study was performed with 3 test concentrations and a control, in 4 replicates each. The test was started with 340 zebrafish eggs per concentration, i.e., 85 eggs/replicate, split to two fry cages (see Figure 2). The first fry cage served to provide samples for gene expression analysis, while the second fry cage was handled in accordance to the guideline and was kept mainly undisturbed.

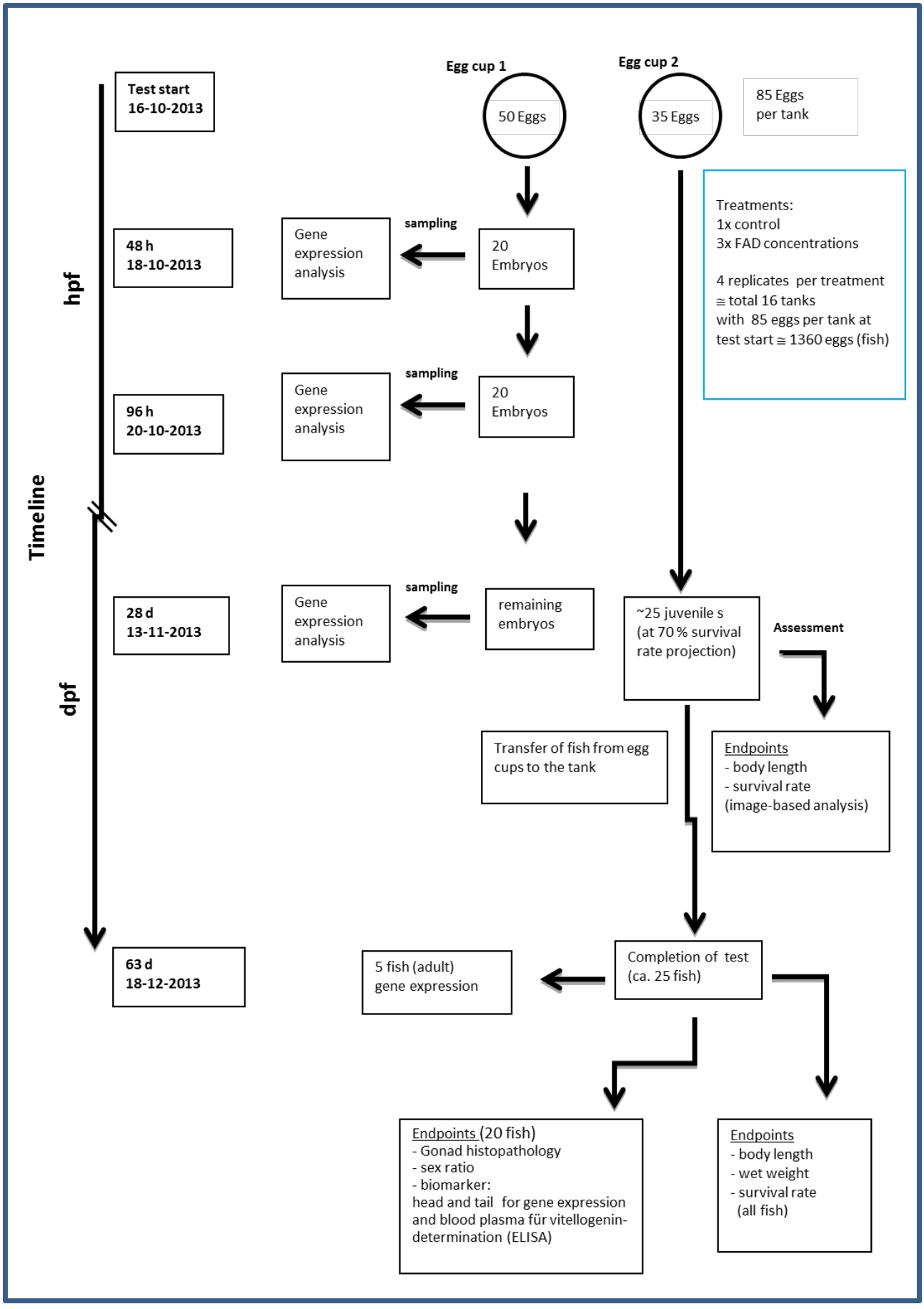
Hatched larvae were fed with breeding food (Tetra Werke, Melle, Germany). Starting from day 7 pf (post fertilization), larvae were additionally fed with brine shrimp nauplii (*Artemia salina*). From day 14 onwards, breeding food was replaced by ground TetraMin flake food.

Into the first fry cage, 50 fertilised eggs were introduced at test start. The first sampling of 20 fish embryos for gene expression analysis was carried out after 48 hours post fertilization (hpf). Accordingly, 20 eggs were sampled after 96 hpf. Remaining eggs were kept for hatching and left in the fry cages for further development. The 20 embryos of each cage were pooled in a 1.5 mL Eppendorf tube, were snap frozen, and stored at -80°C afterwards for later use.

35 fertilised eggs were introduced into the second fry cage at test start. The eggs were kept for hatching and the number of hatched larvae was estimated. After 28 days, the larvae were photographed for computer-based evaluation of survival and growth. Fish remained in the test system for another 5 weeks until test termination after 63 dpf.

Deviating from the original study protocol, only one fry cage instead of two was used for the C-replicate of all treatment levels, as only a reduced number of fry cages was available. The replacement fry cages were approx. twice the size of the original fry cages. Further, the larvae were transferred to the main aquaria already at day 21 pf instead of day 28. The juveniles from the egg cups 1 and 2 were combined in this process. The reason was that an increasing density in egg cups 2 (starting with 35 eggs) was assumed to affect the growth rate if the larvae were to be remained in the cups until day 28. At day 28 pf, excess larvae were randomly selected for gene expression analysis from each treatment tank. Four fish were pooled per replicate tank and snap frozen in a 2.0 ml Eppendorf tube.

Figure 2: FSDT: Timeline and test set-up according to the original study plan



Starting with 25 surviving fish at the end of the in-life test phase at day 63 pf, they were processed as follows: 5 fish were randomly chosen for gene expression analysis. As gene expression analysis may vary to a great extent between individuals, 5 individual fish of each replicate tank were analysed to obtain a statistically meaningful cohort.

The remaining 20 fish were anaesthetised and blood samples were taken (see Section 4.4.3.2). Subsequently, fish were sacrificed by a cut along the neck. Length and wet weight of individual fish was measured.

The fish were fixed for histopathological examinations. Therefore, they were ventrally opened with a precision scissor and the head and tail was removed. The torso was transferred into an appropriate fixative (see Section 4 Materials and Methods). In line with the histopathological examination, the sex of the fish was determined and the effects of substance treatment on gonadal development were evaluated. A schematic drawing of the study protocol is depicted in Figure 2, above).

4.4.3 Standard assessment methods

4.4.3.1 Photography and image analysis

For fry counts and total length measurements, photographs were taken using the digital camera Canon Cybershot. Digital image processing was performed by using the UTHSCSA ImageTool Version 3.0 (University of Texas Health Science Center at San Antonio). Fry were carefully transferred from the fry chamber to a transparent plastic vessel. This was placed on a photo shooting table (light plate with additional illumination from above). After photographing, the larvae were carefully re-introduced in the fry chamber in the aquarium.

4.4.3.2 Blood collection and preparation

Blood samples ranging from approximately 15-30 µL were taken by cardiac puncture. At first the fish were anaesthetised with chloro-butanol (5 g/L). To avoid coagulation of blood and degradation of protein, the samples were collected within phosphate-buffered saline (PBS) buffer containing heparin (1000 units/mL) and the protease inhibitor aprotinin (2 TIU/mL; TIU = trypsin inhibitor unit). As ingredients for the buffer, heparin as ammonium-salt (Sigma) and lyophilised aprotinin (Roth) was used. For blood sampling, a disposable syringe (1 mL) with a fixed thin needle was used (Omnican-F, Braun, Melsungen, Germany). The syringe was pre-filled with buffer (approximately 300 µL) to completely elute the small blood volumes from each fish. Plasma was separated from the blood by centrifugation (30 min; 5000 rpm; 4 °C) and immediately stored at -80°C until further analysis.

4.4.3.3 Vitellogenin measurement

For determination of the vitellogenin (VTG) levels, an enzyme-linked immunosorbent assay (ELISA) raised to zebrafish (*Danio rerio*) VTG (homologous ELISA kit, Biosense, Bergen, Norway) was used. This kit contains pre-coated multiwell plates, detecting zebrafish VTG primary and enzyme labelled secondary antibodies as well as a substrate.

The VTG-analysis is based on a sandwich assay utilizing specific binding between antibodies and VTG. The method is described in Brion et al. (2002). The wells of microtiter plates were coated with a specific capture antibody that binds to VTG in samples added to the wells. Unbound components were washed out, and another VTG-specific antibody (detecting antibody) was added. Unbound detecting antibody was washed out, and an enzyme-labelled secondary antibody was added. After a last wash, the enzyme activity was determined by adding a substrate being metabolised to a coloured product. The enzyme activity (colour intensity) measured by a microplate reader (iEMS Reader, Labsystems) is directly proportional to the concentration of VTG in the sample. The assay was calibrated using purified VTG from zebrafish as a standard. A blank control was run in each assay.

In order to minimise variability generated by the blood sampling methods (e.g. by taking up tissue liquid), the measured vitellogenin concentrations was normalised to the total blood plasma protein content, and data expressed as ng VTG/µg protein. Total protein was quantified by using the BCA Protein Assay Reagent Kit

(Pierce, Rockford, USA). The method of the BCA Protein Assay combines the reduction of Cu^{2+} to Cu^{+} and allows a selective colorimetric detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid. The coloured reaction is formed by chelation of two molecules of BCA with one cuprous ion. This complex shows a strong absorbance at 562 nm which is almost linear with increasing protein concentration. The colour intensities were determined in a microplate reader and the protein concentration quantified by comparison to a protein standard curve (albumin standard provided with the kit).

4.4.3.4 Histopathology

Gonadal differentiation in zebrafish has been described as non-functional protogyny (Maack and Segner, 2003; Takahashi, 1977), with all individuals initially developing an ovarian-like gonad containing only immature oocytes, and later on, about half of the individuals develop into functional females with a mature ovary, and the other half into functional males, with a mature testis. The normal gonad histology of adult, reproducing zebrafish has been described e.g. by Wester and van der Ven (Wester et al., 2003). In the mature testis, the germinal compartments are arranged in anastomosing tubules. The ovary of zebrafish belongs to the asynchronous type, which means that oocytes of all maturation stages are present at the same time. The yolk of zebrafish oocytes is arranged in spherical globules.

The Fish sexual Development Test (FSDT, OECD TG 234) has been designed for a safe evaluation of potential EDCs within an exposure period of 60 days. This covers the sensitive period of sexual differentiation of zebrafish, which is known to be very susceptible to EDCs (Maack and Segner, 2004). This offers the opportunity to use the sex ratio as meaningful endpoint for the evaluation of a suspected EDC. Besides the sex ratio, other meaningful endpoints of the FSDT are VTG, growth, and histopathology. Gonad morphology of fish is considered very sensitive to endocrine-modulating and toxic compounds. A major advantage of gonad histopathology over the molecular endpoints vitellogenin or aromatase is that it enables the identification of different modes of endocrine action and provides insight into the direct consequences for the organism. A disadvantage can be the subjective nature of assessment, even though official diagnostic criteria exist. The OECD guidance document on the diagnosis of endocrine-related histopathology in fish gonads (OECD, 2009) summarises possible effects and criteria for the analysis of fish gonads.

Tissue fixation procedures for histopathology

After blood collection, the fish were opened ventrally by an incision along the midline of the abdomen with dissection scissors taking care not to damage the gonads. Sexing of fish was carried out by macroscopical observation of gonads during preparation where possible. Afterwards the fish were fixated for detailed gonad histopathological evaluations.

Whole fish were placed individually in pre-labelled plastic vials which were filled with at least 30 mL of Davidson's fixative (Davidson's fixative: e.g. for 1 L: 200 mL formaldehyde (37 %), 100 mL glycerol, 300 mL ethanol, 300 mL distilled water and shortly added before use 100 mL pure (100 %) acetic acid). The overnight (approx. for 24 h)fixation was followed by the transfer into 10 % neutral buffered formalin (according to OECD TG 210) on the next day. The histopathological parameters that were analysed are specified in the next section.

Fixation, embedding and sectioning

The histopathological evaluation of the fish torsi was performed at the Centre for Fish and Wildlife Health, University of Bern, Switzerland.

The samples were transferred into tissue cassettes, which were labelled according to the internal coding system of the pathology laboratory. The cassettes were placed into the baskets of a tissue processor and the tissues were dehydrated. Subsequently, they were transferred to the embedding station, properly oriented and embedded in paraffin. For sectioning, the paraffin blocks were placed into the chuck of a rotating microtome in a way that transversal sections proceeding from the ventral side to the dorsal side of the fish were obtained. After trimming of the block, sections at 5 µm thickness were prepared. From each fish, a total of three step sections were prepared and mounted on glass slides. The first section was taken at the point where approximately one third to half of the gonad was removed; the following two sections were taken at 50 – 100 µm distance. The slides were dried, placed into an automated slide stainer and stained using haematoxylin-eosin. After evaluation of the gonads, the number code was used to associate the fish with the treatment levels and sampling intervals. The overall embedding and sectioning procedures followed the OECD Guidance document on histology and histopathology guidelines.

Histopathological classification of gonad development stages

All examined individuals were staged with respect to the maturation status of their gonads. The staging was carried out according to the OECD Guidance document (GD) on the diagnosis of endocrine-related histopathology in fish gonads (OECD, 2009). The occurrence of intersex was recorded. The results were statistically evaluated.

The primary and secondary diagnostic evaluation criteria, as proposed in the OECD GD No. 123, are listed in Table 3.

Table 3: Primary and secondary diagnostic evaluation criteria, as proposed in the OECD GD No. 123

Sex	Primary criteria	Secondary evaluation criteria
Males	• Increased proportion of spermatogonia • Presence of testis-ova • Increased testicular degeneration • Leydig cell hyperplasia/hypertrophy	• Decreased proportion of spermatogonia • Increased proteinaceous fluid • Asynchronous gonad development • Altered proportions of spermatocytes and spermatids • Altered gonadal staging • Granulomatomous inflammation
Females	• Increased oocyte atresia • Perifollicular hyperplasia/hypertrophy • Decreased yolk formation • Change in gonadal staging	• Interstitial fibrosis • Egg debris in oviduct • Granulomatomous inflammation • Altered number of post-ovulatory follicles

4.4.4 Gene expression analysis

Quantitative Real-time PCR (qPCR) was used as the method of choice for gene expression analysis in zebrafish embryos (at 48 hpf) and eleutheroembryos (96 hpf) as well as in the 28 dpf and 63 dpf zebrafish. The iQ5 real-time PCR detection system (BioRad®, Hercules, USA) and the SYBR®GreenER qPCR SuperMix

(Invitrogen, Carlsbad, USA) were used for the qPCR assays at the Fraunhofer IME in Aachen. The procedure is described below.

4.4.4.1 RNA extraction and reverse transcription

Total RNA was isolated from frozen trunk tissue (63 dpf) or pooled embryos/larvae/juveniles by homogenization in TRIreagent (Sigma-Aldrich, T9424) using motor-driven plastic (PBTP-polybutylene terephthalate) pestles (VWR collection, VWR Darmstadt), followed by a centrifugation step to remove insoluble material like cell debris, lipids, polysaccharides or insoluble proteins. The supernatant was transferred to a new 1.5 ml microtube and the RNA extracted by addition of chloroform followed by another centrifugation, which separated the RNA from DNA and proteins. The RNA containing aqueous phase was carefully withdrawn and transferred to a new microtube. To isolate the total RNA, 70% ethanol was added and the RNA was cleaned-up using RNeasy Mini Spin Columns (QIAGEN, Hilden) according to the manufacturers' protocols. After elution from the columns with RNAse-free water, total RNA was stored at –80°C until further analysis.

At the time points 48 hpf and 96 hpf one, samples consisted of the total RNA of 20 pooled embryos and at 28 dpf of 4 pooled juveniles. At 63 dpf fish were sampled individually and the RNA extracted from the trunk section of each fish, head removed. Separation of head and trunk tissue was performed since expression of genes related to endocrine disruption (e.g., *cyp19a1a* and *cyp19a1b* and hypothalamus/pituitary genes) can differ significantly between these tissues. Since most of the target genes are not or very weakly expressed in the brain, the trunk samples were analysed with higher priority. The frozen head samples remain stored for possible later analyses.

RNA concentration measurements and integrity determinations were carried out using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Schwerte). All further processed RNA samples were required to show 260/280 absorbance ratios (measure of protein contamination) between 1.7 and 2.2 (best ≥ 1.9) and 260/230 absorbance ratios (measure of contaminations of e.g. EDTA, carbohydrates or phenol) of -≥ 1.6 and Prior to cDNA synthesis, all RNA was treated with DNAse (Sigma-Aldrich, AMP-D1) to ensure removal of any remaining traces of DNA, which could compromise the PCR results.

After DNAse digestion, total RNA was reverse transcribed using SuperScriptTM III Reverse Transcriptase RT (Invitrogen, Carlsbad, USA) according to the manufacturers' instructions and using random hexamer primers. Negative RT control samples without added reverse transcriptase were included in all RTs and tested for contamination related amplification in the PCR.

4.4.4.2 Quantitative Real-time PCR

Thirty genes were selected for gene expression analysis based on the results of previous microarray studies with zebrafish 48 hpf embryos carried out at the Fraunhofer IME and literature data. Eventually, only 29 genes were measured because three of the originally selected genes were dropped due to the lack of a response in a pre-test with 96 hpf fadrozole exposed embryos. Instead, two additional Gnrhr genes were included. See 4.7 for details.

From the results of an in-house master thesis of Melanie Macherey, in which zebrafish embryos were exposed to the androgenic and anti-estrogenic compounds trenbolone, 17α-methyltestosterone, fadrozole and fulvestrant, and the PhD thesis of Viktoria Schiller, who exposed embryos to the estrogenic and anti-androgenic compounds ethinylestradiol, genistein, bisphenol A, methyl paraben, flutamide, propanil, linuron and prochloraz, genes were extracted, which showed regulation after exposure or which represent regulated pathways . Additional genes were selected according to published data, in particular from one study in which adult zebrafish had been exposed to fadrozole for 24, 48 and 96 h and where brains and gonads were analysed for transcriptome changes (Villeneuve et al. 2009). A list of the selected genes, their corresponding annotation and the reason for the selection can be found in Table 4 below. The primer pairs used for the qPCR analysis of these genes is available in the Annex (Table 39). Most primers were designed using the web-based NCBI PrimerBlast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), a web tool based on the Primer3 program and BLAST. Four primer pair sequences were taken from publications, as indicated in Table 39.

Table 4: List of genes selected for gene expression analysis

Gene	RefSeq accession	Description	ZF Annotation (GO term)	Reason for selection
mvd (NEW: mvda)	NM_001007422.1	mevalonate (diphospho) decarboxylase	isoprenoid biosynthetic process	The terpenoid/isoprenoid biosynthesis pathway was upregulated by fadrozole and mvd significantly down regulated by trenbolone in 48hpf embryos (Macherey 2013).
lss	NM_001083567.1	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	intramolecular transferase activity	The enzyme lanosterol synthase is one of the initiating enzymes of the steroid biosynthesis. It was significantly down regulated by trenbolone and upregulated by methyl testosterone (Macherey 2013), EE_2, genistein and flutamide (Dissertation Schiller 2013) in 48hpf embryos. It was assumed to be also regulated by fadrozole.
sc4mol (NEW: msmo1)	NM_213353.1	msmo1 methylsterol monooxygenase 1	fatty acid biosynthetic process oxidation-reduction process	Was significantly down regulated by trenbolone and upregulated by methyl testosterone (Macherey, 2013) EE_2, genistein and flutamide (Schiller 2013) in 48hpf embryos. It was assumed to be also regulated by fadrozole.
vtg1	NM_001044897.2	Vitellogenin 1	lipid transport response to xenobiotic stimulus cellular response to estrogen stimulus	Vitellogenin is regulated by estrogens and hence, bound to be affected by aromatase inhibition and reduction in estradiol.
star	NM_131663.1	steroidogenic acute regulatory protein	cholesterol metabolic process lipid transport steroid biosynthetic process	Was upregulated in the brain in Villeneuve et al. (2009). However, the lipid transport pathway was down regulated by fadrozole in 48 hpf embryos (Macherey 2013)
apoA1a	NM_131128	apolipoprotein A-1a	steroid metabolic process lipoprotein metabolic process cholesterol metabolic process cholesterol transport cholesterol efflux cholesterol biosynthetic process	A correlation between vitellogenesis and apoA1 was described by Levi et al. (2012).

Gene	RefSeq accession	Description	ZF Annotation (GO term)	Reason for selection
apoEb	NM_131098.1	apolipoprotein Eb	lipid transport lipoprotein metabolic process	Levi et al. 2012 found elevated apoEb in vitellogenic females. The lipid transport pathway was down regulated by fadrozole in 48 hpf embryos (Macherey 2013)
esr1	NM_152959	estrogen receptor 1	steroid hormone mediated signalling pathway regulation of transcription, DNA-dependent	Steroid hormone receptor activity was stimulated in 48hpf embryos (Macherey 2013)
esr2a	NM_180966.2	estrogen receptor 2a	steroid hormone mediated signalling pathway regulation of transcription, DNA-dependent	Was found upregulated in 48 hpf embryos after fadrozole exposure in an in-house study (Macherey 2013).
esr2b	NM_174862.3	estrogen receptor 2b	steroid hormone mediated signalling pathway regulation of transcription, DNA-dependent	Steroid hormone receptor activity was stimulated in 48hpf embryos (Macherey 2013).
ar	NM_001083123.1	androgen receptor	steroid hormone mediated signalling pathway regulation of transcription, DNA-dependent transcription from RNA polymerase II promoter	Was found upregulated in 48 hpf embryos after fadrozole exposure in an in-house study (Macherey 2013).
kiss1rb	NM_001110531	kiss1 receptor b	G-protein coupled receptor signalling pathway neuropeptide signalling pathway synaptic transmission	Kisspeptins and their receptors are associated with puberty onset in zebrafish (Biran et al., 2008). Was found expressed from 7 dpf by Onuma and Duan (2012).
kiss1ra	NM_001105679	kiss1 receptor a	G-protein coupled receptor signalling pathway neuropeptide signalling path-way synaptic transmission	Kisspeptins and their receptors are associated with puberty onset in zebrafish (Biran et al. 2008). Was found expressed from 3 dpf by Onuma and Duan (2012)
npy1r	NM_001102391	neuropeptide Y1 receptor)	G-protein coupled receptor signalling pathway Signal transduction	It was found upregulated in ovaries (not brain) in Villeneuve et al. (2009).
npy8br (previously npyryb)	NM_131436.1	neuropeptide Y receptor Y8b	G-protein coupled receptor signalling pathway Signal transduction	Several neuropeptide y receptor isoforms were found upregulated in gene sets of 48 hpf embryos after exposure to flutamide, linuron and prochloraz (Dissertation Schiller 2013). Villeneuve et al. (2009) found up regulation in ovaries after 96h exposure to 100 µg/L.

Gene	RefSeq accession	Description	ZF Annotation (GO term)	Reason for selection
gnrhr1	NM_001144980	gonadotropin-releasing hormone receptor1		Gonadotropin-releasing hormones have a pivotal function in the HPG-axis and are regulated by estrogens. GnRH neurons are essential regulators of sexual development and differentiation. Both, the transcripts of the hormones as well as the corresponding receptors were upregulated by estrogens (Collard et al., 2013)
gnrhr2	NM_001144979	gonadotropin-releasing hormone receptor2	cellular response to gonadotropin-releasing hormone G-protein coupled receptor signalling pathway Signal transduction	Was found significantly down regulated in 96 hpf embryos after exposure to flutamide (Schiller et al. 2014)
gnrhr3	NM_001177450.1	gonadotropin-releasing hormone receptor3		Gonadotropin-releasing hormones have a pivotal function in the HPG-axis and are regulated by estrogens. GnRH neurons are essential regulators of sexual development and differentiation. Both, the transcripts of the hormones as well as the corresponding receptors were upregulated by estrogens (Collard et al. 2013). The anti-androgen flutamide specifically induced gnrhr3 in 48 hpf embryos (Dissertation Schiller 2013)
gnrhr4	NM_001098193.1	gonadotropin-releasing hormone receptor4		
igfbp5a	NM_001126463	insulin-like growth factor binding protein 5a	regulation of cell growth	Was significantly upregulated by trenbolone in 48 hpf embryos (Macherey 2013), but igfbp1was also upregulated by estrogenic compound EE2, genistein and BPA (Dissertation Schiller 2013)
cyr61	NM_001080987	cysteine-rich, angiogenic inducer, 61	regulation of cell growth	Cyr61 belongs to the CCN proteins, which are involved in diverse biological processes including development, wound healing, inflammation, and tumour growth. Involvement in gpr30 (g-protein coupled estrogen receptor) signalling is speculated from human studies (Pandey et al., 2009)
igf 1	NM_131825	insulin-like growth factor 1	positive regulation of cell proliferation dorsal/ventral pattern formation	It was found upregulated in brain in Villeneuve et al. (2009).
sox9b	AY029578	SRY (sex determining	iridophore differentiation	Was found upregulated in Villeneuve et al. (2009)

Gene	RefSeq accession	Description	ZF Annotation (GO term)	Reason for selection
		region Y)-box 9b	melanocyte differentiation cartilage development embryonic pectoral fin morphogenesis hepaticobiliary system development pancreas development inner ear development otic placode formation otic vesicle formation retina development in camera-type eye	and in in-house EE2 studies (Schiller et al. 2013) It was also deemed important since cyp19a1a and cyp19a1b have sox binding sites in their promoter regions.
prox1(NEW: prox1a)	NM_131405	prospero homeobox 1	regulation of transcription, DNA-dependent negative regulation of sequence-specific DNA binding transcription factor activity neuromast hair cell differentiation neuromast deposition myofibril assembly lymphangiogenesis forebrain neuron development	Was upregulated and discussed as a marker of aromatase inhibition in Villeneuve et al. (2009): It regulates angiogenesis and can repress the transcriptional activity of the liver receptor homologue 1 (nr5a2), which is involved in cyp19a1b activation and gonadotropin regulation. It
cyp19a1a	NM_131154.2	cytochrome P450, family 19, subfamily A, polypeptide 1a	oxidation-reduction process response to xenobiotic stimulus response to steroid hormone response to estradiol	Fadrozole is a known aromatase inhibitor; and the inhibitory effect on the gene expression was confirmed (Fenske and Segner, 2004)
cyp19a1b	NM_131642.1	cytochrome P450, family 19, subfamily A, polypeptide 1b		Fadrozole is a known aromatase inhibitor; and the inhibitory effect on the gene expression was confirmed (Fenske and Segner, 2004)
fshr	NM_001001812.1	follicle stimulating hormone receptor	signal transduction G-protein coupled receptor signalling pathway follicle-stimulating hormone signalling pathway	It was found upregulated in ovaries (not brain) in Villeneuve et al. (2009). There is also the hypothesis that it is induced as compensatory response to impaired vitellogenesis (Ankley et al.2002)
zgc:64022	NM_200365	" ras-like estrogen-regulated growth inhibitor" (Villeneuve et al. 2009) - unconfirmed	signal transduction protein transport small GTPase mediated signal transduction	It was found regulated in brain and ovary in Villeneuve et al. (2009), but trend was inconsistent between concentrations and exposure times.

Gene	RefSeq accession	Description	ZF Annotation (GO term)	Reason for selection
			GTP catabolic process	
Ihc (NEW: lhcgr)	NM_205625	luteinizing hormone/choriogonadotropin receptor	signal transduction G-protein coupled receptor signalling pathway luteinizing hormone signalling pathway	The gonadotropins Lh and Fsh are released from the pituitary in response to estradiol and regulate oocyte development. The lhcgr gene has been identified as an estrogen target gene in 3 dpf embryos (Hao et al., 2013), and the up regulation by E2 was shown for lhcgr also in ovarian cells (Liu et al., 2011)
18s (NEW: zgc:158463)	FJ915075.1	18S rRNA		
rpl8	NM_200713.1	ribosomal protein L8		

For this study, we aimed for a relative quantification of the expression levels of the selected target genes. A relative quantification compares the expression level of a particular gene in different samples. A measured difference in RNA expression levels between two samples is the result of both true biological and experimentally induced (technical) variation. Several variables inherent to a RT-qPCR workflow must be controlled for minimization of technical changes. Influencing parameters are the amount and the quality of starting material, enzymatic efficiencies, and overall transcriptional activity, but also pipetting errors and measurement errors of the PCR cycler. It is recognised that the amount of input cDNA between samples and between PCR reactions varies and needs to be corrected by normalization, typically using one or more reference genes. Run-to-run variation within a series of measurements for a given gene is another type of technical variation that should be avoided or minimised. In order to reduce the inter-run variation, all samples of a given time-point were, whenever possible, analysed for a particular gene in the same run. By this so-called "Sample maximization strategy" (Hellemans et al., 2007) an inter-run calibration can be avoided.

It has been shown that the use of multiple reference genes (often also called housekeeping genes) is preferred to single reference gene normalization. The use of multiple stable reference genes is generally considered as the method of choice for the RT-qPCR data normalization (Bustin et al., 2009; Vandesompele et al., 2002). Practically, this normalization uses the geometric mean of multiple validated reference genes.

For this project, the transcripts coding for the ribosomal protein L8 and the ribosomal subunit S18 rRNA were chosen as reference genes. Both are commonly used reference genes and can be considered stably expressed due to their function. For the 18S gene, the stability of the expression during early development until 120 hpf and in various organs in the adult zebrafish has previously been confirmed (McCurley and Callard, 2008).

Specifically, two µL of cDNA equivalent to 40 ng of RNA, were used as starting template for each PCR-reaction and added to Sybr®Green qPCR Super Mix (Life Technologies/Invitrogen) in one well of a white 96-well PCR plate. Two technical replicates of each cDNA sample and target gene respectively reference gene were integrated in each PCR run, which consisted of 96 PCR reactions. Negative controls (minus RT and minus template controls) were included repeatedly but not in every run. On each 96-well plate, between six and twelve different cDNA samples were measured, depending on the number of target genes included per run. For each cDNA sample on a 96-well plate, the two reference genes, *18s* and *rpl8*, were run in parallel on the same plate. The expression values of these reference genes were used to normalise the expression values of the target genes.

The PCR-cycler protocol was as follows: After incubating for 2 min at 50°C and for 10 min at 95°C (for DNA polymerase activation), targets were amplified using the following cycle: 95°C for 15 s, then 54, 54.5 or 60°C for 30 s (which of the three annealing temperatures was used for a particular target can be seen in Table 39 in the Annex under 8.3) and 60°C for 30s (for acquisition) (40 cycles); the run was finalised by a melt curve analysis: 95°C for 60s, then 55°C for 60s (80 cycles, with +0.5°C increment each cycle and a 10s acquisition). The BioRad iQ5 real-time PCR detection system (BioRad™, Hercules, USA) was used for the analyses.

After each run, a report was generated by the BioRad cycler software, which included the RFU (relative fluorescence unit) and Ct (threshold cycle) values for each well of the 96 well plate. These data were exported to Excel and the files saved for further analysis. From the exported Excel data files, the Ct values were extracted and used for the calculations of the relative expression levels of the target genes on the corresponding 96 well plate.

The plate layouts were variable but the same set of genes was measured together on the same plate whenever possible. In order to minimise well-variation within one plate, the technical replicates of each sample and gene were assigned to different locations on the plate and not measured in adjacent wells.

For the analysis of the data, 2'-ΔΔCt (ddCt), fold-change respectively fold induction values (i.e. control normalised 2'-ΔΔCt values, which correspond to the mean relative mRNA amount of a corresponding gene of a biological replicate sample) were calculated based on the threshold cycle (Ct-values) obtained for each

reaction/well, using the comparative "Delta Delta Ct method" (ΔΔCt) (Pfaffl, 2001). This analysis method is based on the assumption that the amplification efficiencies of the reference control genes and the target gene of interest are approximately equal. Specifically, ΔΔCt assumes that each PCR cycle will exactly double the amount of material in the sample (i.e., amplification efficiency = 100%). We can hence calculate the ratio of our target gene in our treated sample relative to our untreated sample by taking 2'-ΔΔ*Ct.* Each gene is normalised to two reference gene within each sample to ensure that no systematic errors due to differences between each sample are introduced.

4.5 Chemical analysis

One sample of an appropriate volume of test water (10 mL) was taken from each vessel from the mid water body at test start, and thereafter, once a week for the first two weeks. Afterwards, samples were taken alternating from one of the two test vessels served by one dosing pump. Water samples were stabilised by addition of acetonitrile (1:1; v+v) containing 0.2% formic acid prior to storage. A second sample was taken in the same way and stored as a retained sample. The retained sample was stored at ≤18°C. Stock solution samples were analysed on the day of preparation and application to the test systems. In any other case, samples of the stock solution were sampled and kept frozen at ≤ -18°C until analysis. A control sample was taken for matrix calibration purposes.

4.5.1 Analytical method

Fadrozole substance analysis was performed by high performance liquid chromatography tandem mass spectrometry with negative ionization. Data were collected on a Waters 2695 separations module coupled to a Quattro-Micro tandem mass spectrometer (Waters). Separation was performed on a binary gradient of a 20 mmol ammonium acetate solution in methanol (A) and a 20 mmol ammonium acetate solution in a 90:10 water: methanol mixture (B), starting with 60% A and 40% B, increasing to 100% A and 0% B within 3 min, returning to 60% A and 40% B after 6.1 min, and holding initial conditions for 3 min. A 50-µL aliquot of the sample was injected into a Gemini C18 high-performance liquid chromatography column (150 mm x 3 mm, 5 µm particle size; Phenomenex) at a flow rate of 0.5 mL/min and a column temperature of 30°C. Matrix-free procedural blanks were run with the complete analytical system each working day to exclude possible cross-contaminations during laboratory work. The concentrations of the calibration solution were in the range of 0 µg/L to 250 µg/L (0 µg/L; 1.25 µg/L; 2.5 µg/L; 5 µg/L; 10 µg/L; 25 µg/L; 50 µg/L; 125 µg/L; 250 µg/L). Coefficient of correlation (r2) of the calibration function was estimated as ≥ 0.99. Samples exhibiting fadrozole concentrations beyond the highest calibration standard (250 µg/L) were diluted to meet the range of the calibration function. The limit of quantification (LOQ) is defined as the lowest validated spike level meeting the method performance acceptability criteria (mean recoveries for each representative commodity in the range 70-120%, with a $RSD_r \leq 20\%$). The limit of quantification (LOQ) for the test item was determined to be 5 µg/L.

All details of the analytical method can be found in the Annex under 8.4.

4.6 Treatment of the Results

4.6.1 Data evaluation

In this report, numerical values are frequently rounded to a smaller degree of precision (number of digits) than used in the actual calculation. Minor differences in results obtained from calculations with such rounded values in comparison to those obtained with higher precision values are possible. They are, however, well within the limits of the experimental accuracy and thus of no practical concern.

4.6.2 Analysis of qPCR data

The qPCR data were normalised (Vandesompele et al., 2002) and the relative change in expression for each target gene calculated according to the ΔΔCt (ddCt) method of Livak and Schmittgen (2001). Based on the Ct values generated by the PCR cycler software according to default settings, the 2'-ΔΔCt (ddCt) and fold

induction values (i.e. control normalised 2'-ΔΔCt values) were calculated in Excel sheets (Version 2010 for Windows). These data were then transferred to GraphPad Prism (Version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com) for the statistical analysis and for the graphing of the data as bar charts. To express up regulation as positive fold-change data and down regulations as negative fold-change data, the fold induction values were log2 transformed.

At 48 hpf, 96 hpf and 28 dpf four replicate samples were available for each treatment group (control, 10 µg/L 32 µg/L and 100 µg/L), at 63 dpf it was 15 replicate samples for each treatment group (three times five fish per replicate tank). For 48 hpf, 96 hpf and 28 dpf all 29 target genes were measured in all four replicate samples of each treatment but only three replicates were used for the analysis because the fourth replicates generated deviating results. We were unable to ascertain the reason for these deviating results but one contributing factor was presumably the delay in the measurement of the forth replicate samples, which were analysed between 10 and 12 months after the first three replicates.

For the calculations of the 2'-ΔΔCt (ddCt) values modifications were in some cases necessary in case of missing Ct values due to technical issues, related either to the PCR reaction mix or fluorescence measurement of the PCR cycler (i.e. no RFU obtained). Obvious outliers of the two technical replicates of each sample and gene (Ct difference >> 1.0) on a PCR plate were removed manually from the analysis. As a consequence sometimes only one Ct value was used for the calculations or Ct values of replicate samples were used to calculate an average Ct value for a single sample and gene. Another possible deviation was the removal or the lack of an average Ct value of one replicate sample. In this case replicate was excluded from the calculation of the mean ΔCt value. All these deviations are highlighted in yellow or red in the Excel sheets, which supplement this report.

4.6.3 Statistical evaluations

4.6.3.1 Apical effect data

All apical endpoints were analysed using ToxRat® Professional 2.10. Normal distribution of data was confirmed by the Shapiro-Wilks test, followed by Levene's test for homogeneity. A one-way ANOVA was performed, with the Williams t-test as post-hoc test, applying a significance level of $p \leq 0.05$.

Data on histopathology were evaluated by Dr Lisa Baumann, Centre of Fish and Wildlife Health, University of Bern, Switzerland. The applied software (GraphPad Prism) as well as the statistical tests are indicated in the respective results section 5.3.

Details of the statistical tests used for the analysis of the endocrine disruption relevant apical endpoints are outlined in the Annex under section 8.2.

For the biological results, No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) were determined, whereby the LOEC was the lowest concentration of the three tested fadrozole concentrations at which a statistically significant difference from the control group occurred. The NOEC was the concentration just below the LOEC.

4.6.3.2 Gene expression data

As described earlier, for the analysis of the relative gene expression changes was conducted according to ΔΔCt (ddCt) method of Livak and Schmittgen (2001) and a normalization to two internal reference genes by geometric averaging (Vandesompele et al. 2002) . The statistical evaluation was performed on the mean reference gene normalised ΔCt values, the 2'-ΔΔCt values. For statistical determination of significant differences of the fadrozole treatments compared to the controls, one-way ANOVAs followed by Dunnett's multiple comparison tests ($p<0.05$), were performed. As for the error propagation for ΔΔCt and log2 transformed fold-change values, the error is the sum of multiple additive errors, i.e. the error had to be calculated for the average ΔCt of each target and reference gene and then the geometric means determined to calculated all ΔCt values plus and minus the error. In case of the log2-transformed data, errors were calculated prior to log2-transformation, i.e., the errors of the mean 2'-ΔΔCt values were determined and then added to and subtracted

from the mean. The resulting three values were then separately log2-transformed. In order to obtain log2-transformed errors, the log2-transformed values of the mean subtracted/ added from/to the error were subtracted from log2-transformed value of the mean.

4.7 Deviations from the study protocol and the OECD234 TG

The following deviations occurred during the time course of the study:

- At test start, each test vessel should be equipped with two fry cages, analytical sieves of stainless steel with a diameter of 10 cm and a brim height of 4.5 cm (approx. 75 cm^2). Due to a limited number of fry cages of this size, the two smaller fry cages were replaced by one fry cage with an approx. two-fold size (diameter approx. 13.5 cm; 150 cm^2) in replicate C of all treatment levels.
- Early transfer of juveniles from the egg cups to the tank on day 21 instead of day 28. The juveniles from the egg cups 1 and 2 were united in this process. Reason: Increasing density in egg cups 2 (start with 35 eggs) was assumed to affect the growth rate if the larvae were to be remained in the cups until day 28.
- Reduction of fish to 25 in each tank: 25 fish were selected at random and the surplus fish collected and stored for gene expression analysis. This procedure was necessary to even out and correct the fish numbers according to the original study plan.
- Of the originally selected 30 genes as listed in the study plan only 27 were measure because the genes *igfbp1a*, *igfbp1b*, *igfbp5b* were dropped since they failed to show a response to fadrozole exposure in a pre-test on 96 hpf embryos. Instead, the genes *gnrhr3* and *gnrh4* were included; hence 29 target genes (plus the two reference genes) were measured altogether.

These deviations were considered not having an effect on the overall integrity of the study.

5 Results

5.1 Test conditions

The physical test parameters temperature, oxygen saturation, and pH were recorded during the time course of the study. The mean temperature in the test vessels was in the range between 24.5°C and 25.1°C, the mean oxygen saturation was in the range between 99.0 % and 103.4 %; and the mean pH varied between 8.08 and 8.17, with minor deviations (Table 5).

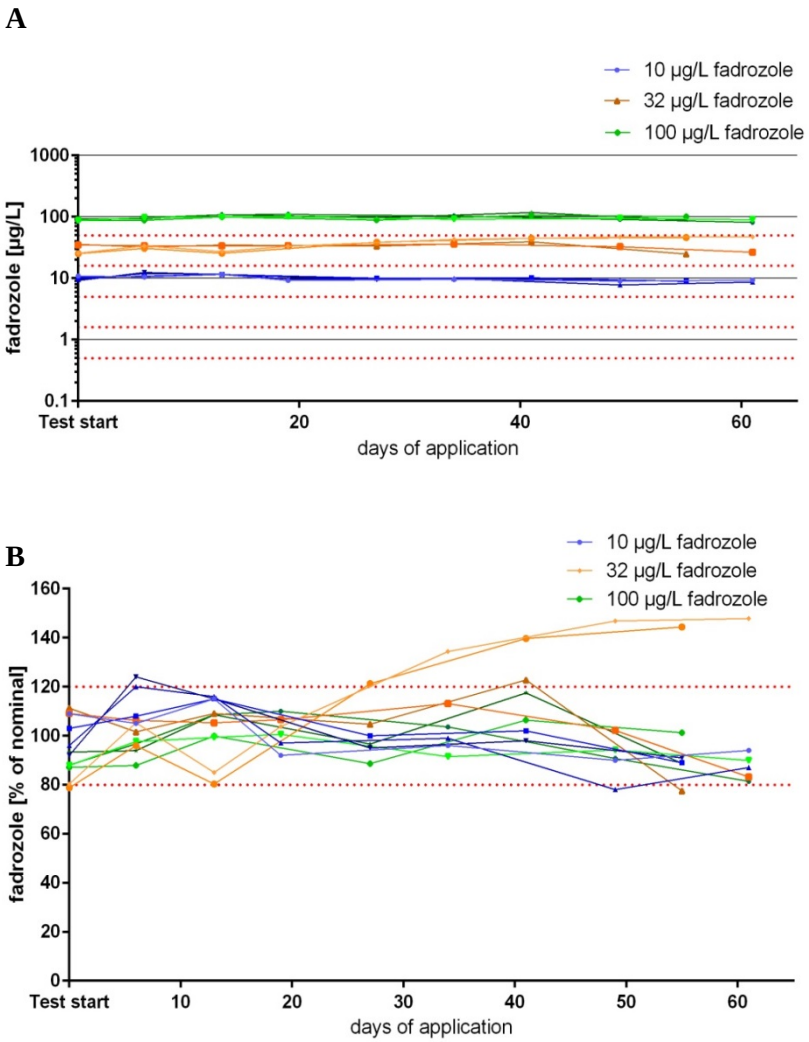
Table 5: Test conditions (temperature [°C]; oxygen saturation [%]; pH) over the time course of the study, from 0 to 63 dpf. Presented are the mean values of each test vessel and the total mean value of each treatment group.

		Temperature [°C]				Oxygen saturation [%]				pH			
Nominal conc. [µg/L]	Vessel	Mean	SD	SD [%]	Mean total	Mean	SD	SD [%]	Mean total	Mean	SD	SD [%]	Mean total
control	A	24.5	0.3	1.3	**24.8**	103.1	3.1	3.0	**102.5**	8.11	0.17	2.1	**8.09**
	B	24.7	0.3	1.0		102.7	2.8	2.7		8.09	0.14	1.7	
	C	24.9	0.3	1.0		102.7	4.0	3.9		8.08	0.14	1.7	
	D	24.9	0.2	0.9		101.5	4.3	4.2		8.09	0.13	1.6	
10	A	24.6	0.2	0.9	**24.7**	102.8	4.5	4.4	**102.7**	8.17	0.14	1.8	**8.16**
	B	24.8	0.3	1.0		102.8	3.8	3.7		8.17	0.14	1.7	
	C	24.6	0.3	1.1		103.4	3.9	3.7		8.16	0.12	1.5	
	D	24.7	0.3	1.0		102.1	3.5	3.5		8.16	0.12	1.5	
32	A	25.0	0.3	1.2	**24.9**	100.2	6.2	6.2	**101.3**	8.14	0.14	1.7	**8.15**
	B	25.1	0.4	1.4		99.8	5.7	5.7		8.13	0.14	1.7	
	C	24.8	0.3	1.3		103.2	3.4	3.3		8.16	0.13	1.6	
	D	24.7	0.3	1.3		102.0	2.5	2.5		8.17	0.12	1.5	
100	A	24.7	0.3	1.3	**24.9**	101.5	5.0	4.9	**100.6**	8.17	0.14	1.7	**8.15**
	B	25.1	0.3	1.2		100.9	4.6	4.5		8.15	0.13	1.6	
	C	24.9	0.3	1.4		101.1	3.0	3.0		8.13	0.13	1.7	
	D	25.1	0.3	1.3		99.0	3.9	3.9		8.15	0.13	1.7	

5.2 Test item concentrations

The mean measured concentrations of the application solutions of fadrozole between day 1 and day 61 of the study ranged between 94.4% and 114.8% per treatment (Table 6). Test concentrations during the time course of the study did not overlap (Figure 3 A) and remained within the 20 % deviation limits for most vessels. The exception were two vessels (replicates A and B) of the nominal 32 µg/L test concentration, which displayed average concentrations of 144.3 % and 147.7 % of nominal at test end (compare Figure 3 and raw data in the Annex in Section 8.1.1). The slight but constant concentration increase was likely caused by a technical error affecting the water supply of the two vessels which was provided by the same dosing pump. However, as the system should not be influenced, it was decided not to counter-regulate the water supply and disturb the system since the concentration increase indicated stabilisation and did not exceed 150 %. Apart from these two vessels, the measured concentrations were in good accordance with the nominal concentrations over the whole test duration (Table 6). However, all effect data presented following are referred to the nominal test concentrations.

Figure 3: Fadrozole concentrations during the time course of the study (in [µg/L] and [% of nominal]



Fadrozole concentrations displayed on a logarithmic scale; no overlap of concentrations during the time course of the study was observed. (B) Fadrozole concentrations presented as % of nominal. Note that two test vessels of the 32 µg/L test concentration, which were served by the same pump, displayed increasing test concentrations in the second half of the study. Legend: blue: 10 µg/L; orange: 32 µg/L; green: 100 µg/L.

Table 6: Mean measured concentrations of the test item fadrozole in [µg/L] and [%]

		Mean measured concentration per vessel		Mean measured concentration per treatment	
Nominal conc. [µg/L]	**Vessel**	**[µg/L]**	**[%]**	**[µg/L]**	**[%]**
Control	A	<LOQ		<LOQ	
	B	<LOQ			
	C	<LOQ			
	D	<LOQ			
10	A	10.0	100.0	10.1	**101.1**
	B	10.3	102.8		
	C	9.9	99.1		
	D	10.3	102.5		
32	A	36.7	114.8	34.6	**108.2**
	B	35.2	110.0		
	C	33.2	103.7		
	D	33.4	104.5		
100	A	94.4	94.4	96.6	**96.6**
	B	95.2	95.2		
	C	97.0	97.0		
	D	99.8	99.8		

5.3 Standard biological endpoint results of the FSDT

For the endpoints hatching success, post-hatch survival, total body length at 28dpf, and total body length wet weight, survival at test termination data are presented as mean plus standard deviation (total and [%]) values per treatment group. Statistically significant differences are indicated by asterisks (*). Results of each endpoint are summarised in individual tables, with fadrozole concentrations shown as nominal concentrations. The endpoints are presented following the key steps of zebrafish development, i.e. the early life stage (ELS; ≤ 35 dpf), sexual differentiation, and sexual maturation.

5.3.1 Early life stage (ELS)

5.3.1.1 Hatch

The study was initiated by introducing 1 x 35 and 1 x 50 fertilised eggs into the two fry cages of each test vessel. This resulted in a total number of 340 fertilised eggs each for the control and exposure groups. In the replicate test vessel C of each treatment group, only one fry cage with a total number of 85 eggs was introduced.

Hatching was observed starting from day 3 pf, and a hatching rate > 95 % of remaining eggs (340 eggs minus 20 eggs per sampling point and replicate = 180 eggs) in all concentrations was observed at day 6 pf (Table 7).

5.3.1.2 Survival

Another endpoint during the early life stage phase was the determination of the post hatch survival rate at day 28 pf. The survival was estimated based on digital images of each vessel acquired at the indicated time points by computer aided counting. The number of hatched larvae at day 6 pf in each test vessel of the control and the treatments was defined as 100%.

Of the control, 81% of larvae survived until day 28 pf and post-hatch survival was therefore in the valid range of at least 70 % at 28 dpf. The survival rate of the treatments decreased concentration-dependent to a value of 71 % at a fadrozole concentration of 32 µg/L and to 68 % at the highest fadrozole concentration of 100 µg/L (Table 7). This decrease was statistically significantly different from the control (William's t-test; $p<0.05$; one-sided smaller). Therefore, the NOEC for post-hatch survival during the ELS was 10 µg fadrozole/L. The detailed statistic can be found in the Annex, Section 8.2.

5.3.1.3 Growth in terms of standard length

The growth in terms of lengths of the fish larvae was determined on day 28 pf. The lengths were measured based on digital images of each vessel acquired at the indicated time points by computer aided measurements. The average length of the control was 1.01 cm (Table 6). The mean lengths of the treatments varied between 1.06 cm (100 µg/L) and 1.07 cm (10 and 32 µg/L). No significant differences between control and treatments could be observed (Table 7).

Table 7: Summary of evaluated test endpoints during the early life stage

		Nominal concentration fadrozole [µg/L]			
		control	10	32	100
Hatch [n] day 4 pf	**Total**	80 (of 260)	39 (of 260)	27 (of 260)	68 (of 260)
Hatch [n] day 6 pf	**Total**	180	180	180	180
Survival [n] day 28 pf	**Total**	146	140	127	122
Post hatch survival [%] day 28 pf	**Mean**	80.6	77.8	70.6*)	67.8*)
	SD	2.1	3.1	3.3	5.3
	SD%	2.6	4.0	4.7	7.8
Mean length [cm] day 28 pf	**Mean**	1.01	1.07	1.07	1.06
	SD	0.07	0.04	0.05	0.08
	SD%	6.5	3.9	4.4	7.4

Percentages of post hatch survival were based on the number of larvae counted in each test vessel after the last sampling point of the embryonic test phase.
*) statistically significant deviation from the control, $p<0.05$, Williams test, one sided smaller, nominal data arcsine-transformed

5.3.2 Juvenile Growth (JG)

5.3.2.1 Survival

Survival of juvenile fish was not affected by fadrozole treatment. After reduction to 25 fish per replicate at day 28, no further mortality was observed.

5.3.2.2 Growth in terms of standard length and weight

The growth during the juvenile phase was determined by measurements of the standard length and weight at day 63 pf (test termination). Therefore, individual fish were weighted (wiped wet weight) and the lengths measured.

Control fish and exposed fish showed a mean length of 2.8 cm (Table 7). The mean weights slightly increased with increasing exposure concentration, from 0.183 g for the controls and to 0.196 g in the highest treatment condition. However, the differences in the mean values were not significant between treatments.

Table 8: Summary of evaluated test endpoints at test termination

		Nominal concentration fadrozole [µg/L]			
		Control	10	32	100
No. of fish [n] day 63 pf	**Total**	98	100	100	99
Mean length [cm] day 63 pf	**Mean**	2.8	2.8	2.8	2.8
	SD	0.1	0.0	0.1	0.0
	SD%	2.8	0.5	2.1	1.5
Mean weight [g] day 63 pf	**Mean**	0.183	0.186	0.188	0.196
	SD	0.011	0.003	0.010	0.012
	SD%	5.9	1.7	5.3	6.2

5.3.3 Sex ratio

The determination of the gonadal sex was carried out during the histopathological evaluation, which is described in the following section 5.3.4). The macroscopic classification of gonadal sex (where realised) agreed with the subsequent histological classification.

For ten fish the sex could only be classified as unknown because their inner organs were lost during the sampling process or due to damage of the gonads caused during preparation. However, these fish were equally distributed across all treatment groups (i.e. two in controls; four at 10 µg fadrozole/L; three at 32 µg fadrozole/L; one at 100 µg fadrozole/L) and thus the impact on the data quality can be considered low.

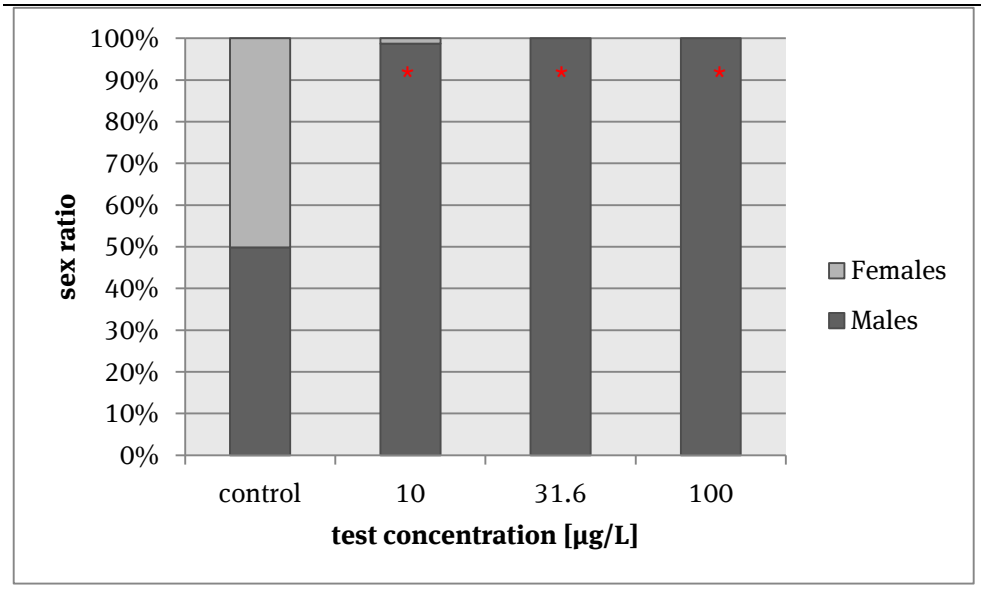
The controls display a mean proportion of males and females of almost 50 %, with an average of 2.5 % remaining undetermined (Table 9). At the lowest concentration of fadrozole, all fish were identified as males, except for one female in replicate A. In the higher concentrations, only males were identified (compare Table 9 and Figure 4). Few fish remained histopathologically undetermined due to damage of the gonads caused during preparation.

Table 9: Sex ratio of control and treatments at test termination

	Nominal concentration fadrozole [µg/L]							
	Control		10*)		32*)		100*)	
	[%] of total							
	male	female	male	female	male	female	male	female
A	52.6	47.4	94.7	5.3	100.0	0.0	100.0	0.0
B	38.9	61.1	100.0	0.0	100.0	0.0	100.0	0.0
C	50.0	50.0	100.0	0.0	100.0	0.0	100.0	0.0
D	57.9	42.1	100.0	0.0	100.0	0.0	100.0	0.0
Mean	**49.9**	**50.1**	**98.7**	**1.3**	**100.0**	**0.0**	**100.0**	**0.0**
SD	**8.0**	**8.0**	**2.6**	**2.6**	**0.0**	**0.0**	**0.0**	**0.0**
SD%	**16.1**	**16.0**	**2.7**	**200.0**	**-**	**-**	**-**	**-**

*) statistically significant deviation from the control, $p<0.05$, Williams test, two-sided, nominal data arcsine-transformed; nd = not determined: During preparation of fish carcasses, 10 fish tissue got damaged, resulting in loss of gonads. Thus, histopathological evaluation was not possible.

Figure 4: Sex ratio [%] of control treatments compared to fadrozole treatments



Sex ratios at 63 dpf upon treatment with fadrozole: In the 10 µg/L treatment group, only one female fish was found whereas in the other treatment groups the sex ratio was completely shifted towards males. Shown are percentages of females, males; * denotes statistically significant deviation from the control, $p<0.05$, Williams test, two-sided, nominal data arcsine-transformed.

5.3.4 Histopathology

The sex ratio in the control vessels was found to be close to 50 % males and 50 % females, which was anticipated for unexposed zebrafish (Maack and Segner, 2003). In contrast, no females were found after exposure to 32 µg/L and 100 µg/L fadrozole, except for one female at 10 µg/L. Consequently, only results for males are presented for the fadrozole treatment groups.

5.3.4.1 Sexual development

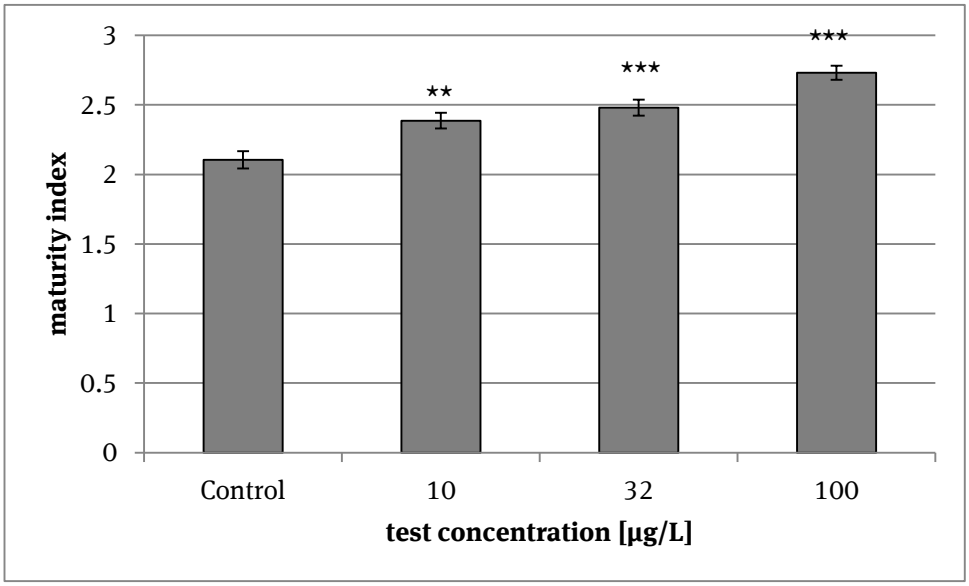
Female gonad development

The ovarian development of the control fish was characterised by an early maturity stage with all females exhibiting gonads consisting of early stage oocytes. Only one gonad had reached a maturation stage of 2, all other ovaries were at stage 1 (see histopathology data in Table 10 and Table 26, and in the Annex, Section 8.1.5).

Females of the control group had an average gonad maturity index of 1.8, which is comparably low for the age. Zebrafish females at the age of 60 dpf normally show an average maturity index of approx. 3 (Baumann et al., 2013). One female of group 1 had immature gonads. As ovarian maturation in fish is a function of growth, the low maturity index may reflect slow growth of the experimental fish. The delay in ovarian maturation was not associated with any pathological alterations of the gonads.

Male gonad development

Figure 5: Maturity index of male fish gonads after fadrozole exposure



An increasing degree of maturation with increasing fadrozole concentrations was observed in male gonads. For treatments with 10 and fadrozole/L highly and for treatment with 32 µg and 100 µg fadrozole/L most highly significant differences compared to the controls were observed.
Shown are means ± SEM of the maturity indices of male fish gonads after fadrozole exposure; **p<0.01; ***p<0.001; GraphPad PRISM, one-way ANOVA with Dunnett's multiple comparison test

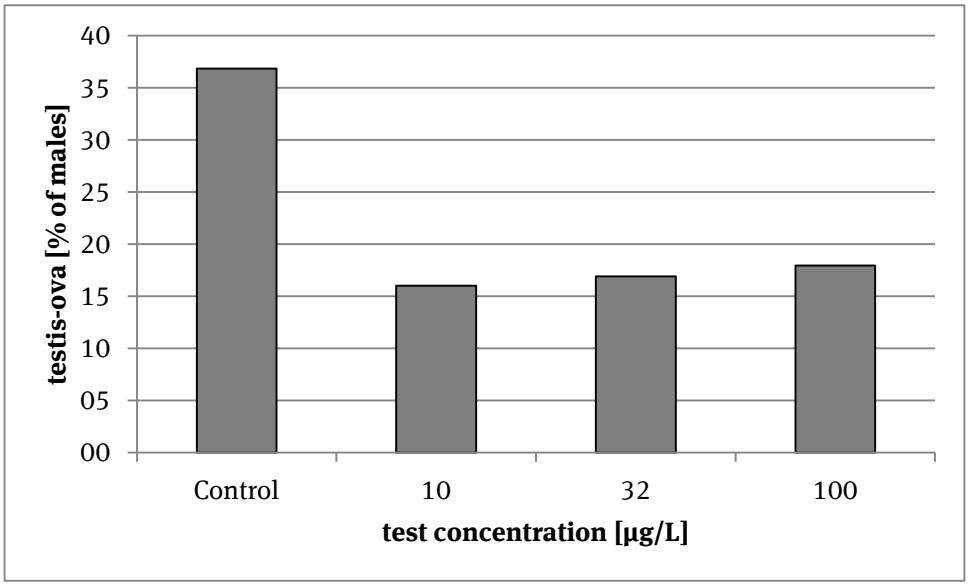
Males of the control group had an average testis maturity index of 2.1, which is low for63 dpf. Average maturity indices of males at the age of 60 dpf found in the literature were in a range of 2.5 -3.0 (Baumann et al., 2013). The average gonad maturity of males increased with increasing fadrozole concentrations (Figure 5; Table 10). Compared to the controls, this effect was statistically significant already at 10 µg fadrozole/L. The

highest concentration (100 µg fadrozole/L) showed an average maturity index of 2.7 (Table 10). As shown by the skewed sex ratios (Figure 4), the aromatase inhibition had a strong masculinizing effect on the exposed fish.

Among control males, 36.8 % exhibited “testis-ova” (Figure 6; Table 10), which means that the testicular tissue was interspersed with small immature oocytes. This is characteristic of the normal transition phase in the gonad development of zebrafish, where the early gonad of genetic males transforms from an ovary-like stage into the male testis. This phase normally occurs earlier and is accomplished after day 60 pf. This was not the case in the current study and was in line with the gonad histopathological observation of a generally low gonad maturity, which could be an indication of a general delay in the development. The incidence of testis-ova was over 20 % lower in the 10 µg/L fadrozole exposed fish and remained at this rate with increasing exposure concentrations (Figure 6). The severity grade of testis-ova was on average non-significantly higher in the control males than in the fadrozole exposed fish (see Table 10, Table 27, and in the Annex section 8.1.5).This was in agreement with the increasing gonad maturity indices of the treatment groups, which imply a faster and thus, further progressed gonad development of exposed fish.

Besides, no other alterations of testicular morphology or any other adverse effects were found in the pathology.

Figure 6: Males with testis-ova [% of males]



Shown are the percentages of males with testis-ova after fadrozole exposure. Note that the percentage of males with testis-ova is › 15% lower in the fadrozole-treated than in the control fish.

In summary, the fadrozole exposure had a strong masculinizing effect on developing zebrafish and gonad development was skewed completely towards males (compare sex ratio in Section 5.3.3). The average male gonad maturity was significantly increased and occurrence of testis-ova, as an indicator of a delayed development, was decreased. The histopathological results are summarised in Table 10, below.

Table 10: Summary of histopathological effects

Nominal concentration fadrozole [µg/L]			Control	10	32	100
Observed effect	**Maturity index of testis**	**A**	2.10	2.37	2.42	2.70
		B	2.00	2.33	2.45	2.83
		C	2.20	2.56	2.55	2.70
		D	2.10	2.30	2.50	2.70
		Mean	**2.10**	**2.39**	**2.48**	**2.73**
		SD	**0.08**	**0.12**	**0.06**	**0.06**
		SD%	**3.89**	**4.89**	**2.30**	**2.38**
		SEM	**0.04**	**0.06**	**0.03**	**0.03**
	Males with testis-ova [%]	**A**	40.0	26.3	21.1	15.0
		B	57.1	11.1	20.0	22.2
		C	10.0	5.6	15.0	10.0
		D	45.5	20.0	11.1	25.0
		Mean	**38.1**	**15.7**	**16.8**	**18.1**
		SD	**20.1**	**9.2**	**4.6**	**6.8**
		SD%	**52.6**	**58.6**	**27.5**	**37.8**
		SEM	**10.0**	**4.6**	**2.3**	**3.4**
	Severity of testis-ova	**A**	2.00	1.40	1.25	1.00
		B	1.50	1.50	1.25	2.00
		C	1.00	1.00	1.67	1.00
		D	1.40	1.25	1.50	1.20
		Mean	**1.48**	**1.29**	**1.42**	**1.30**
		SD	**0.41**	**0.22**	**0.21**	**0.48**
		SD%	**27.88**	**16.89**	**14.50**	**36.62**
		SEM	**0.21**	**0.11**	**0.10**	**0.24**
	Maturity index of ovary	**A**	1.89	1.00	ND	ND
		B	1.64	ND	ND	ND
		C	1.90	ND	ND	ND
		D	1.75	ND	ND	ND
		Mean	**1.80**	**1.00**	**ND**	**ND**
		SD	**0.12**	**ND**	**ND**	**ND**
		SD%	**6.91**	**ND**	**ND**	**ND**
		SEM	**0.06**	**ND**	**ND**	**ND**

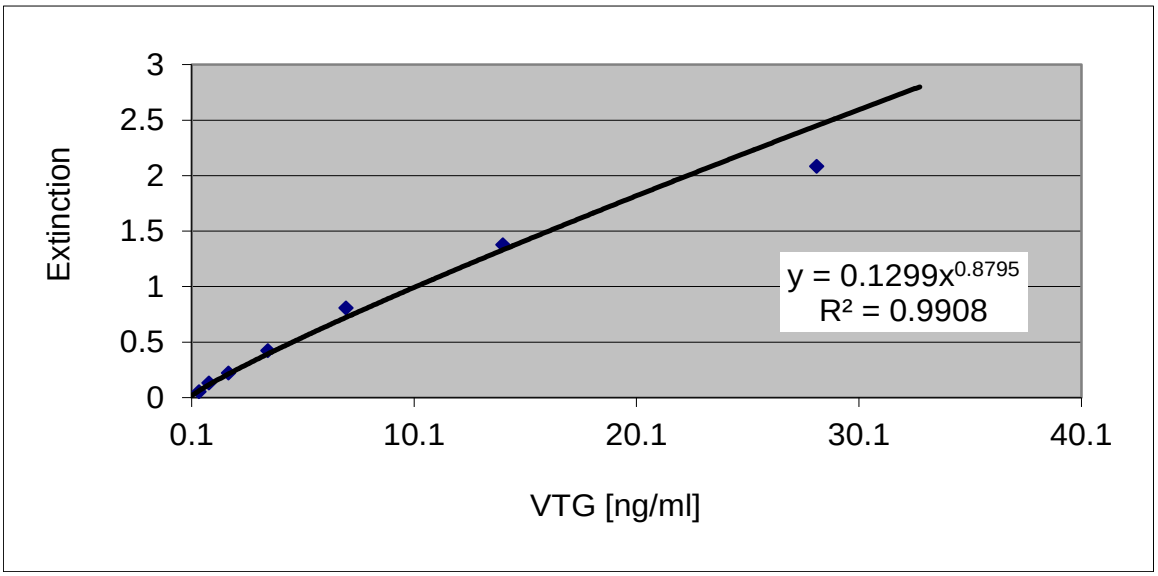
ND = not determinable, as no females were present in the fadrozole treatments

5.3.5 Vitellogenin measurements

5.3.5.1 Assay calibration

A calibration curve was generated for every vitellogenin (VTG) assay performed (example shown in Figure 7). Table 28, presented in the Annex in section 8.1.6, summarises the equation parameters (a and b) of all regressions, the regression coefficients as well as the percentages of non-specific binding (NSB) absorbance compared to the absorbance of the highest standard concentration.

Figure 7: Calibration curve; exemplarily shown for a representative assay



5.3.5.2 Limit of Detection (LOD)

According to the OECD test guideline 229, the limit of detection (LOD) is defined as the concentration of the lowest analytical standard, and limit of quantitation (LOQ) is defined as the concentration of the lowest analytical standard multiplied by the lowest dilution factor. LOD and LOQ were determined for each VTG assay and are shown in the annex in section 8.1.6 (Table 29).

5.3.5.3 Results of VTG measurements

The analysis of VTG provides information on the estrogenic state of the exposed fish and may help to interpret the effects observed for the population relevant endpoints.

VTG concentrations were generally low, as analysis was performed early in development at the beginning (i.e., 63 dpf) rather than the end of the reproductive phase, when this biomarkers is usually evaluated in other study types). Effects of fadrozole on vitellogenin plasma concentrations of females could not be determined due to the lack of female fish in the treatment groups. Only one female was found in replicate A of the 10 µg fadrozole/L treatment, which displayed a vitellogenin concentration of 0.01 ng VTG/µg protein. This concentration was by a factor of 1000 lower than the mean VTG concentration found in control females (12.18 ng VTG/µg protein; Table 11). However, the plasma concentrations of the control females displayed high variation, and females with low amounts in the range ≤ 0.01 ng/µg were also found (Table 30, Annex Section 8.1.6).

Male fish displayed VTG concentrations below or close to the LOD. A tendency to lower concentrations in treatment conditions than in the controls could be observed. However, it has to be noted that small measurement inaccuracies close to the detection limit had a comparably greater influence on the VTG concentration determined. Thus, the differences in the VTG levels of exposed males were statistically not significantly different from the control males (Table 12).

Table 11: Summary VTG levels [ng/µg protein] in females

Females	Nominal concentration fadrozole [µg/L]			
Replicate	**Control**	**10**	**32**	**100**
A	9.73	0.01	ND	ND
B	11.62	ND	ND	ND
C	18.07	ND	ND	ND
D	9.30	ND	ND	ND
Mean	**12.18**	**0.01**	**ND**	**ND**
SD	**4.06**	**ND**	**ND**	**ND**
SD%	**33.3**	**ND**	**ND**	**ND**

ND = not determinable, as no females were present due to fadrozole treatment

Table 12: Summary VTG levels [ng VTG/µg protein] in males

Males	Nominal concentration fadrozole [µg/L]			
Replicate	**Control**	**10**	**32**	**100**
A	0.008	0.005	0.003	0.006
B	0.006	0.006	0.003	0.006
C	0.006	0.006	0.003	0.003
D	0.009	0.005	0.004	0.002
Mean	**0.007**	**0.006**	**0.003**	**0.004**
SD	**0.002**	**0.001**	**0.001**	**0.002**
SD%	**22.8**	**13.3**	**18.1**	**46.1**

5.4 Gene expression results

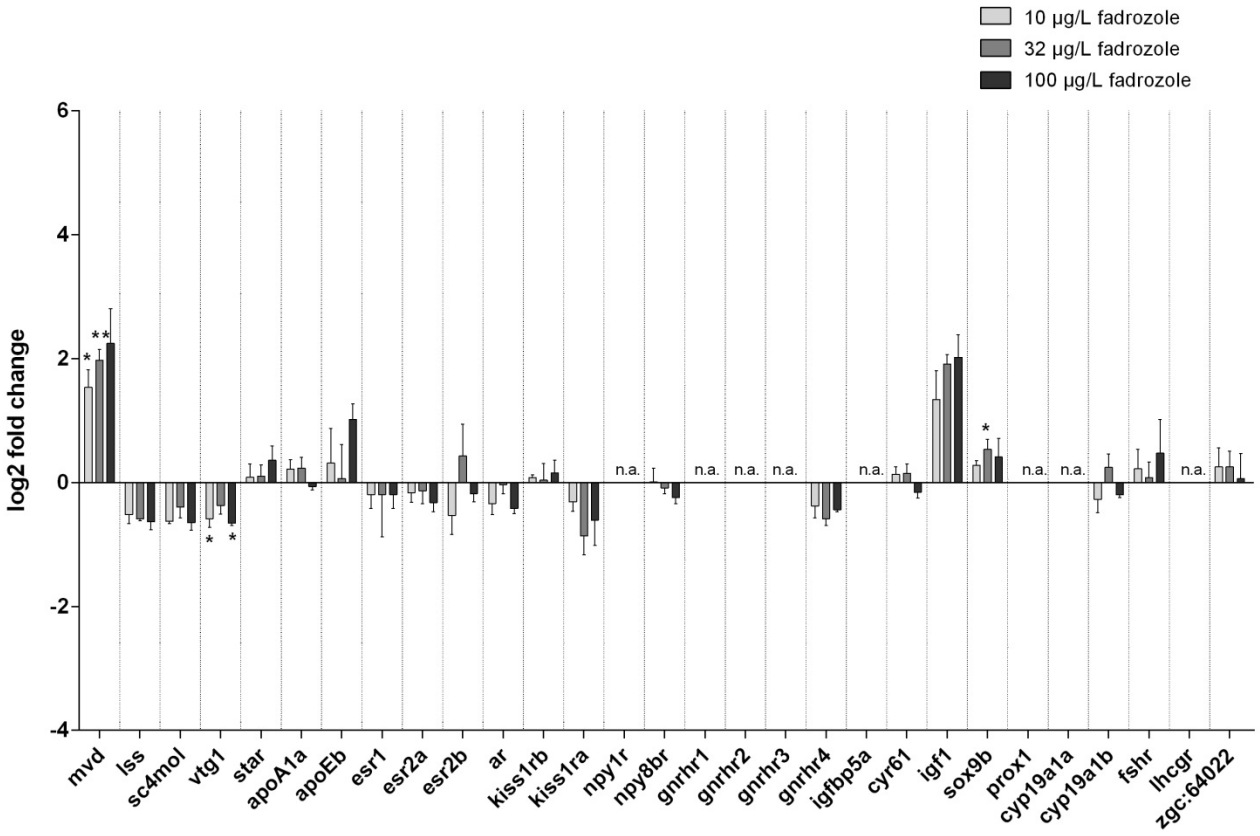
29 Target genes were selected according to criteria described in section 3.5 and in (Table 4, and which previously indicated regulation in 48 hpf or 96 hpf zebrafish (in-house studies). Expression of 29 selected genes were measured in 48 hpf and 96 hpf embryos and larvae, in 28 dpf juvenile and 63 dpf pre-adult fish using quantitative RT-PCR (SYBRGreen-based). The aim of this investigation was to analyse the expression and possible regulation of these genes by fadrozole in order to evaluate their potential application as early biomarkers of endocrine disruption caused by aromatase inhibition.

5.4.1 Gene expression in 48 hpf embryos

The analysis was performed on cDNA samples of 20 pooled embryos at 48 hpf, and one sample was available per replicate tank, thus 16 samples altogether. In consideration of the substantial costs and expenditures of real-time PCR, only three of the four replicates of each treatment were measured at first. The forth replicate samples were measured several months later. However, due to significant deviations of the Ct values and fold-changes from the other three replicates, the data were not included in the results.

Overall, all genes except for *npy1r*, *gnrhr1*, *gnrhr2*, *cyp19a1a* and *ihcgr* were found expressed in the embryos (Figure 8). Significant regulation was confirmed for *mvd* with up-regulation at 10 µg/L and 32 µg/L, for *vtg1* with down regulation at 10 µg/L, 32 µg/L and 100 µg/L and for *igf1* with up-regulation at 32 µg/L of fadrozole. Up-regulation in a similar fold-change range than *vtg1* was also found for *apoEb* (100 µg/L) and *sox9* (all concentrations) but in both cases the changes were non-significant. Down-regulation in the same fold-change range as *vtg1* was measured for *lss* and *sc4mol* (all concentrations), *kiss1ra* (32 µg/L and 100 µg/L), *esr2b* (10 µg/L) and *gnrhr4* (all concentration), but also here the data proved non-significant.

Figure 8: Gene expression changes in 48 hpf fadrozole exposed embryos, depicted as fold-change against controls



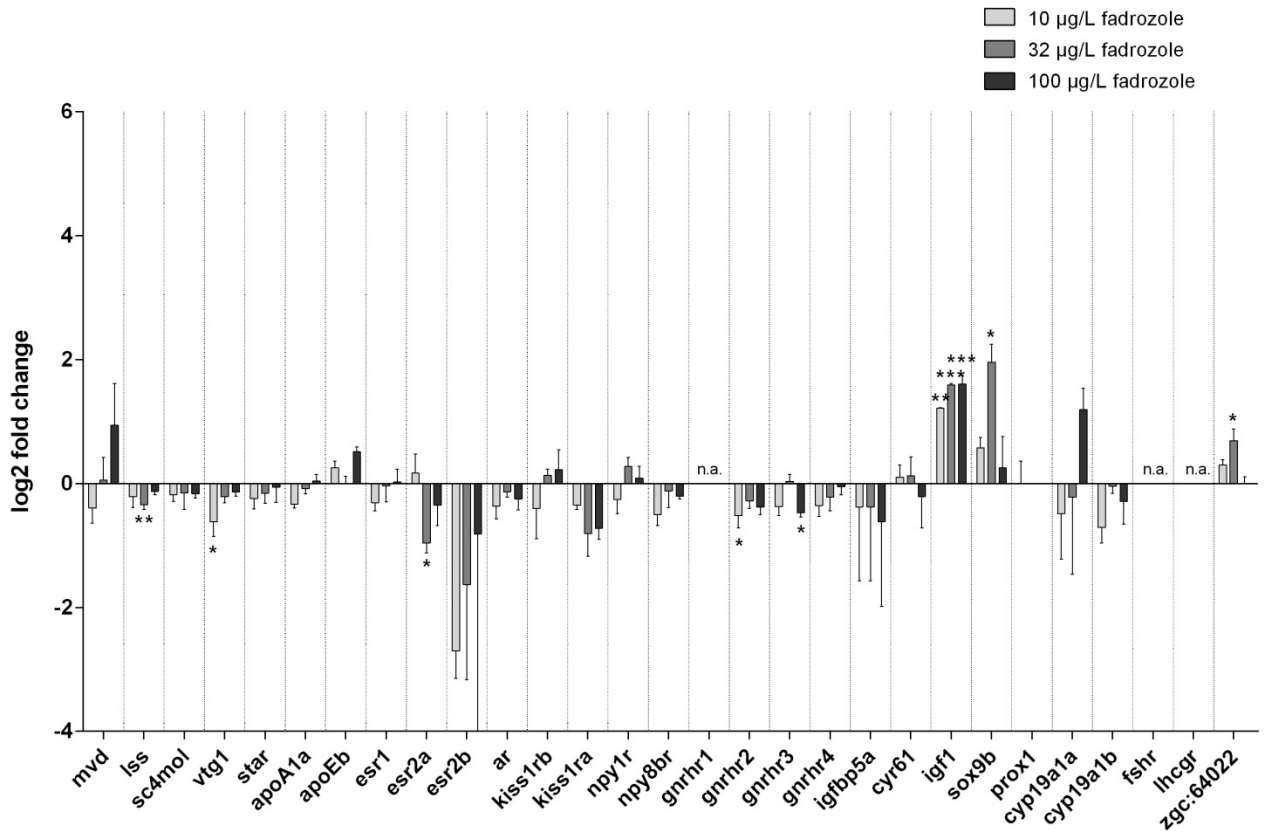
Changes in gene expression of fadrozole exposed 48 hpf zebrafish compared to the 48 hpf control fish, determined by quantitative real-time RT-PCR. Results are shown as bars ± SEM depicting the log2 fold change of the mRNA level in relation to the level of the control. For each gene, the relative mRNA amount was determined by the ΔΔCt Method (Livak and Schmittgen, 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values express the mRNA fold-change in relation to the relative mRNA amount of the corresponding controls. The 2'-ΔΔCt values were log2 transformed to show the fold-changes of the treatment groups. Each bar represents the mean of three replicate samples and each replicate sample consisted of 20 pooled embryos. Statistical evaluation was based on the geometric means of the 2'-ΔΔCt values (i.e. relative mRNA amounts before normalization to the controls) and was performed by ANOVA and unpaired t-tests to compare individual treatment groups to the controls. Significance values are indicated by asterisks* according to the significance levels: *p ‹ 0.05; **p ‹ 0.01; ***p ‹ 0.001. Genes with no bars showing were either not expressed (*npy1r, gnrhr 1*, *gnrhr2, cyp19a1a* and *ihcgr)* or the expression was not dissimilar to the controls (gnrhr3, igfbp5a and prox1).

5.4.2 Gene expression in 96 hpf larvae

The expression of the target genes was measured after hatch of the embryos at 96 hpf. Again, pooled samples (20 larvae per sample) with one sample per replicate tank were used for the analysis. For the same reasons as mentioned for the 48 hpf samples, only three replicates were analysed at first and the fourth replicate was measured at a later time point. The data are available and will be provided as a supplement to this report but were not included in the results. In general, the range of expression changes of the target genes compared to the controls similar to the 48 hpf but the profile was different (Figure 9). The largest fold changes in the range of ≥ 2and ≤ -2 were measured for *esr2b*, *igf 1* and *sox9b*, with *igf1* being significantly up-regulated at all fadrozole concentrations and *sox9b* at 32 µg/L. Also up-regulated was *zgc64022* at 32 µg/L. Significant

down-regulation was found for *lss* at 32 µg/L, *vtg1* and *gnrhr2* (both at 10 µg/L), *esr2a* (at 32 µg/L) and *gnrhr3* (100 µg/L), but the fold-changes remained ≤ -1. Besides, *kiss1ra, npy8br* and *cyp19a1b* showed a concentration independent inhibitory trend, whereas *mvd* and *cyp19a1a* indicated up regulation at 100 µg/L of fadrozole, although these changes were not significant. No expression was found for *gnrhr1*, *fshr* and *ihcgr*.

Figure 9: Gene expression changes in 96 hpf fadrozole exposed larvae, depicted as fold-change against controls



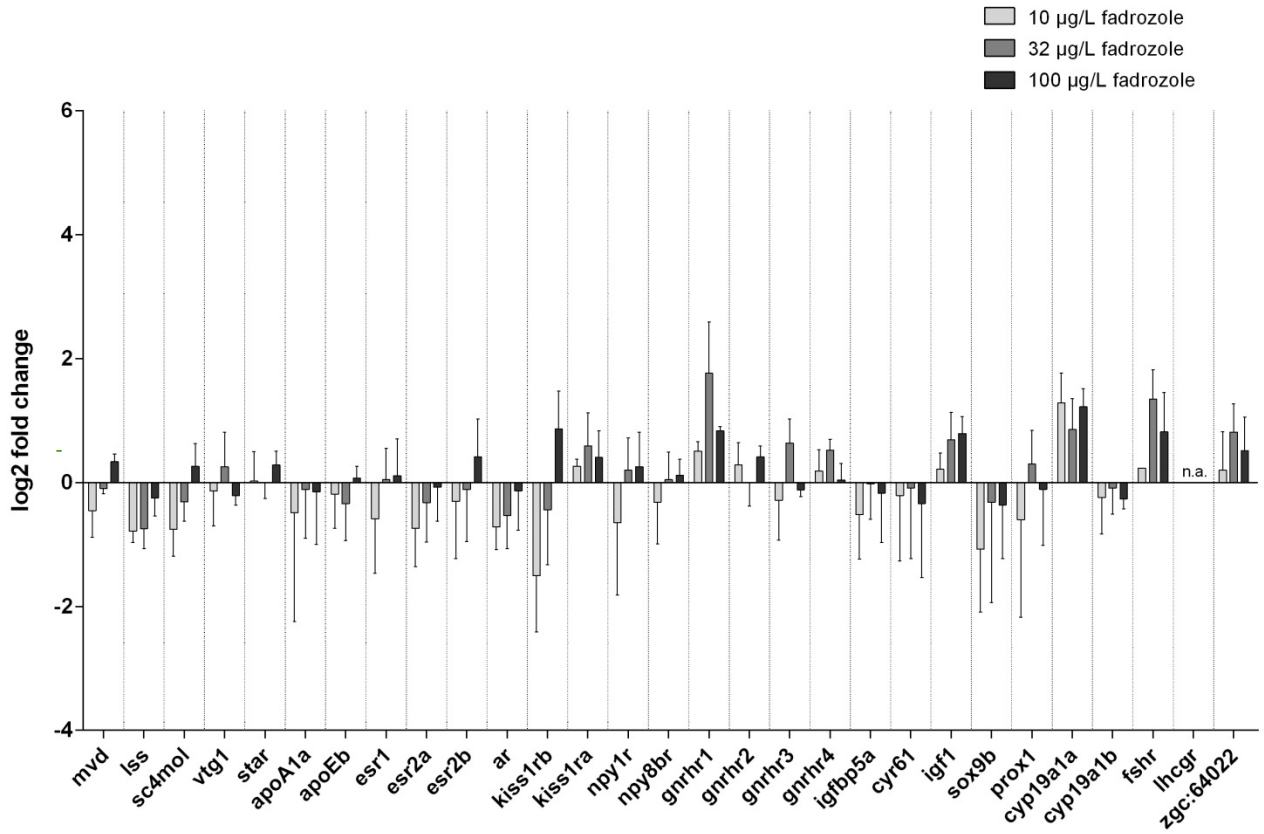
Changes in gene expression of fadrozole exposed 96 hpf zebrafish compared to the 96 hpf control fish, determined by quantitative real-time RT-PCR. Results are shown as bars ± SEM depicting the log2 fold change of the mRNA level in relation to the level of the control. For each gene, the relative mRNA amount was determined by the ΔΔCt Method (Livak and Schmittgen, 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values express the mRNA fold-change in relation to the relative mRNA amount of the corresponding controls. The 2'-ΔΔCt values were log2 transformed to show the fold-changes of the treatment groups. Each bar represents the mean of three replicate samples and each replicate sample consisted of 20 pooled larvae. Statistical evaluation was based on the geometric means of the 2'-ΔΔCt values (i.e. relative mRNA amounts before normalization to the controls) and was performed by ANOVA and unpaired t-tests to compare individual treatment groups to the controls. Significance values are indicated by asterisks* according to the significance levels: *p ‹ 0.05; **p ‹ 0.01; ***p ‹ 0.001. Genes with no bars showing were not expressed (*gnrhr1, fshr* and *ihcgr*).

5.4.3 Gene expression in 28 dpf juvenile fish

At this time point, four fish per treatment and replicate tank were pooled together as samples, resulting in 16 samples in total. Due to the same reasons mentioned for the 48 and 96 hpf samples, only three replicates were analysed at first and the fourth replicate was measured at a later time point. The data are available and will be provided as a supplement to this report but were not included in the results.

The juvenile fish at 28 dpf showed expression of all genes in a similar range of log2 fold-changes than the earlier developmental stages, in particular the 48 hpf (Figure 8). The most notable result was the lack of any significant regulation of all genes despite of log2 fold-changes of > -1.5 (*kiss1rb* at 10 µg/L) and 1.5 (*gnrhr1* at 32 µg/L). An up regulation trend was found for *kiss1ra*, *gnrhr1*, *igf1*, *cyp19a1a*, *fshr* and *zgc:64022*, with a concentration-dependency indicated for *igf1*. Fold changes ≤ -1 may suggest down-regulation, which was measured for *kiss1rb* and *sox9b* at 10 µg/L of fadrozole, but these changes were again non-significant. It should be noted that the samples of the replicate groups A of all treatments (including controls) showed deviating results from the other replicate groups, with notably higher Ct values for many genes including the house keeping genes. Therefore, the data of these samples negatively impacted on the error propagation of the ΔΔ Ct (ddCt) analysis, with obvious consequences for the SEM values and the statistical analysis. The cause of the deviation was investigated but no obvious evidence was found neither technical issue identified. The deviations also occurred in the controls, what excluded a potential exposure effect. The A-replicates samples neither differed in RNA quality nor amount, and the size of the fish in terms of body length was not different (see Table 20 of the Annex) from the other replicate groups. The only possible but speculative source of a technical error was the RT-reaction, which may have been less efficient in these samples lowering the concentration of template cDNA. Also possible was a calculation error for the RNA dilution prior to the RT, so that lower starting concentrations entered the RT.

Figure 10: Gene expression changes in 28 dpf fadrozole exposed fish, depicted as fold-change against controls



Changes in gene expression of fadrozole exposed 28 dpf zebrafish compared to the 28 dpf control fish, determined by quantitative real-time RT-PCR. Results are shown as bars ± SEM depicting the log2 fold change of the mRNA level in relation to the level of the control. For each gene, the relative mRNA amount was determined by the ΔΔCt Method (Livak and Schmittgen 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values express the mRNA fold-change in relation to the relative mRNA amount of the corresponding controls. The 2'-ΔΔCt values were log2 transformed to show the fold-

changes of the treatment groups. Each bar represents the mean of three replicate samples and each replicate sample consisted of 4 pooled embryos. Statistical evaluation was based on the geometric means of the 2'-ΔΔCt values (i.e. relative mRNA amounts before normalization to the controls) and was performed by ANOVA and unpaired t-tests to compare individual treatment groups to the controls. Significance values are indicated by asterisks* according to the significance levels: *p ‹ 0.05; **p ‹ 0.01; ***p ‹ 0.001. The gene *ihcgr* was not expressed.

5.4.4 Gene expression in 63 dpf pre-adult fish

The expression of the target genes was measured in trunk samples of individual fish of 63 dpf. Five fish per replicate and treatment were available, resulting in a total number of 80 trunk samples. For each treatment group, five fish of three replicates were measured. The analysis of the forth replicate was shelved due to time and budget constraints.

5.4.4.1 Discrimination of 63 dpf control fish into a low *vtg1* and a high *vtg1* groups

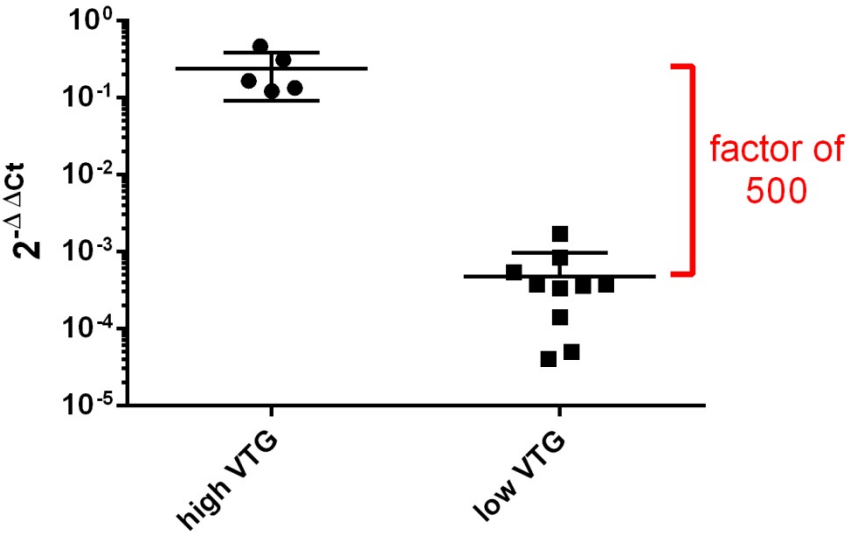
The 63 dpf fish dedicated to PCR analysis were not available for gonad morphology and histopathology since these analyses require complete gonadal tissue, which could not be sacrificed. Therefore, the morphological sex of the fish was unknown. During the analysis of the gene expression data, a divergence in the *vtg1* relative mRNA amounts of the control samples (after normalization to the reference genes) became apparent (Table 13; Figure 11) and a sex-dependent expression of *vtg1* was assumed. Consequently, the control fish were divided into a low and a high level *vtg1* group.

Table 13: Levels of *vtg1* expression (2'-ΔΔCt) in 63 dpf control fish normalised to the reference genes

2'-ΔΔCt	
high *vtg1*	low *vtg1*
0.13219	0.00037
0.30840	0.00014
0.16332	0.00033
0.12024	0.00004
0.46311	0.00084
	0.00054
	0.00170
	0.00005
	0.00036
	0.00037

The ratios of the target genes in the fadrozole treated fish were calculated relative to either the untreated controls with low *vtg1* levels (Figure 13 A) or with high *vtg1* levels (Figure 13 B). The differences in gene expression of the control fish exhibiting either low or high *vtg1* expression were analysed by comparing the mean relative expression changes of all other genes in these two control groups (Figure 12A) and by calculating the fold change differences of the high *vtg1* - versus the low fish (Figure 12B).

Figure 11: Expression variation of *vtg1* in 63 dpf control fish

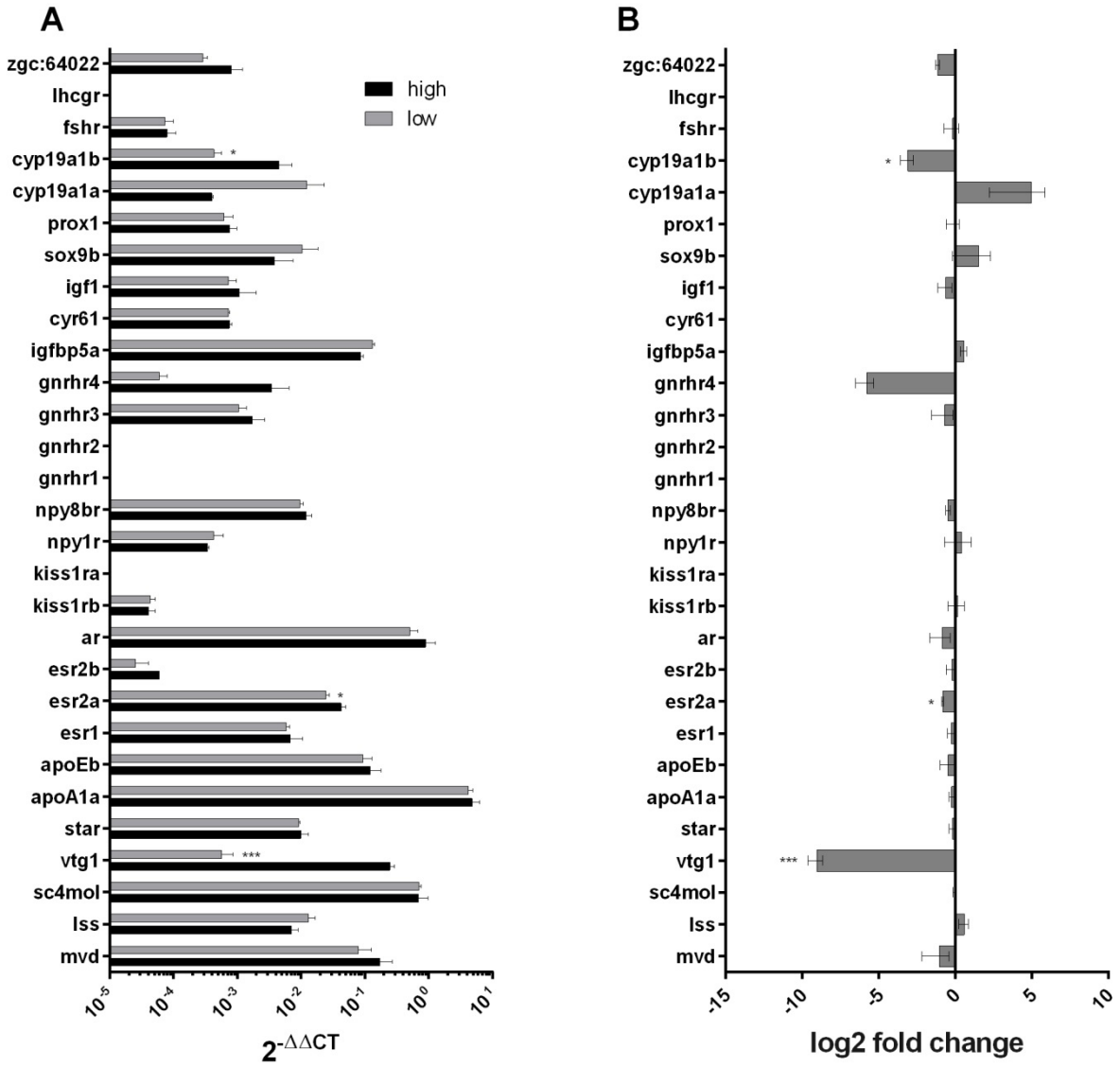


During data evaluation, a divergence in the relative *vtg1* amounts among 63 dpf pre-adult control fish was observed, which allowed discrimination between high and low *vtg1* "expressers" (representing presumable female respectively male fish).

5.4.4.2 Comparison of gene expression in low *vtg1* and high *vtg1* controls

The expression of the *vtg1* gene was highly variable in the control fish samples and the values clustered into two statistically different expression groups, one exhibiting changes in expression > 8-fold and the other one <1.0-fold. The data suggested that these two groups represent male (with low *vtg1* levels) and female (with high *vtg1* levels) fish. These two groups were analysed separately or differential expression of the other 28 target genes (see Figure 12A). Consequently, the log2 fold-changes for all other genes expressed in either low *vtg1* or high *vtg1* controls were calculated and compared separately within these groups (Figure 12B).

Figure 12: Differences in gene expression of unexposed 63 dpf control pre-adult fish: Comparison of relative mRNA amounts (2'-ΔΔCt values) of low *vtg1* against high *vtg1* controls (A); and fold change differences (log_2-transformed) between low *vtg1* and high *vtg1* controls (B)



Relative gene expression of 63 dpf control fish with either low or high *vtg1* levels (A) depicted as relative mRNA amounts (2'-ΔΔCt values ± SEM) of the low (grey bars) and high (black bars) *vtg1* controls in comparison. (B) shows the differences in expression between the low and the high *vtg1* control groups, depicted as the log2 fold change of the mRNA level of the low *vtg1* controls in relation to the level of the high *vtg1* controls. For each gene, the relative mRNA amount was determined by the 2'-ΔΔCt Method (Livak and Schmittgen 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values were log2 transformed to shown the fold-change differences of the low level *vtg1* fish versus the high level group. Each bar represents the mean of three replicate groups and each replicate group consisted of 5 individual fish (trunks), i.e. n=15. Five of the 15 control fish expressed *vtg1* at a high-level. Genes with no bars showing were not expressed *(kiss1ra, gnrhr1, gnrhr2* and *lhcgr*).

Apart from *vtg1*, the analysis revealed significant differences in the expression levels also for the genes *esr2a* and *cyp19a1b* (Figure 12A). This could similarly imply a sex-dependent expression of these two genes, which we were unable to confirm with the present data.

The largest differences in the log2 fold-change values of the low level *vtg1* versus the high level *vtg1* control group (Figure 12B) were found for *gnrhr4* and the two aromatase genes *cyp19a1a* and *cyp19a1b*.

5.4.4.3 Gene expression in fadrozole exposed 63 dpf pre-adult fish

Since the control fish divided into two groups, the low *vtg1* and the high level *vtg1* controls, the calculation of the fold-changes and thus, the normalization of the $2^{-\Delta\Delta Ct}$ values of the fadrozole treatments against the control were performed in two different ways:

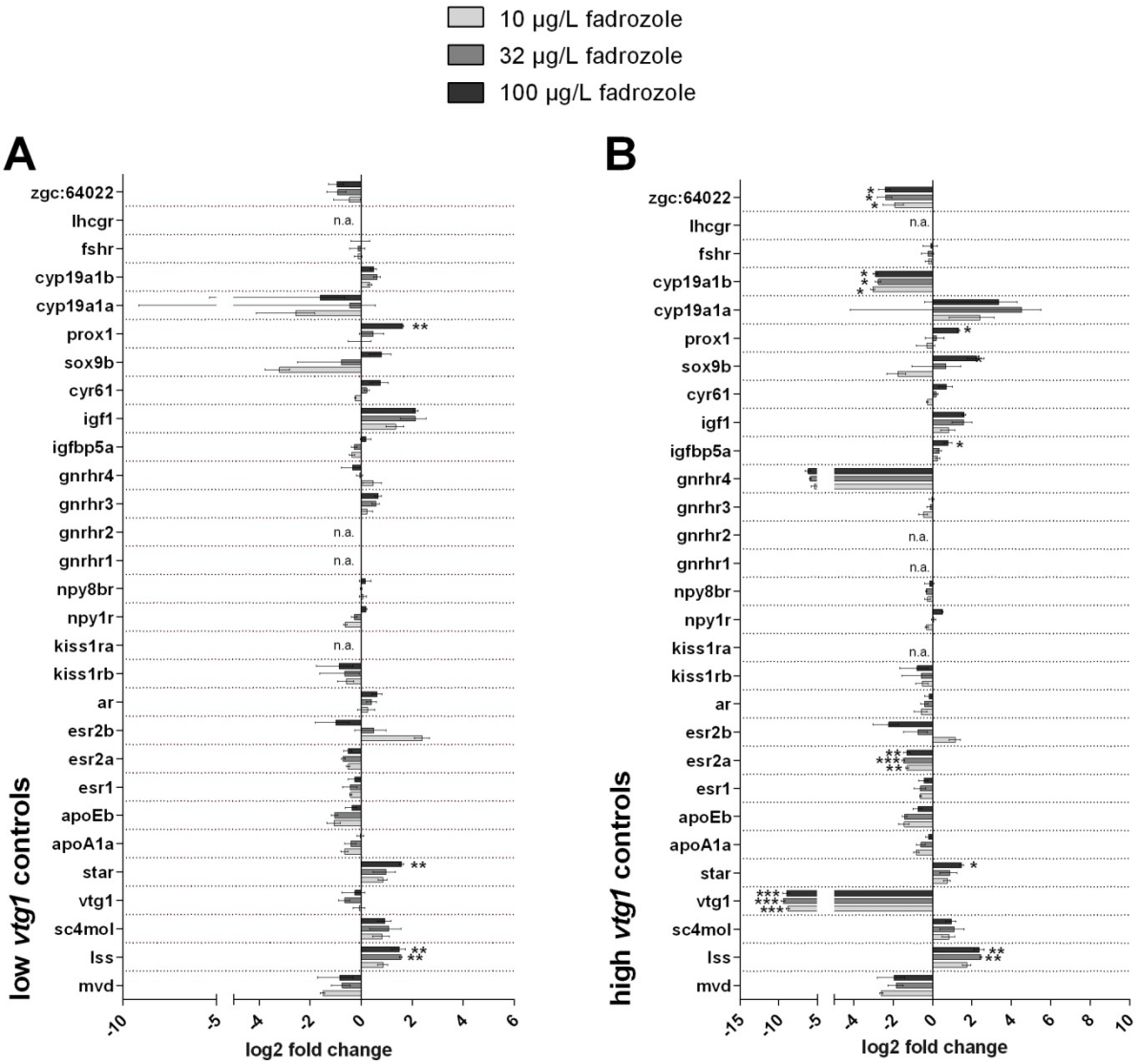
Fadrozole treatment group compared to low *vtg1* control group

Independent of the normalization, the log2-fold change range was wider than for the earlier developmental stages and down regulation values up to 10 (for *vtg1*) and up regulation of ≥ 4-fold (*cyp19a1a*) were reached. Significant fold-change differences to the low *vtg1* level control fish (n = 10) (Figure 13) were detected for the genes *lss*, *star* and *prox1*. The gene *lss* was up-regulated at 32 µg/l and 100 µg/L, *star* and *prox1*were up-regulated at the fadrozole concentration of 100 µg/L. The highest but non-significant changes > 2-fold was measured for *esr2b*, and < -2-fold for *sox9a* and *cyp19a1a* at 10 µg/L. A concentrations-dependent tendency to a fold-change increase was indicated for *ar*, *kiss1rb* (down), *gnrhr3*, *igf1* and *zgc: 64022* (down), and a concentration-dependent decrease trend for *mvd*, *apoA1a*, *apoEb* and *cyp19a1a* (all down). These trends remained statistically unconfirmed.

Fadrozole treatment group compared to high *vtg1* control group

Fold-change differences to the high *vtg1* level control fish (n = 5) showed up-regulation for *lss* at 32 µg/l and 100 µg/L and *star*, *igfbp5a* and *prox1* at 100 µg/L of fadrozole. Significant down regulation was measured for the genes *vtg1*, esr2a, *cyp19a1b* and *zgc:64022* at all exposure concentrations, and this change was highly significant for *vtg1*. A very strong (> 5-fold) but non-significant down regulation at all concentrations was also seen for *gnrhr4*, and also mvd showed up to 2-fold non-significant down regulation. A concentration-dependent up-regulation trend, which was again not significant, was found for *igf1* and *cyp19a1a* (reaching a mean fold-change of >4 at 32 µg/L). Down-regulation trends in an inverse concentration related manner were indicated for *apoA1*, *apoEb* and *ar*, and concentration-dependent tendency for *kiss1rb*.

Figure 13: Gene expression changes in 63 dpf fadrozole exposed pre-adult fish, depicted as fold-change against low *vtg1* controls (A) or high *vtg1* controls (B)



Changes in gene expression of fadrozole exposed 63 dpf zebrafish relative to control fish exhibiting either (A) low *vtg1* (n = 5) or (B) high *vtg1* expression (n = 10), determined by quantitative real-time RT-PCR. Results are shown as bars ± SEM depicting the log2 fold change of the mRNA level in relation to the level of the control. For each gene, the relative mRNA amount was determined by the ΔΔCt Method (Livak and Schmittgen 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values express the mRNA fold-change in relation to the relative mRNA amount of the corresponding controls. The 2'-ΔΔCt values were log2 transformed to shown the fold-changes of the treatment groups. Each bar represents the mean of three replicate groups and each replicate group consisted of 5 individual fish (trunks), i.e. n=15 (for the fadrozole treatment groups). Statistical evaluation was based on the geometric means of the 2'-ΔΔCt values (i.e. relative mRNA amounts before normalisation to the controls) and was performed by ANOVA and unpaired t-tests to compare individual treatment groups to the controls. Significance values are indicated by asterisks* according to the significance levels: *p ‹ 0.05; **p ‹ 0.01; ***p ‹ 0.001. Genes with no bars showing were not expressed *(kiss1ra, gnrhr1, gnrhr2* and *lhcgr*).

5.4.4.4 Changes in gene regulation over time

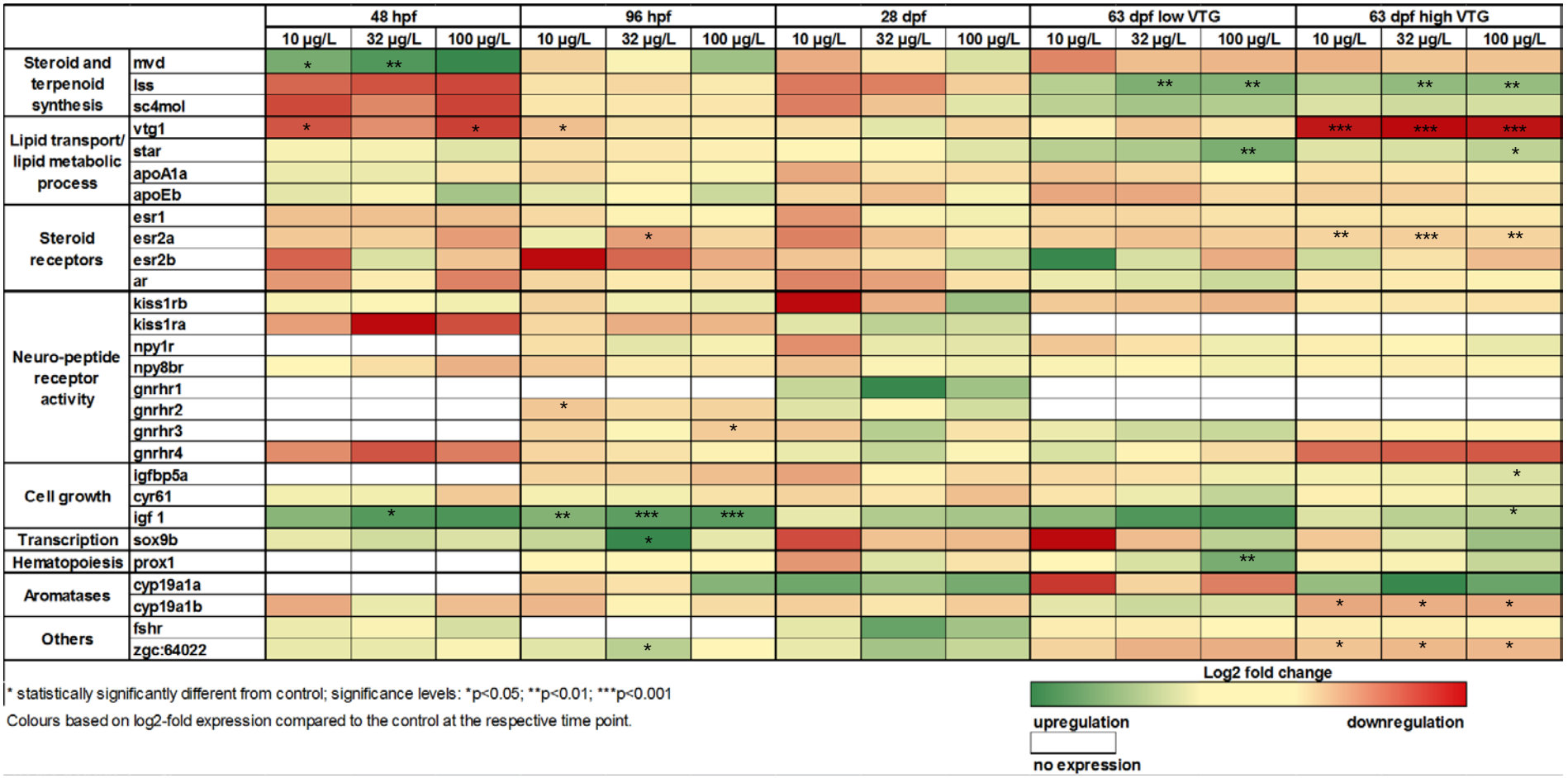
The qPCR analysis of the RNA samples obtained at the different time points revealed regulation of 17 out of the 29 target genes. Since these genes responded differently depending on the developmental stage, the changes in expression were evaluated over the 63d duration of the FSDT separately for each gene.

The colour coded heatmap-like overview table (Figure 14) summarises and illustrates the expression patterns of all 29 target genes across the different sampling time points. For better visualisation, up regulation of a gene was translated into shades of green and down regulation into shades red. White colour cells indicate that the gene was not expressed. Overall, the panel demonstrates that the pattern of gene regulation in the embryonic (48 hpf) and larval (96 hpf) stages differed from the later developmental stages. Altogether, 17 genes were regulated in at least one exposure group at any of the four time points, and seven of these 17 genes were found regulated in more than one exposure group at a given time-point. No gene was significantly regulated at every time points, and only *vtg1, star, igf1* and *zgc:64022* were significantly regulated at more than one time-point. A high number of genes regulated was found at 96 hpf, with four genes (*vtg1, esr2a, gnrhr2, gnrhr3*) being down and three genes (*igf1, sox9b, zgc:64022*) being upregulated and with an apparent exposure concentration dependent persistent up regulation for *igf1*. Significant up respectively down regulation at more than one exposure concentration both at 48 hpf and 96 hpf was found for *vtg1* and *mvd* and *igf1*. Notable was the decrease in the down regulation level of *lss* and *sc4mol* from 48 hpf to 96 hpf, which was not significant but persistent across all fadrozole concentrations. Also, *mvd* suggested opposite regulation at 48 hpf, where it was upregulated, versus 96 hpf, at least at 10 µg/l and 32 µg/L.

At 28 dpf gene regulation and expression levels showed no coherence with the earlier time points and little agreement with the 63 dpf time point. The only exception was the gene *igf1*, which was consistently upregulated at all time points (from 28 dpf on in a non-significant manner) across all t fadrozole exposure concentrations. No genes were found significantly regulated at 28 dpf.

Gene regulation at 63 dpf was different for several genes in the low vtg1 group compared to the high *vtg1*groups. The steroidogenesis related genes were in agreement as well as *star, esr2a, igf1, prox1*and *zgc:64022.* Significantly regulated in both groups were *lss* and *star*. In the low *vtg1*control group, *lss, star* and *prox1* were found significantly upregulated in at least one of the two highest fadrozole exposure concentrations. In the high *vtg1* group, significant down regulation in all exposure groups was determined for *vtg1* (highly significant), *esr2a, cyp19a1b* and *zgc:64022* whereas up regulation occurred only in the high fadrozole concentrations for *lss* and *star*.

Figure 14: Colour coded “heatmap” illustration of the expression of all target genes in fadrozole exposed fish at the different sampling time points



Heatmap-like illustration showing the comparative expression fold-changes versus the control fish of all target genes at the different time points, categorised and assigned to different colour shades. Colour and intensity of the shading of a cell reflect the mean log2 fold-change values of each gene at a certain sampling time point and of a certain treatment group, with red shades representing down regulation (negative log2 values) and green shades representing up regulation (positive log2 values); light yellow represents no regulation. White cells indicate that no expression of the corresponding gene was measured. For the 63 dpf time point, the up- or down regulation of genes is shown either in relation to the low *vtg1* control or the high *vtg1* controls. The columns of the table are labelled accordingly. Asterisks (*)in the cells denote levels of significance according to the legend below the table.

5.4.4.5 Identification of potential (bio-)marker genes

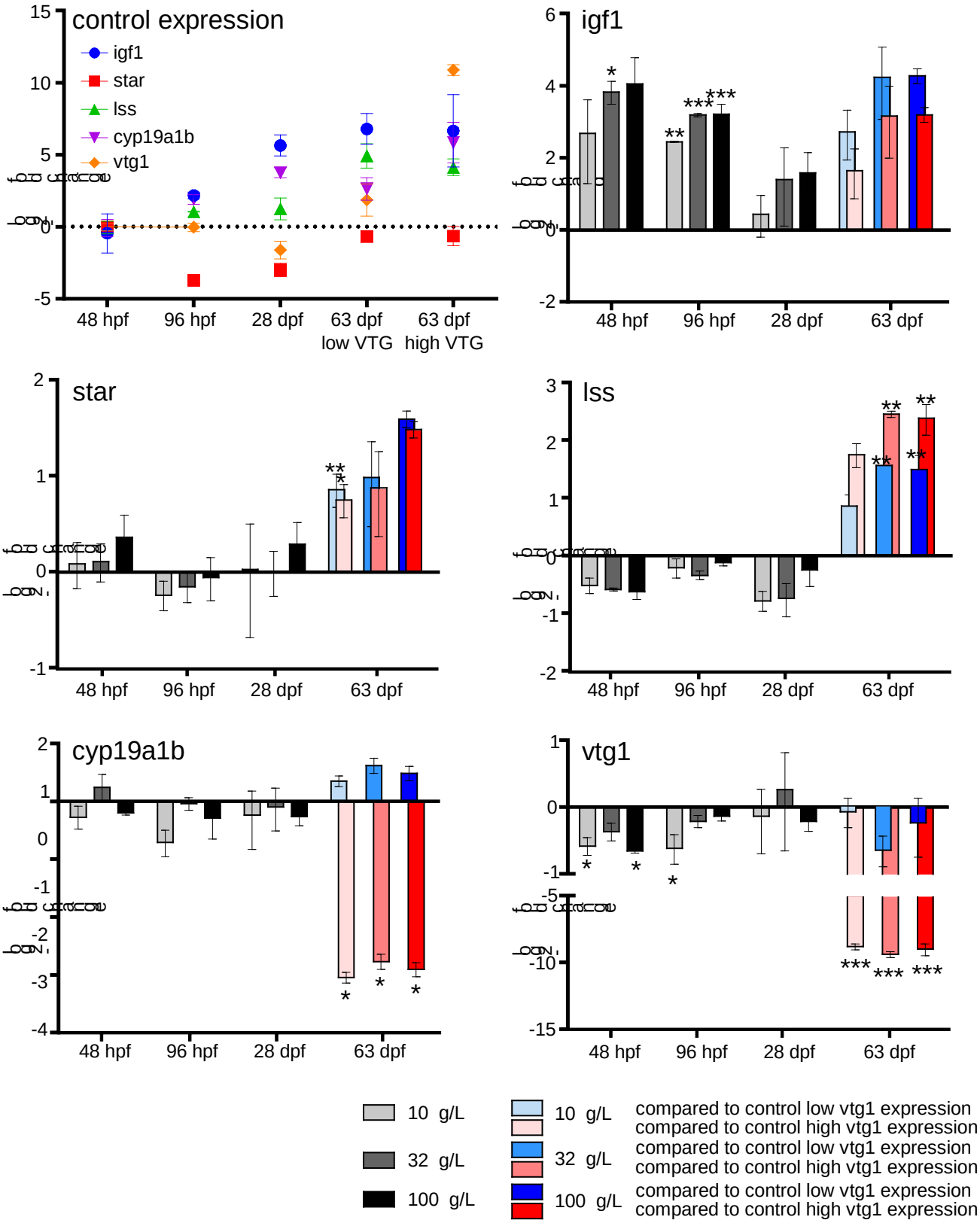
The strongest and most significant changes in gene expression in more than one fadrozole exposure concentration at least one time point were found for estrogen regulated genes *mvd, lss, vtg1, star, esr2a, igf1 cyp19a1b* and *zgc:64022*. These genes represent different biological functions related to steroidogenesis, lipid transport, cell growth and aromatase activity, and belong to regulative pathways known to be differently affected by endocrine disruptors. These genes would therefore be predestined for use as biomarker gene. Since *mvd* and *lss* represent the same pathways and *esr2a* and *zgc:64022* were comparatively weak in their expression levels at all time points, it was decided to focus on the five remaining genes *lss, vtg1, star, igf1* and *cyp19a1b* as the most likely potential (bio-) marker genes suitable for the detection and identification of aromatase inhibition in the context of the FSDT. Subsequently, the potential marker genes were analysed in more detail in terms of their individual patterns of expression over time at fadrozole exposed as well as unexposed control conditions (see Figure 15 and Table 14).

At unexposed control conditions (Figure 15, panel top left), *vtg1* and the steroidogenesis regulating gene *star* showed down regulation during early life and puberty and the expression only started to go up with sexual maturation at 63 dpf. The increase in gene expression was markedly weaker for *star* than *vtg1*, where the expression peaked in the presumed female high *vtg1* fish.

The genes *lss, igf1* and *cyp19a1b* showed a steady increase in expression during development all at similar rates. Differences in the expression level between the high *vtg1* and low *vtg1* controls at 63 dpf were apart from *vtg1*, only displayed by *cyp19a1b.*

Fadrozole exposure (Figure 15, all bar charts) affected only two of these five genes during early life, namely *vtg1* which responded by up regulation already at 48 hpf, and the growth factor *igf1* which was upregulated at 96 hpf. A different response was seen for the steroidogenic genes *star* and *lss* and the aromatase gene *cyp19a1b*, since their response only occurred at 63 dpf, specifying the stimulation of steroid hormone biosynthesis and inhibition of the brain-specific aromatase *cyp19a1b* due to fadrozole exposure as a delayed response. Notably, *cyp19a1b* was down regulated in the trunks of the 63 dpf exposed fish only in relation to high *vtg1* controls (presumable females). Strongly down regulated in relation to high *vtg1* controls at the 63 dpf time point was also the estrogenic biomarker *vtg1*.

Figure 15: Expression profiles of the potential (bio-) marker genes



The panel top left shows the time course of gene expression change of *vtg1, cyp19a1b, lss, star* and *igf1* in unexposed control zebrafish in relation to the expression level at 48 hpf, which was set to zero. The other panels of the figure depict the meanlog2 fold-changes as bar charts for *vtg1, cyp19a1b, lss, star* and *igf1* in fadrozole exposed fish at the different time points in the s in relation to the respective controls. At 63 dpf, the log2 fold-changes were calculated in relation to either f low *vtg1* controls (n = 10; blue bars) or high *vtg1* controls(n = 5; red bars). For each gene, the relative mRNA amount was determined by the ΔΔCt Method (Livak and

Schmittgen 2001) and normalised to two reference genes according to Vandesompele et al. (2002). The expression of the reference genes *18s* (18S ribosomal RNA) and *rpl8* (ribosomal protein L8) were used for normalization. The 2'-ΔΔCt values were log2 transformed to show the fold-changes of the treatment groups. Each bar represents the mean of three replicate groups and each replicate group consisted of either of one samples of 20 pooled embryos (48 hpf and 96 hpf) or of 4 pooled fish (28 dpf) or 5 individual fish (trunks), thus n=15 for each fadrozole treatment group (63 dpf). Statistical evaluation was based on the geometric means of the 2'-ΔΔCt values (i.e. relative mRNA amounts before normalization to the controls) and was performed by ANOVA and unpaired t-tests to compare individual treatment groups to the controls. Significance values are indicated by asterisks* according to the significance levels: *p ‹ 0.05; **p ‹ 0.01; ***p ‹ 0.001

Table 14: Summary of the relative gene expression values (mean 2'-ΔΔCt; relative to reference gene expression; fold changes ± SEM relative to control expression) of the selected (bio-) marker genes

Gene & time-point	Nominal fadrozole concentration (µg/L)											
	Control			10			32			100		
	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM
igf1												
48 hpf	0.000006	1.000	0.561	0.000014	2.529	0.968	0.000021	3.764	0.411	0.000023	3.833	1.174
96 hpf	0.000026	1.000	0.092	0.000060	2.331	0.010	0.000077	3.013	0.046	0.000078	3.044	0.308
28 dpf	0.000309	1.000	0.280	0.000358	1.160	0.231	0.000500	1.617	0.581	0.000535	1.732	0.365
63 dpf *vtg1* low	0.000734	1.000	0.330	0.001883	2.564	0.603	0.003191	4.344	1.452	0.003229	4.396	0.306
63 dpf *vtg1* high	0.001068	1.000	0.842	0.001883	1.763	0.414	0.003191	2.988	0.999	0.003229	3.024	0.210
star												
48 hpf	0.014660	1.000	0.121	0.015538	1.059	0.174	0.015758	1.075	0.146	0.018800	1.282	0.221
96 hpf	0.001132	1.000	0.088	0.000954	0.844	0.090	0.001015	0.897	0.096	0.001088	0.959	0.147
28 dpf	0.001903	1.000	0.196	0.001933	1.015	0.395	0.001900	0.998	0.161	0.002317	1.217	0.206
63 dpf *vtg1* low	0.009216	1.000	0.043	0.016650	1.807	0.215	0.018180	1.973	0.585	0.027700	3.005	0.177
63 dpf *vtg1* high	0.009925	1.000	0.316	0.016650	1.677	0.200	0.018180	1.832	0.543	0.027700	2.790	0.165
lss												
48 hpf	0.000389	1.000	0.172	0.000271	0.697	0.065	0.000259	0.664	0.011	0.000251	0.645	0.055
96 hpf	0.000805	1.000	0.011	0.000696	0.863	0.099	0.000635	0.788	0.040	0.000737	0.917	0.035
28 dpf	0.001005	1.000	0.296	0.000584	0.580	0.068	0.000600	0.596	0.118	0.000846	0.842	0.154
63 dpf *vtg1* low	0.013120	1.000	0.277	0.023790	1.812	0.256	0.038770	2.954	0.114	0.036890	2.811	0.518
63 dpf *vtg1* high	0.007087	1.000	0.276	0.023790	3.357	0.475	0.038770	5.470	0.211	0.036890	5.206	0.960
cyp19a1b												
48 hpf	0.000063	1.000	0.258	0.000052	0.828	0.113	0.000075	1.185	0.192	0.000055	0.872	0.024
96 hpf	0.000240	1.000	0.125	0.000147	0.611	0.097	0.000233	0.972	0.073	0.000198	0.819	0.183
28 dpf	0.000866	1.000	0.130	0.000734	0.847	0.285	0.000812	0.938	0.235	0.000722	0.834	0.087
63 dpf *vtg1* low	0.000431	1.000	0.305	0.000549	1.274	0.082	0.000664	1.541	0.139	0.000604	1.401	0.120
63 dpf *vtg1* high	0.004530	1.000	0.601	0.000549	0.121	0.007	0.000664	0.146	0.013	0.000604	0.133	0.011

Gene & time-point	Nominal fadrozole concentration (µg/L)											
	Control			10			32			100		
	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM	Mean 2´-ΔΔCt	Fold change	SEM
vtg1												
48 hpf	0.000128	1.000	0.097	0.000086	0.667	0.061	0.000100	0.774	0.071	0.000081	0.633	0.013
96 hpf	0.000128	1.000	0.120	0.000083	0.652	0.099	0.000110	0.862	0.054	0.000116	0.908	0.041
28 dpf	0.000045	1.000	0.223	0.000041	0.909	0.293	0.000054	1.196	0.561	0.000039	0.862	0.085
63 dpf *vtg1* low	0.000575	1.000	0.476	0.000548	0.953	0.146	0.000367	0.639	0.099	0.000487	0.847	0.252
63 dpf *vtg1* high	0.246500	1.000	0.183	0.000548	0.002	0.001	0.000367	0.001	0.001	0.000487	0.002	0.001

6 Discussion

6.1 Aim of the study and study design considerations

In this study, an FSDT (OECD TG 234) with the aromatase inhibitor fadrozole was performed using zebrafish (*Danio rerio*) as test species. The study aimed to investigate the value of additional molecular endpoints, like gene expression, for the identification of endocrine disrupting chemicals (EDCs) and potentially also the mode of endocrine action, early prior to the manifestation of adverse effects. In addition to the endpoints required by the OECD FSDT test guideline, the expression of 29 genes was analysed, which were known from previous studies to be affected by aromatase inhibition in zebrafish. The hypothesis was that (bio-) markers at gene expression level can specify molecular initiating events which lead to adverse effects defined as endocrine disruption. With such gene (bio-) markers, the establishment of links between molecular event caused by exposure to toxicants, and resulting adverse effect at apical level would be facilitated. The knowledge of the molecular initiating events and the mode of action as well as how they relate to a particular adverse outcome is important to predict, endocrine disruption effects of unknown toxicants at an earlier and more reliably than with an apical test in combination with physiological endpoints (like the FSDT) alone. Within the context of the adverse outcome pathway (AOP) paradigm (Ankley et al. 2010), the study demonstrated that a FSDT including molecular mechanistic endpoints such as gene expression could be instrumental in defining new AOPs or in the refinement of existing AOPs related to endocrine disruption.

An AOP for aromatase inhibition in adult female fish has already been established (https://aopkb.org/aopwiki/index.php/Aop:25) and hence, aromatase inhibition can be considered a good example for a proof of concept. However, the selective non-steroidal aromatase inhibitor fadrozole was originally chosen for the study because its endocrine disrupting effects were already reasonably well defined for fish due to numerous published studies and because the knowledge base about the substance and the effects on gene expression gained from in-house zebrafish studies was sound. Based on the knowledge of adverse effect (in particular sex ratio and gonad morphology) and gene expression effect threshold concentrations of fadrozole, suitable exposure concentrations were selected (i.e., 10 µg/L, 32 µg/L, and 100 µg/L), taking the prevention of systemic toxicity effects in the ELS fish into account. The time points chosen for the analysis of gene expression defined key developmental steps, i.e. embryonic development and organogenesis (48 hpf), post-hatch larval development and onset of swim bladder inflation (96 hpf), onset of puberty (28 dpf) and end of sexual development and gonad differentiation (63 dpf). The availability of large scale gene expression data for EDC exposed 48 hpf zebrafish embryos favoured the 48 hpf time point, and the 96 hpf corresponds to the duration of the fish embryo toxicity test FET according to the OECD TG 236.

6.2 Fadrozole effect on apical endpoints

Fadrozole significantly reduced survival during the early life stage until 28 dpf from 81% in the controls to 71% and 68% respectively, at 32 and 100 µg/L. This observation suggested low systemic toxicity, reflecting an elevated stress susceptibility known for larval stages of zebrafish. During the early life stage, zebrafish are especially sensitive to chemical and physical stimuli (Belanger et al., 2010; Diekmann and Nagel, 2004; Korwin-Kossakowski, 2008; Woltering, 1984), as the metabolism changes from intrinsic absorption of yolk nutrients to external feeding, which often results in an increased mortality also under control conditions. This assumption was supported by observations that zebrafish embryos (rather than larval zebrafish) exposed to even higher fadrozole concentrations did not display increased mortality (concentration range of 0.1 – 1 mg fadrozole/L; unpublished results). Nevertheless, the increase in mortality due to fadrozole treatments was statistically significant and systemic toxicity cannot be ruled out. Systemic toxicity can be described as an adverse effect on the body in general rather than in an endocrine-specific manner, and test concentrations resulting in such systemic toxicity (e.g. mortality) might also influence endpoints known as endocrine indicators in a non-endocrine manner (Wheeler et al., 2013). Therefore, it is advised to choose test concentrations in a range between baseline effects and systemic toxicity in order to reduce false-positive results for endocrine disruption (Ankley and Jensen, 2014; Wheeler et al., 2013). Even though the chosen test concentrations

of the current study obviously exceeded the recommended range slightly, the results obtained for the apical endocrine endpoints sex ratio and gonad differentiation argue that they were most likely caused by the specific aromatase inhibiting mode of action of fadrozole. It was therefore deemed unlikely that the observed effects on gonadal histopathology, sex ratio, vitellogenin, and also on expression of genes of the HPG axis in the surviving fish were influenced by systemic toxicity.

The interpretation of results of other endpoints, which are not directly linked to endocrine disruption, was not self-evident. For example, the weight of fish at test termination indicated a slight elevation compared to the controls (Table 8), although statistically non-significant. It can be speculated whether this observation is relevant and related to endocrine specific effects due to fadrozole. Gonad histopathology revealed that the gonads of the treated fish were further developed than of the controls (compare section 5.3.4), which suggested a generally more progressed male developmental stage of the treated male fish. For treated female fish no conclusion can be drawn, as very few females were found in treated condition. As the developmental stage is related to growth, this growth promotion would have also increased the weight of fish. Accordingly, Liu et al. (2014) describe a delayed maturation stage of the gonads of zebrafish exposed to the triazole fungicide triadimefon during a full-life cycle study, associated with a decreased weight for.

6.3 Fadrozole influences the sexual development of zebrafish and alters the sex ratio

Fadrozole exposure had a major effect on sexual development, shifting the gonadal sex ratio completely towards males already from the lowest treatment concentration (compare 5.3.3). This gonadal shift can be attributed to the specific aromatase inhibitor MOA of fadrozole. Fadrozole is a pharmaceutical originally designed to competitively inhibit the cytochrome P450 aromatase (CYP19) enzyme and hence the synthesis of estrogen for the treatment of post-menopausal breast cancer.(Brodie, 1994; Miller, 1997). Meanwhile, many studies have demonstrated that it inhibits CYP19 aromatase and concordantly estrogen biosynthesis, in a diverse range of animals, including mammals, birds, and also fish (Afonso et al., 2000, 1999; Elbrecht and Smith, 1992; Schieweck et al., 1988; Steele et al., 1987). In vertebrates, the CYP19 is the key enzyme for the conversion of C19-androgens to C18-estrogens (Callard et al., 1978), and therefore an important modulator of sex steroid hormone concentrations. The unequivocal evidence of the strong effect of fadrozole on the reproduction success of small fish species together with its specific MOA on the aromatase enzyme are the reason why fadrozole has been used as a reference compound in many EDC fish studies. The examination of aromatase inhibition in the context of EDC testing strategies was broadly explored by Ankley et al. (2002).

Zebrafish are particularly sensitive to aromatase modulations during gonadal sex differentiation, as they are undifferentiated gonochorist which develop via a stage described as non-functional protogyne gonad (Maack and Segner, 2003). The transformation of the undifferentiated “bi-potential” stage of the gonad (Siegfried and Nüsslein-Volhard, 2008) into either functional ovary or testis is easily influenced by an imbalance between estrogens and androgens e.g., caused by endocrine disruptors. Inhibition of aromatase causes testosterone and 11-keto testosterone levels to increase and promotes the maturation of the gonads into testes and thus, the development of a male phenotype, as e.g., shown by Fenske and Segner (2004). In the present study, already 10 µg fadrozole/L was potent enough to induce a 99% masculinisation, confirming the potency of fadrozole as an aromatase inhibitor.

The histopathological results verified the masculinizing effect of fadrozole. It also demonstrated as a more rapid development of the gonads. The average maturity index of the male gonads increased gradually from just over 2 in the control groups to approximately 3.7 at the highest treatment concentration (compare Fig. 5). This effect on the maturity index was described by Baumann et al. (2013) for other masculinizing substances. The increased male maturity index after fadrozole treatments underpins our assumption that the aromatase inhibition induced elevated levels of androgen in the fish. Further evidence strengthening this assumption is provided e.g., by Seki et al. (2006), who reported elevated gonadosomatic indices in zebrafish after exposure to 17ß-trenbolone (a strong androgen). The same stimulating effect on testis maturation was also observed by Morthorst et al. (2010), who exposed zebrafish to 17ß-trenbolone for 60 days. Inhibition of

the aromatase enzyme leads to an accumulation of unconverted androgens. Additionally, the synthesis of androgens may be stimulated via a positive feedback loop induced by low-levels of estrogens, resulting in even higher testosterone levels. Such feedback loop control mechanisms of steroidogenesis have been described and discussed for fish (Ankley and Villeneuve, 2015; Villeneuve et al., 2013, 2009).

In the present study, the maturity index of females was determined for the controls only, since no females, except for one found in the lowest treatment concentration, were identified in the fadrozole treatments. The gonad of this female displayed an undeveloped stage, corresponding to a zero maturity index. This would be anticipated for females treated with fadrozole. However, since zero maturity indices were also assigned to several females of the control condition, a generally low developmental stage of the test fish was assumed (compare Baumann et al. (2013) for normal maturity indices at this age). Therefore, the maturity index of the remaining female did not allow any conclusions regarding the effects of the aromatase inhibition.

Regarding the VTG plasma levels determined by ELISA, we were unable to show a fadrozole related reduction in the VTG concentrations of exposed 63 dpf fish. The lack of females in the fadrozole treatment conditions disallowed a comparison of VTG concentrations of control and fadrozole exposed females. On the other hand, the VTG concentrations of control males were already close or below the detection limit which made the determination of a further decrease in VTG in exposed males technically impossible. This shortcoming of the study reflected the minor error in the planning of the study in terms of the grading and range of the fadrozole concentrations, which were chosen too high. A reduction of the VTG plasma level in fish exposed to fadrozole could therefore be demonstrated in other studies (Ankley et al., 2002).

6.4 Fadrozole alters the expression of genes involved in steroidogenesis and estrogen-mediated signalling

Expression of 29 genes (see list in Table 4) was measured in 48 hpf and 96 hpf embryos and larvae, in 28 dpf juvenile and 63 dpf pre-adult fish using quantitative RT-PCR. The aim of this investigation was to study the fadrozole induced regulation of these genes in order to evaluate their potential value as early indicative (bio-) markers. The selected genes are involved in key regulatory events of the HPG signalling, during steroidogenesis, steroid signalling, and signalling responses

We identified five of the 29 genes as promising candidates for early biomarkers. The genes *igf1*, *star*, *lss*, *cyp19a1b*, and *vtg1* showed expression changes which demonstrated disruption of steroid biosynthesis at several stages along the pathway (compare Figure 15). Gene expression responses to fadrozole were not restricted to the above mentioned genes but the selected genes displayed strong and highly significant regulation at several time points. Furthermore, *cyp19a1b* and *vtg1* were selected since their expression is estrogen-dependent due to an ERE in their promoter region. Therefore, an early response of these two genes was considered likely, even though this was only verified for *vtg1*. However, *cyp19a1b* proved an important late maker gene, which could show a sex dimorphic inhibitory effect of fadrozole also on the coding gene. Several genes *(lss, mvd, sc4mol*) involved in steroid/terpenoid synthesis displayed significant regulation due to fadrozole exposure, but it was decided to only include *lss* as a representative of this function. However, in terms of an application of the identified gene biomarker in the context of a fish embryo based screening test, what will be discussed at a later point, the replacement of *lss* by *mvd* would be recommended since *mvd* responded early at 48 hpf whereas *lss* only at the end of the test at 63 dpf.

Following, gene expression upon fadrozole treatment at the individual time points will be discussed in more detail.

6.4.1 Gene expression differences at key steps of zebrafish development and sexual maturation caused by aromatase inhibition

Ankley and Villeneuve (2015) considered the time point at which gene expression analysis takes place as equally important as the dose to which the fish was exposed. Since immediate molecular responses upon endocrine disruption are rapid, they can be detected already after a few hours or days. Ankley and Villeneuve

(2015) furthermore postulated that low concentrations exposure responses can recover to control level or overcompensate in the opposite direction via compensatory feedback mechanisms. Higher concentrations instead result in a more sustained response and only recover slowly to control levels. Taken these assumptions into consideration, it seems likely that a number of genes reacted in a non-monotonous manner over time and with no clear concentration-response relationship. Direct effects with only low compensatory influence most likely occurred at 48 hpf and 96 hpf.

An immediate effect of fadrozole on steroid synthesis was seen by the up regulation of *mvd* at 48 hpf, a gene related to isoprenoid synthesis. In coherence with the overcompensation hypothesis postulated by Ankley and Villeneuve (2015), we found a significant regulation at the two lower concentration levels, while treatment with 100 µg/L fadrozole was not significantly different from control. Furthermore, expression returned to control levels already at 96 hpf, which might suggest compensatory feedback mechanisms regulating steroid concentrations.

6.4.1.1 Gene expression differences at 48 hpf (embryos)

In total, three genes, *mvd*, *igf1* and *vtg1*, were significantly regulated at 48 hpf. In accordance with the mode of action of fadrozole to inhibit estrogen synthesis, the inhibition of estrogen-responsive genes like *vtg1* was anticipated and the immediate response of *vtg1* to endocrine disrupters at 48 hpf has been shown by our laboratory before (Schiller et al. 2014). The expression of the gene encoding the egg yolk precursor protein vitellogenin was down regulated upon fadrozole treatment of 10 µg/L and 100 µg/L, reproducing the previously demonstrated exposure concentration independent response (Schiller et al. 2013). *Igf1* was upregulated at 32 µg/L fadrozole at 48 hpf, indicating an effect of aromatase inhibition on cell growth. Igf1 is related to the growth hormone (GH)/ insulin-like growth factor (IGF) axis and is essential for normal growth and development in vertebrates. However, it is also postulated that *igf1* expression is influenced by estrogen-receptor activity (Hewitt et al., 2010, a study performed in mouse), and the expression of *igf1* is known to be essential for normal gonadal function in fish (Brown et al., 2011; Reinecke, 2010). Furthermore, it was shown that Igf1 stimulated aromatase activity and also *cyp19a1* gene expression in red sea bream (Kagawa et al., 2003). This may explain the up regulation in the present study as a compensatory mechanism to the aromatase inhibition of fadrozole. A study performed with brown trout (*Salmo trutta fario*) (Marca Pereira et al., 2011) demonstrated that *igf1* was upregulated also upon treatment with prochloraz, an imidazole fungicide known to act as aromatase inhibitor.

6.4.1.2 Gene expression differences at 96 hpf (early larvae)

At 96 hpf, we observed again regulation of *vtg1* and *igf1*, substantiating their key role during signalling pathways affected by aromatase inhibition. This is especially interesting for *igf1*, which function is not restricted to steroidogenesis and which is not directly estrogen regulated like *vtg1*.

Besides, we observed down regulation of the estrogen receptor *esr2a* at 32 µg fadrozole/ml and additionally a tendency for down regulation of *esr2b.* This observation hinted at a regulation of the steroid receptors by aromatase inhibition at this stage of larval development, which was vaguely indicated already at 48 hpf. Aromatase inhibition alters levels of the steroids estrogen, testosterone and 11-keto-testosterone, which suggests a link to steroid receptor expression regulation. Such a link was described by other studies, e.g. by Caspillo et al. (2014), who demonstrated an effect of EE2 on steroid receptor expression in male zebrafish in a non-monotonous manner, or by Overturf et al. (2014), who demonstrated effects of the progestin levonorgestrel on steroid receptor mRNA expression during an early life stage test with fathead minnow (FHM) as well as alteration of steroid levels in sexually mature female FHM.

The effect on the estrogen receptor genes faded at 28 dpf presumably due to compensatory mechanisms. Interestingly, *esr2a* was significantly down regulated at 63 dpf and at all exposure concentrations in relation to the high *vtg1* controls.

We also observed significant differences in transcription of genes involved in the HPG axis, namely *gnrhr2* and *gnrhr3*, at 96 hpf. Both genes were found down regulated, *gnrhr2* at 10 µg fadrozole/L, and *gnrhr3*

at100 µg fadrozole/L. Gonadotropin-releasing hormones and their receptors are expressed in zebrafish as early as 1 dpf. Regulation of the *gnrhr* genes by EDCs was previously shown by us and others (Dang et al., 2015; Schiller et al., 2014, 2013b). However, no concentration-dependent response relationship and no regulation of *gnrhrs* at other time points were observed in our study or elsewhere. This suggests that genes of the gonadotropins as well as the corresponding receptors are not massively influenced by aromatase inhibition and therefore have little value as biomarkers.

Two further genes were found regulated at 96 hpf, namely the transcription factor *sox9b* and the ras-like estrogen-regulated growth inhibitor *zgc:64022*, which were both upregulated at the medium test level of 32 µg fadrozole/L. These genes were chosen based on observations by Villeneuve et al. (2009) who demonstrated up regulation of these genes upon fadrozole treatment of adult zebrafish. Our study confirmed these results already as early as 96 hpf. Transcription of *zgc:64022* was also regulated at 63 dpf, as described later.

6.4.1.3 Gene expression differences at 28 dpf (juvenile fish)

We found no significantly regulated genes at this time point (compare Figure 14). Genes related to neuropeptide receptor activity, i.e. *kiss1rb* and *npy1r*, indicated stimulation at 32 µg fadrozole/L. For kisspeptin (Kiss1) and the Kiss1 receptor, a central role in the endocrine control of reproduction in vertebrates is described. It is demonstrated that Kiss1-Kiss1R regulates HPG axis signalling and puberty (Ohga et al., 2015; Onuma and Duan, 2012), and its expression is influenced by steroids (Wang et al., 2013).

One possible reason for the lack of effects on gene expression at this time point discussed was the pooling of several fish. The 28 dpf time point coincides with the early phase of sexual and gonadal differentiation of zebrafish (Maack and Segner 2004, Baumann et al. 2013) and it can thus be assumed that these fish had already entered this phase (although this was not studied histopathologically or genetically in this study). Consequently, the onset of sexual differentiation in these fish might have also impacted the expression of sexual dimorphic genes. This effect became more evident at the 63 dpf time point (section 5.4.4). Based on this assumption it was hypothesised that the variation in the gene expression levels in the controls and the fadrozole treatments was higher at the 28 dpf than the earlier time points, which led to a statistically non-significant result. The height of the error bars of Figure 10 substantiates this hypothesis. The measurement of gene expression in individual fish would have resolved the high variation, but the pooling of four fish instead most likely obscured individual effect on gene expression. The n-number (number of replicates), on the other hand, was too low to reduce the variation based on an average value.

6.4.1.4 Gene expression differences at 63 dpf (pre-adult fish)

The time point with the strongest informative value was 63 dpf. No sex determination was performed on these fish prior to PCR analysis but it was possible to discriminate individuals with high *vtg1*expression levels from individuals with low *vtg1* levels in control condition. Firstly, this apparent sex-dimorphic expression of *vtg1* was used to differentiate suspected young male from female zebrafish towards the end of sex differentiation. Secondly, we were able to compare fadrozole-induced differences in genotypic and phenotypic males.

Significant differences in gene expression between controls with high *vtg1* compared to controls with low *vtg1* mRNA levels were observed for *vtg1, esr2a*, and *cyp19a1b*. However, down regulation of *esr2a* in low *vtg1* expressers was in a low log2-fold range, and significance resulted from low inter-replicate differences. It was therefore not considered physiologically relevant. A tendency to either down- or up regulation was additionally observed for the genes *gnrhr4*, and *cyp19a1a*. Other genes were either only moderately or not regulated.

The two genes encoding the two aromatase isoforms were found regulated in opposing directions in low *vtg1* expressers; *cyp19a1b* was down regulated, while *cyp19a1a* was upregulated, very likely as a result of their diverging routes of regulation. Expression of the brain aromatase *cyp19a1b* is controlled by an ERE in its promoter region. It is therefore directly regulated by estrogen-receptor activation, and thus, by estrogen levels, which should be low in males. An up regulation of *cyp19a1a* in putative males is in contradiction to the

literature, as it is supposed that its down regulation is the only necessary step to induce masculinisation (for review, Guiguen et al. (2010); Trant et al. (2001)). However, its expression is regulated by a diverse set of mechanisms related to sex differentiation. Analysis of not yet fully developed pre-adults (indicated by the low maturity index at 63 dpf also in controls) might result in high variability of gene expression depending on the maturation stage, even in fish of the same genetic sex. This assumption is further supported by the mechanism of gonad formation in zebrafish. Males initially develop a "juvenile ovary", which only later develops into functional testes, and thus, juvenile zebrafish are phenotypically indistinguishable. Final differentiation might be influenced by environmental as well as polygenic factors (Baroiller et al., 2009; Liew and Orbán, 2014; Traut and Winking, 2001). The differences in *cyp19a1a* expression between the presumable control males and females of this study may have become apparent only later after further maturation (Kallivretaki et al., 2007).

In the fadrozole exposed fish at 63 dpf, genes involved in steroid and terpenoid synthesis as well as in lipid metabolism were mostly upregulated, i.e. *lss*, *star* and *prox1* relative to the low *vtg1* controls. This can be interpreted as a compensatory mechanism to elevated testosterone and decreased estrogen levels. The up regulation of *star*, which was significant at 100 µg/L in relation to low *vtg1* and high *vtg1* controls, may hint at an increased cholesterol demand for steroidogenesis to overcome decreased estrogen levels caused by aromatase inhibition rather than from naturally occurring low estrogen levels in genotypic males. This observation suggested that upstream regulation of steroid biosynthesis was affected similarly in genotypic males and females, and that the response was exposure concentration dependent. Similar was the result for *prox1*, which was also upregulated at 100 µg/L of fadrozole in relation to both *vtg1* control groups. Villeneuve et al (2009) found up regulation of this gene by fadrozole in adult zebrafish and discussed its possible value as a marker of aromatase inhibition. It regulates angiogenesis and can repress the transcriptional activity of the liver receptor homologue 1 (*nr5a2*), which is involved in *cyp19a1b* activation and gonadotropin regulation.

Compared to high *vtg1* controls, we found down regulation of the ERE-possessing genes *vtg1* and *cyp19a1b*, and *esr2a*, and up regulation of *lss* and *star*, *prox1* and *igfbp5a*, but only at the highest exposure concentration. Other than in the low *vtg1* control correlation, all gene biomarkers but *igf1* were affected in relation to high *vtg1* controls, The down regulation of the genes clearly signify a response to reduced estrogen levels, as the expression of these genes is directly dependent on estrogen-receptor activation. A reduced estrogen level due to aromatase inhibition in presumable females demonstrated that the aromatase inhibition obviously negatively feedback on *cyp19a1b* expression and down regulates *vtg1*.

6.4.2 Differential gene expression in female and male zebrafish

The molecular mechanisms of sex determination in zebrafish are not yet fully understood, and genetic markers are lacking. Thus, it was of special interest to evaluate whether a discrimination between males and females would be possible early from the onset of sexual maturation (i.e. 28 dpf), based on gene markers differentially regulated upon fadrozole treatment. Further, the question was raised whether potential gene marker responses would depend on the phenotypic rather than the genotypic sex. Several studies have evaluated sex-related expression for zebrafish at the mRNA (Rodríguez-Marí et al., 2005; Schulz et al., 2007; Siegfried and Nüsslein-Volhard, 2008) and protein level (Groh et al., 2013), and differences between phenotypic males and females were described. The literature identified candidate genes for sex discrimination during early gonadal differentiation, including e.g. the anti-Müllerian hormone (*amh*) and the doublesex and mab-3 related transcription factor 1 (*dmrt1*), which were demonstrated to be upregulated in future males as early as 28 or 31 dpf (Rodríguez-Marí et al., 2005; Schulz et al., 2007). Furthermore, Schulz et al. (2007) demonstrated an estrogen-induced suppression of these genes, favouring them as potential biomarkers for early determination of sex differentiation. However, neither Villeneuve et al., (2009) nor our in-house study data indicated a regulation of the mentioned genes during early development or upon fadrozole treatment; hence they were excluded from the present analysis. Based on the findings of the study, we would now suggest including them in future investigation, especially for the time points representing the onset of sexual differentiation, i.e. 28 dpf.

Furthermore, the literature describes differential expression of *sox9b* in male and female zebrafish. *Sox9b* was shown to be expressed only in the ovaries but not in testes (Rodríguez-Marí et al., 2005). In mammals, the expression of *sox9* is followed by the expression of *amh*, leading to an inhibition of aromatase expression, resulting in masculinisation (Groh et al., 2011; Koopman, 1999). Moreover, Rodríguez-Marí et al. (2005) demonstrated co-expression of *amh* and *sox9a* in testes of 31 dpf male zebrafish, while female zebrafish of the same age displayed a co-expression of *sox9b* and *cyp19a1a*. Based on this evidence, a down regulation of *sox9b* upon fadrozole treatment as early as 28 dpf as well as a differential expression in putative males and females at 63 dpf should have taken effect, but we were unable to confirm the described effects for *sox9b*. At 28 dpf, differences in expression between individuals were most likely obscured due to the fact that four fish were pooled together. Furthermore, differential expression of *sox9b* was only described for the gonads, during craniofacial development and for the brain (Chiang et al., 2001; von Hofsten and Olsson, 2005). *Sox9b* is also involved in neural crest formation (Li et al., 2002). We therefore hypothesised that elucidation of *sox9b* differential expression in this study would have required analysis of individuals at 28 dpf and /or mRNA isolation from the gonads, because Villeneuve et al. (2009) were able to demonstrate the effect of aromatase inhibition on *sox9b*.

6.4.3 Vitellogenin gene expression as reliable marker for endocrine disruption

Synthesis of the egg yolk precursor protein vitellogenin is widely accepted as the most specific biomarker of exposure to estrogenic and anti-estrogenic EDCs (Fenske et al., 2001; Heppell et al., 1995), as it is produced in the liver in response to estrogenic stimulation (for review, compare Lazier and MacKay (1993)). It is highly abundant in plasma of adult females, but its expression was shown to be highly sensitive to (xeno-) estrogens also in males, resulting in increased vitellogenin levels. It appears equally sensitive to androgens, resulting in decreased vitellogenin levels in females (Ankley et al., 2002; Baumann et al., 2013). Due to its high sensitivity and specificity it has become an important biomarker endpoint in several fish testing strategies for endocrine disruptors (Ankley and Johnson, 2004; Knacker et al., 2010).

The reduction of *vtg1* mRNA as early as 48 hpf and 96 hpf, as observed in this study, provided therefore unequivocal evidence that this gene is a good candidate for early detection of endocrine disruption. Other studies, detecting significant regulation of *vtg1* mRNA as early as 48 hpf upon treatment with norgestrel (Liang et al., 2015) or 17α-ethinylestradiol (Muncke and Eggen, 2006) support this evidence.

A regulation of *vtg1* was not observed at 28 dpf. At this time point, sexual development is already initiated (von Hofsten and Olsson, 2005), and it is likely that individual fish genetically determined to one sex already display differences in steroidogenesis and thus, in the amount of *vtg1* mRNA. This observation was probably disguised since four fish were pooled into one sample, which was discussed already earlier. In this study, the informative value of *vtg1* was revealed only at 63 dpf when trunks of individual fish were analysed. Despite the lack of morphological sex determination was only an issue in control animals, *vtg1* expression of the control fish allowed discrimination between putative males and females. Log2-fold up regulations of approx. 10 in several of the analysed animals, a sex-dimorphic expression seemed self-evident. The high abundant expression of *vtg1* in presumable females suggested sensitivity to aromatase inhibition, what indeed repressed *vtg1* in relation to the high *vtg1* controls at all exposure concentrations of fadrozole, in line with the histopathological data confirming the absence of females (despite of one individual at a treatment concentration of 10 µg/L fadrozole). Although the complete sex shift towards males did not allow a comparison of expression levels of phenotypic females to control females, the study demonstrated that the determination of *vtg1* mRNA expression was more sensitive and informative than the VTG plasma concentrations and that this gene can also help discern genetic male from female fish.

The obtained results regarding *vtg1*, together with the evidence from the literature, substantiate the status of vitellogenin as a sensitive, reliable biomarker for endocrine disruption, and especially at the mRNA level it is also a very early indicator of ED, as shown here for aromatase inhibition.

6.5 Conclusion - for endocrine disruption

6.5.1 Adverse outcome pathway (AOPs) of aromatase inhibition during sexual development

An AOP is generally described as a “sequence of events from the exposure of an individual or population to a chemical substance to the adverse health effect at the individual level (for human health) or population level (for ecological health)”, according to a definition provided by the OECD (OECD, 2013b). In the present study we analysed the effects of the well-known aromatase inhibitor fadrozole on gene expression as well as apical endpoints in the context of a FSDT. This approach set the framework for the development and definition of an AOP for aromatase inhibition during sexual development of fish. An AOP of aromatase inhibition leading to reproductive dysfunction of female fish is already described in the knowledge base of AOP Wiki (https://aopkb.org/aopwiki/index.php/Aop:25) and there is a long list of publications providing evidence for the described mechanisms. Briefly, the molecular initiating event (MIE) of aromatase inhibition in adult females leads to a number of key events (KE), i.e. a reduced estrogen synthesis in granulosa cells of the ovaries and thus, to reduced circulating estrogen levels. Consequently to the reduced estrogen levels a reduction in VTG expression and production in hepatocytes, and a reduction of circulating VTG levels occur. The reduced VTG levels lead to an impaired oocyte development and egg quality. At the individual level, aromatase inhibition results in reduced fecundity and leads to a declining trajectory at the population level.

As a modification of the described AOP of aromatase inhibition, we defined an AOP specific for the early life and sexual development phase. This AOP is very similar in terms of the MIE, several KE and the effect at the population level, and the declined trajectory. However, also differences are described, which can nevertheless be explained by the MIE of aromatase inhibition, which in this case occurred at an earlier stage of fish development.

Figure 16: The Adverse Outcome Pathway (AOP) of aromatase inhibition during sexual maturation

This figure presents a schematic drawing of the AOP of aromatase inhibition during sexual maturation. Principally, the AOP starts with a Molecular Initiating Event (MIE), which leads to a chain of Key Events (KE) at the cellular or organ level. Ultimately, an Adverse Outcome (AO) at the organism or population level is observed. Hence, the MIE is still the aromatase inhibition. The first KEs are most likely an altered steroid and reduced estradiol synthesis, resulting in reduced estradiol levels, causing reduced transcription of ERE-containing genes like *vtg1* and *cyp19a1b*.Consequently, VTG concentrations in liver and blood plasma will decrease. The AO at the organism level is a skewed sex ratio towards males causing a declining population trajectory.

Like for the AOP of aromatase inhibition in adult females, the MIE of fadrozole exposure during fish sexual development is the effective inhibition of the P450 aromatase (CYP19), which regulates the conversion of C19-androgens to C18-estrogens (Callard et al., 1978). This conversion is a key event in the steroid synthesis pathway and its inhibition leads to a reduction of estrogen synthesis and levels, to a down regulation of ERE-containing genes like *vtg1* and *cyp19a1b* and in final consequence, to reduced VTG levels. In adult females the reduced VTG concentration leads to the impaired egg quality, reduced estrogen. In our case, a skewed sex ratio towards males reduced the VTG concentrations during sexual development. However, both AOPs result in a declining population trajectory at the rendering aromatase inhibition as detrimental for aquatic fish populations independent of the developmental stage affected by exposure. A schematic overview of the AOP during sexual development is presented in Figure 16.

In accordance to the described AOP, we found regulation of genes as early as 48 hpf, confirming regulation of genes involved in the steroid synthesis pathway (*mvd*, *lss*, *sc4mol*), very likely in order to compensate for altered steroid levels. Down regulation of *vtg1* as a direct consequence of low estrogen level due to aromatase inhibition, demonstrated that the described AOP already manifests at a very early stage.

At 63 dpf, the adverse outcome (AO) of aromatase inhibition at the organism level was exhibited by a complete shift of the sex ratio towards males, with obvious consequences for reproduction and survival of the population. Expression of genes that were differentially expressed between putative males and females (low vs. high *vtg1* expressers) in controls, displayed a comparable expression in fadrozole treated fish to putative males in controls, thus confirming the skewed sex ratio at the mRNA level (*vtg1*, *cyp19a1b*, *gnrhr4*).

6.5.2 New (bio-) markers for the identification of EDCs and their AOPs

The present study aimed at identifying new (bio-) markers for the identification of endocrine disrupting chemicals, ideally irrespective of their mode of action. However, to obtain specific results with no/ minor influencing side effects, a substance with a very specific mode of action was chosen as reference substance. Fadrozole as an aromatase inhibitor proved as good choice, since we were able to confirm the already described MOA and to propose an AOP. We identified regulated genes that were clearly associated with steroid biosynthesis, and selected biomarkers that represent different key steps during steroid biosynthesis (e.g. regulation of transcription factors, steroid/terpenoid synthesis, cholesterol transport, direct regulation of expression by estradiol), of which regulation upon endocrine disruption was already described in the literature. The genes include the insulin-like growth factor 1 (*igf1*), the steroidogenic acute regulatory protein (*star*), lanostyryl synthase (*lss*), the brain aromatase (*cyp19a1b*) and vitellogenin 1 (*vtg1*). The regulation of these genes at the different sampling time points was already described in the previous chapters. Their involvement in steroid biosynthesis is depicted in Figure 17. Igf1plays a central role in a diverse range of physiological processes, including growth, differentiation, and immune responses (e.g. Perez-Sanchez, 2000). Its function in endocrine processes and its sensitivity to endocrine disruption in fish is known but reports inconsistence in terms of up- or down regulation among fish species or MOA of endocrine disruption (Filby et al., 2007; Shved et al., 2008). We assume an involvement in aromatase inhibition, since we found a constant up regulation upon fadrozole treatment, though not always significant. Activation of IGF receptors by Igf1 leads to an induction of transcription factors, which ultimately regulate expression of *star*. Star is responsible for the transport of cholesterol to the inner mitochondrial membrane, which is considered a rate-limiting step during steroidogenesis. *Star* is differential expressed upon treatment with EDCs (Johns et al., 2011; Sharpe et al., 2007). The production of cholesterol itself from fatty acids involves genes related to the steroid/terpenoid synthesis like *lss*, *sc4mol* or *mvd*, which were all found to be regulated at one or the other sampling time point, and which were described to be regulated by estrogenic and anti-androgenic substances (Schiller et al., 2014). At the end of the steroid biosynthesis pathway, the conversion of testosterone to estrogen is regulated by aromatase, which is directly inhibited by fadrozole. This also reduces expression of *cyp19a1b*, which is furthermore influenced by estrogens, as it is also the case for v*tg1*. Therefore, expression levels of *cyp19a1b*, as well as *vtg1*, are sensitive sensors of estrogen levels and indirectly indicators of aromatase inhibition.

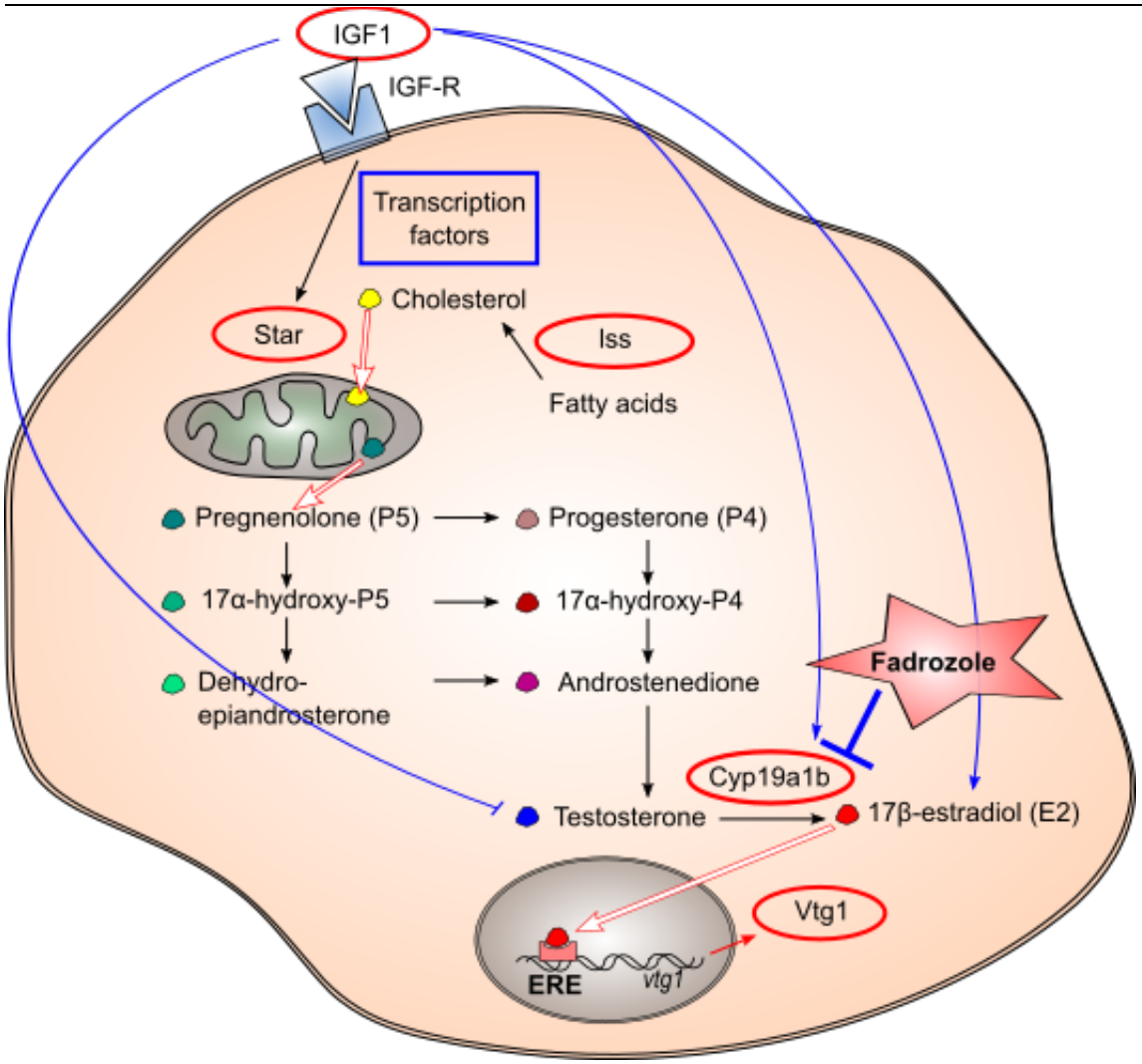
Table 15: Summary information of potential (bio-) markers

Potential biomarker gene	Description	Results, gene regulation	Function during steroidogenesis
igf1	Insulin-like growth factor 1	• Up regulation at all investigated time points (significant at 48 hpf and 96 hpf)	• Central role in a diverse physiological processes, incl. growth, differentiation, and immune response (Perez-Sanchez, 2000) • Sensitivity to endocrine disruptors described (Filby et al., 2007; Shved et al., 2008) • Induction of transcription factors regulating expression of *star*
star	Steroidogenic acute regulatory protein	• Significant up regulation at 63 dpf • No regulation at other time points	• Transport of cholesterol to the inner mitochondrial membrane → rate-limiting step during steroidogenesis (Johns et al., 2011) • Differential expression upon treatment with EDCs already described (Johns et al., 2011; Sharpe et al., 2007)
lss	Lanostyryl synthase	• Down regulated at 48 hpf, 96 hpf, 28 dpf • Up regulation at 63 dpf	• Steroid/terpenoid synthesis → cholesterol from fatty acids • Regulation by EDCs previously described (Schiller et al., 2014) • Other genes involved in this pathway: *mvd*, *sc4mol*
cyp19a1b	Cytochrome P450, family 19, subfamily A, polypeptide 1b Brain aromatase	• Negligible regulation at 48 hpf, 96 hpf, 28 dpf, and 63 dpf (low *vtg1* group) • Significant down regulation at 63 dpf (high *vtg1* group)	• Conversion of androgens to estrogens (Callard et al., 1978) • Expression regulated by the ERE in the promoter region • Regulation by EDCs described in several studies (e.g. Filby et al., 2007; Wang et al., 2010)
vtg1	Vitellogenin 1	• Significant down regulation at 48 hpf and 96 hpf, and 63 dpf (high *vtg1* group) • No regulation at 28 dpf and 63 dpf (low *vtg1* group)	• Expression regulated by the ERE in the promoter region • Regulation by EDCs described in several studies (e.g. Heppell et al., 1995; Schiller et al., 2014)

Three of these genes annotated as potential biomarkers (*igf1*, *star*, *vtg1*) were already described by Johns et al., 2011), who used these genes for gene expression analyses in FHM early development after xenoestrogen

treatment. Wang et al. (2010) performed a study with larval zebrafish and a set of xenoestrogens and found regulation of *vtg1* and *cyp19a1b* starting from 4 dpf. Thus, four of five genes chosen as potential biomarkers during this study were previously considered also by other researchers. This corroborates our conclusions about the potential gene biomarker and also nourishes the assumption that these biomarker genes are also suitable for the identification other MOA. Information on the potential biomarker genes and their regulation is summarised in Table 15.

Figure 17: The Steroid Biosynthesis Pathway



This figure shows the steroid biosynthesis pathway and the specific roles of the selected (bio-) marker genes. Fadrozole exposure directly influenced the activity of the aromatase (i.e. subsequently also *cyp19a1a* and *cyp19a1b*), and thus, the conversion of testosterone to estradiol. As a direct consequence, ERE-responsive genes are down regulated (*vtg1*, *cyp19a1b*). Aromatase inhibition also influences other key steps of the steroid biosynthesis pathway, e.g. IGF-R signalling, the synthesis of fatty acids to cholesterol or the cholesterol transport to the inner membrane of the mitochondrion. Involved genes were *igf1*, *lss*, and *star*. In contrast to the ERE-responsive genes, regulation of these genes might be subject to feedback loops mechanism, which makes the prediction of the direction of their expression regulation difficult.

6.6 Outlook

6.6.1 Limitations of the methodological approach

6.6.1.1 The impact of systemic toxicity on gene expression results

Systemic toxicity influences gene expression but can also affect endpoints of endocrine disruption (compare Ankley and Jensen, 2014; Wheeler et al., 2013). The fadrozole highest exposure concentration of this study was chosen primarily based on the information gained from previously conducted in-house zebrafish embryo tests, which showed no lethal or other morphological effects of fadrozole exposure up to 500 µg/L (Master thesis M. Macherey 2013, unpublished results). Literature also indicate potency of fadrozole at this concentration on apical endpoints (Andersen et al., 2005; Ankley et al., 2002; Panter et al., 2004). Further, this concentration was successfully applied as positive control for validation studies for the OECD 21-day screening assay. It was therefore unfortunate that the present test indicated minor unanticipated systemic toxicity, displayed in a slight increase in ELS mortality for 32 and 100 µg/L fadrozole at 28 dpf. Since there was no evidence of systemic effects at 48 hpf and 96 hpf, no negative impact on the development of the embryos and larvae and on gene expression was assumed for these developmental stages. However, for the gene expression results of the 28 dpf fish, a potential negative impact due to systemic toxicity cannot be ruled out. The rather tentative evaluation of the gene expression results of the 28 dpf time point due to the technical issues discussed (i.e. pooling of fish and deviating Ct values obtained from the A-replicate samples), we are confident that the conclusion drawn from the study were not impacted. The developmental stages 48 hpf and 96 hpf were considered more valuable in terms of an early effect detection, and the late time point 63 dpf the most informative in terms of mechanistic information. At 63 dpf, underlying toxicity was not implied by the fitness parameter growth and mortality.

6.6.1.2 Issues with gene expression analysis at early life stages

Adding on to what has been discussed in the previous section, the gene expression at 28 dpf may be of particular interest for the identification of EDs, as this time point marks the onset of sexual differentiation. Consequently, the onset of (genetic) sex dependent gene regulation can also be envisaged, with individual fish likely to express diverging levels of genes involved in sex differentiation. The pooling of fish at this time point resulted in inadvertent increase in gene expression variation among samples of the same treatment. Pooling of few fish, as happened in this study, disallowed the discrimination of differentially expressed genes among individuals (Wang et al., 2008) and moreover, the gene expression was measured for whole body homogenates, which may have diluted a tissue specific response, in particular given the known tissue and /or cell specific expression of some of the target genes like the two aromatase isoforms *cyp19a1a* and *cyp19a1b*. Other studies identified further genes that are differentially expressed in developing gonads, and whose expression is essential for developmental processes like chondriogenesis or neural crest formation (e.g. the *sox9* genes, compare Rodríguez-Marí et al., 2005). This aspect has likely contributed to the weak and non-significant responses of the 29 target genes at 28 dpf, although this developmental stage (onset of sexual development and gonad differentiation) is recognised as prone to disruption (Maack and Segner, 2004). The sampling procedure for follow-up investigation leaves room for improvement, and the dissection of the relevant organs like liver, gonads and brains, should be considered where ever technically possible. Dissection of the gonads and other organs for tissue-specific mRNA analysis at 28 dpf would have been technically very challenging due to the small size of the fish at this time point. However, just the separation of the head from the trunk section and the sampling of individual fish would have probably achieved a significant improvement in data resolution. There is also little doubt that a tissue-specific mRNA analysis at 63 dpf would have provided a more comprehensive and meaningful dataset of differential gene expression in control and fadrozole exposed fish and facilitated the interpretation of the presumed sex-dimorphic gene responses in the fadrozole induced all male cohorts.

In terms of influencing factors which may have impacted the gene expression data obtained at the early life stages 48 hpf and 96 hpf, is the generally high and dynamic overall transcriptome activity, characteristic for an organism undergoing development and in particular embryogenesis. From extensive experiences gained

from transcriptomic studies on 48 hpf embryos in-house (partly published), we are aware that this can cause a lot of gene expression "background noise" which makes it difficult to discern a clear gene response. The intrinsically high gene activity of developmental regulatory pathways in embryo and larval stages leads to a high data variation already at control conditions and complicates interpretation results.

6.6.2 Possible follow-up projects

The present study aimed at demonstrating the benefits of gene expression integration into existing test guidelines for endocrine disruption by a proof-of-principle using the FSDT. Therefore, an extensive literature review was performed to obtain a comprehensive overview of existing approaches to use gene expression as an indicative endpoint of endocrine disruption effects. Based on the information from this data base and considering available in-house data, the aromatase inhibitor fadrozole was chosen as the most suitable test compound and 29 target genes were selected for which regulation by endocrine disruptors was suggested by previous studies.

Resulting from the study, five potential (bio-) marker genes for aromatase inhibition were identified, which are likely to be susceptible to other endocrine disruption MOAs.

Despite the successful completion of the study, issues and questions arose from the results and the experiences gained, which should be addressed by further investigations. The test chemical fadrozole was known to act rather specific, which was confirmed by the clear effects on the apical endpoint gonad differentiation and sex ratio and reflected by the results of the gene expression. However, in order to verify these results, a less specific acting compound should be tested as well. A not exclusively as aromatase inhibitor acting compound or an EDC of another endocrine modality, which knowingly cause similar endocrine relevant apical effects would be recommended to test whether a similar response of the marker genes can be obtained. Finally, for the validation of the approach, an unknown substance suspected to act as endocrine disruptor, preferably by a mechanisms not strictly related to the sex hormone activity, has to be tested. Early endocrine regulatory processes induced by progestins and gestagens would be possible targets.

Other issues and shortcomings of the present study (summarised in section 6.6.1) which encourage follow-up studies, were the inclusion of additional marker genes and the exploration of systemic toxicity effects on gene expression as a source of error in EDC testing. Another interesting task for a follow-up study would be an in-depth investigation of different sampling procedures and target organs for mRNA expression analysis.

A potential test design for a follow-up should again be based on a FSDT with fadrozole as positive control and an additional EDC of known aromatase inhibition activity but with a wide range of other mechanisms of action. As the gene expression analysis at 96 hpf yielded promising results, an explorative study, which would focus on the fish embryo toxicity test (FET) period of 96 hpf, could help addressing issues related to high false negative rate often suspected for screening assays. The integration of a larger set of biomarker genes into the FET could prove very valuable for a whole organism screening approach for endocrine disruption. The possible integration of such an enhanced FET as screening tool into regulatory processes is discussed in section 6.6.3.

6.6.3 Implementation of gene expression analysis in regulatory processes

Another important question that arose from the study was how such mechanism or MOA indicative molecular biomarkers could be applied in a regulatory context. Based on the results of this study, three possible approaches were discussed:

- A gene biomarker enhanced fish embryo test as EDC screening tool
- Extension of existing test guidelines by gene expression analysis
- Use of gene expression analysis for biomonitoring purposes

A biomarker enhanced fish embryo test as EDC screening tool

Especially early life stages, i.e. embryonic and early larval stage, respond highly sensitive to exposure, and the effects on gene expression are quick and comparably easily measurable. The already existing test guideline for the Fish embryo toxicity test FET (OECD 236) can easily be supplemented with gene expression analysis. Such an extended fish embryo test could provide the basis for a tiered testing strategy. The advantage of the fish embryo test over the currently promoted cell-based screening assays (Conceptual Framework of the OECD 2010) is the possible evaluation of endocrine-specific biomarker genes in a whole organism. The significance of data obtained *in-vivo* can certainly be considered higher than *in-vitro,* since cell-based systems lack the complexity of whole organisms and cell-cell interactions. Consequently, the achievement of a higher reliability of screening assays through the reduction of false negative results could be a factor also promoting the avoidance of unnecessary fish tests (i.e. animal tests). Knacker et al. (2010) proposed a tiered testing strategy for the detection of endocrine disrupters in fish, which considers at the basic tier data from existing toxicology studies, data from literature reviews and *in-silico* from e.g. QSAR calculations. Additionally, *in-vitro* test data which focus on receptor interactions in cell lines are included. These cell assays are prone to false negative as well as false positive results. A positive result in any of these tests, however, triggers *in-vivo* screening assay at the next tier, like the fish short term reproduction assay (OECD 229). The profound evidence in the literature (e.g., Busch et al., 2011; Kosmehl et al., 2012; Sawle et al., 2010; Voelker et al., 2007; Weil et al., 2009) combined with the extensive experiences regarding gene expression analysis in embryos in-house (Schiller et al., 2014, 2013a, 2013b; Turner et al., 2012), could be exploited to develop a FET based screening approach specifically for endocrine disruptors.

Extension of existing test guidelines by gene expression analysis

Due to an increasing number of applications for authorization of chemical and pharmaceutical compounds of uncertain mode of action in non-target organisms, the demand for and relevance of EDC test methods will continue to increase. The integration of gene biomarkers into existing test guidelines could improve the identification of EDC also in the context of non-EDC tests. This may be of particular interest for pharmaceutical compounds which often target specifically the hormone system of humans or of livestock. The reported study demonstrated that gene expression has indicative function for the identification of endocrine disruption in fish. For the well described mode of action of aromatase-inhibition, the applicability of gene biomarkers was demonstrated by this study. The genes identified the aromatase modulation as well as the effects on estrogen regulated genes. Moreover, aromatase inhibition induced feedback loop regulations via terpenoid, cholesterol- and steroid hormone biosynthesis related genes were identified, and these are common targets of endocrine disruptors affecting estrogen and androgen signalling. However, the FSDT was originally developed to detect ED mechanisms like aromatase inhibition or androgen disruption, which manifest at organism and population level as masculinization and a shift in sex ratios towards an increased number of males. The applicability of gene expression enhanced fish test guidelines for endocrine disruption should therefore also be investigated.

Use of gene expression analysis for biomonitoring purposes

A broad field of applications for gene expression analysis is provided by biomonitoring projects and the examination of biological matrices. The analysis of biota samples taken from contaminated habitats, which is routinely performed by chemical analysis of key contaminants (like pharmaceuticals or pesticides), could be supplemented by MOA based gene expression analysis in the context of an effect-directed analysis (EDA). Gene expression measured in exposed organism could provide an additional analytical tool for the identification of potentially adverse endocrine disrupting effects of exposure to groups of pollutants (mixtures).

As an addition, the value of gene expression for the analysis of cryo-preserved samples of environmental specimen banks is indisputable and opens up numerous opportunities for retrospective exposure evaluation.

7 Literature

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8 Annex

8.1 Raw data

8.1.1 Physical water parameters

Table 16: Water temperature [°C] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	Control				10			
Replicate	A	B	C	D	A	B	C	D
day	Temperature [°C]							
0	24.6	24.9	24.9	24.9	24.7	24.8	24.7	24.7
1	25.0	25.0	25.0	25.1	25.0	24.9	25.1	25.0
2	25.0	25.0	25.1	25.0	25.0	25.0	25.0	25.0
5	24.8	24.9	24.9	24.9	24.8	24.9	24.9	24.9
6	24.7	24.7	24.7	24.8	24.7	24.7	24.8	24.9
7	24.5	24.6	24.8	24.7	24.4	24.6	24.4	24.6
8	24.6	24.8	25.0	24.9	24.6	24.6	24.6	24.8
9	24.5	24.7	24.8	24.8	24.5	24.6	24.6	24.7
12	24.5	24.5	24.8	24.6	24.6	24.5	24.6	24.6
13	24.3	24.6	24.7	24.6	24.4	24.6	24.4	24.6
14	24.6	24.7	24.8	24.8	24.6	24.7	24.6	24.7
15	24.7	24.7	24.9	24.8	24.7	24.7	24.7	24.6
19	24.5	24.7	24.9	24.8	24.6	24.7	24.6	24.7
20	24.5	24.6	24.9	24.7	24.5	24.7	24.6	24.6
21	24.9	25.1	24.9	24.7	24.7	24.7	24.5	24.6
22	24.4	24.6	25.0	24.7	24.6	24.6	24.6	24.6
23	24.2	24.5	24.8	24.7	24.5	24.6	24.4	24.6
26	24.9	25.1	25.3	25.4	25.1	25.2	25.1	25.1
27	24.7	24.7	25.1	25.0	24.8	24.8	24.8	24.8
28	24.8	24.8	25.2	25.1	24.9	24.9	24.9	24.8
29	24.7	24.7	25.1	25.0	24.7	24.8	24.7	24.7
30	24.6	24.8	25.0	25.0	24.6	24.8	24.6	24.7
33	24.8	25.1	25.3	25.4	25.0	25.4	25.1	25.2
34	24.6	24.8	25.3	25.1	24.9	25.1	24.9	25.0
35	25.5	24.7	25.1	25.0	24.6	25.0	24.7	24.9
36	24.6	24.8	25.2	25.1	24.8	25.0	24.9	25.0
37	24.5	24.8	25.2	25.1	24.7	25.1	24.8	25.0
40	24.4	24.5	25.1	24.8	24.6	24.6	24.8	24.6
41	24.8	24.8	25.2	25.1	24.7	24.9	24.8	24.8
42	24.6	24.8	25.1	25.0	24.6	25.0	24.6	24.8

Table 16 (continued): Water temperature [°C] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	Control				10			
Replicate	A	B	C	D	A	B	C	D
day	Temperature [°C]							
43	24.5	24.8	25.1	25.1	24.6	25.0	24.7	24.7
44	24.7	24.8	25.1	25.2	24.8	25.0	24.8	24.9
47	24.6	24.8	25.2	25.0	24.8	24.9	24.8	24.8
48	24.4	24.7	25.1	25.0	24.6	24.9	24.7	24.8
49	24.2	24.4	24.8	24.8	24.4	24.6	24.4	24.5
50	24.6	24.8	25.2	25.2	24.8	25.1	24.9	25.0
51	24.2	24.4	24.9	24.9	24.4	24.7	24.4	24.6
54	24.0	24.3	24.7	24.8	24.3	24.6	24.3	24.4
55	24.0	24.3	24.7	24.7	24.2	24.5	24.2	24.3
56	24.1	24.1	24.4	24.5	24.1	24.3	24.1	24.1
57	24.1	24.2	24.4	24.5	24.1	24.3	24.1	24.2
58	24.0	24.1	24.4	24.4	24.2	24.2	24.0	24.1
61	24.1	24.4	24.9	24.9	24.5	24.8	24.5	24.5
62	24.2	24.3	24.8	24.8	24.4	24.7	24.4	24.6
63	24.1	24.2	24.1	24.9	24.8	24.2	24.8	24.1
Mean	**24.5**	**24.7**	**24.9**	**24.9**	**24.6**	**24.8**	**24.6**	**24.7**
SD	**0.31**	**0.26**	**0.26**	**0.22**	**0.23**	**0.26**	**0.26**	**0.26**
SD%	**1.27**	**1.04**	**1.05**	**0.88**	**0.95**	**1.03**	**1.06**	**1.05**

Table 16 (continued): Water temperature [°C] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	32				100			
Replicate	A	B	C	D	A	B	C	D
day	Temperature [°C]							
0	24.8	24.6	24.9	24.8	24.8	25.0	24.8	25.0
1	25.0	25.0	25.1	25.1	25.0	25.1	25.2	25.2
2	25.0	25.1	25.0	25.0	25.0	25.2	25.2	25.2
5	24.9	24.8	24.9	24.9	25.0	25.1	25.2	25.2
6	24.7	24.8	24.7	24.8	24.8	25.0	25.0	25.0
7	24.6	24.5	24.7	24.5	24.6	24.9	24.8	25.0
8	24.8	24.8	24.8	24.8	24.8	25.2	25.0	25.2
9	24.6	24.5	24.9	24.7	24.8	25.0	25.1	25.0
12	24.5	24.4	24.7	24.6	24.5	25.0	24.8	25.1
13	24.4	24.5	24.6	24.6	24.5	25.0	24.8	25.1

Table 16 (continued): Water temperature [°C] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	32				100			
Replicate	A	B	C	D	A	B	C	D
day	Temperature [°C]							
14	24.7	24.6	24.8	24.7	24.8	25.1	25.1	25.2
15	25.0	24.8	24.9	24.8	24.9	25.2	25.0	25.2
19	24.9	24.9	24.9	24.9	24.7	25.1	25.1	25.2
20	24.8	24.8	24.8	24.8	24.6	25.1	24.9	25.2
21	24.9	24.9	24.9	24.8	24.8	25.1	25.0	25.3
22	24.9	24.8	24.9	24.7	24.6	25.2	25.0	25.2
23	25.0	24.9	24.7	24.6	24.5	24.9	25.0	25.1
26	25.4	25.4	25.4	25.3	25.3	25.7	25.6	25.7
27	25.3	25.3	25.1	25.0	25.1	25.3	25.1	25.4
28	25.2	25.2	25.0	24.9	25.1	25.2	25.1	25.3
29	25.3	25.3	25.0	24.9	24.8	25.3	25.0	25.4
30	25.3	25.3	25.0	24.9	24.8	25.3	25.1	25.2
33	25.5	25.5	25.4	25.3	25.2	25.4	25.4	25.4
34	25.4	25.5	25.1	24.9	24.9	25.4	25.1	25.5
35	25.3	25.3	24.9	24.9	24.7	25.2	24.9	25.2
36	25.3	25.4	25.1	25.0	24.8	25.4	25.1	25.5
37	25.3	25.5	25.0	25.0	24.9	25.3	25.1	25.5
40	25.3	25.3	24.9	24.6	24.7	25.1	25.0	25.2
41	25.5	25.5	25.1	25.0	24.9	25.4	25.1	25.4
42	25.4	25.5	24.9	24.9	24.8	25.3	25.0	25.4
43	25.4	25.6	25.0	25.0	24.8	25.4	24.9	25.3
44	25.6	25.6	25.1	24.9	24.8	25.3	24.9	25.3
47	25.4	25.5	25.1	24.8	24.9	25.2	24.9	25.1
48	25.2	25.4	24.7	24.9	24.7	25.1	24.7	25.1
49	25.1	25.1	24.6	24.3	24.4	24.7	24.5	24.6
50	25.5	25.5	24.9	24.9	24.6	25.2	24.7	25.2
51	25.0	25.0	24.6	24.6	24.2	24.8	24.3	24.9
54	24.8	25.0	24.5	24.3	24.0	24.7	24.3	24.8
55	24.9	24.9	24.0	24.1	24.1	24.6	24.2	24.6
56	24.8	24.8	24.1	24.2	24.1	24.4	24.1	24.4
57	24.7	24.9	24.1	24.2	24.1	24.5	24.1	24.4
58	24.6	24.8	24.1	24.2	24.1	24.4	24.1	24.4
61	24.9	25.0	24.2	24.1	24.4	24.9	24.7	24.9
62	24.9	25.0	24.2	24.1	24.3	24.8	24.5	24.8
63	24.9	24.5	24.8	24.2	24.8	24.1	24.9	24.2

Table 16 (continued): Water temperature [°C] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	32				100			
Replicate	A	B	C	D	A	B	C	D
day	Temperature [°C]							
Mean	**25.0**	**25.1**	**24.8**	**24.7**	**24.7**	**25.1**	**24.9**	**25.1**
SD	**0.31**	**0.35**	**0.33**	**0.32**	**0.31**	**0.31**	**0.34**	**0.33**
SD%	**1.23**	**1.40**	**1.33**	**1.28**	**1.25**	**1.25**	**1.37**	**1.30**

Table 17: Oxygen saturation [%] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	Control				10			
Replicate	A	B	C	D	A	B	C	D
day	Oxygen saturation [%]							
0	97	99	99	100	101	99	99	99
2	102	102	102	102	102	102	102	102
5	103	100	103	100	100	100	100	98
8	106	104	106	105	102	100	103	104
12	107	107	109	107	106	106	104	105
15	108	109	110	109	108	108	107	107
19	107	107	106	107	107	107	107	104
22	102	102	104	107	108	108	106	105
26	106	102	104	103	112	109	106	105
29	106	104	106	105	103	103	106	105
33	106	103	105	103	105	105	108	107
36	103	101	103	99	107	103	107	102
40	102	105	106	97	105	107	111	105
43	104	104	101	102	103	102	102	103
47	100	100	96	95	96	99	98	98
50	102	103	102	101	101	102	103	101
54	100	101	98	97	97	99	101	100
57	103	102	99	100	100	102	102	100
61	98	99	96	96	95	98	99	96
63	100	100	98	95	97	96	96	95
Mean	**103.1**	**102.7**	**102.7**	**101.5**	**102.8**	**102.8**	**103.4**	**102.1**
SD	**3.1**	**2.8**	**4.0**	**4.3**	**4.5**	**3.8**	**3.9**	**3.5**
SD%	**3.0**	**2.7**	**3.9**	**4.2**	**4.4**	**3.7**	**3.7**	**3.5**

Table 17 (continued): Oxygen saturation [%] from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	32				100			
Replicate	A	B	C	D	A	B	C	D
day	Oxygen saturation [%]							
0	100	100	101	100	100	99	99	98
2	103	102	103	102	103	103	103	102
5	99	99	99	99	99	99	99	98
8	103	103	104	102	102	100	102	101
12	105	106	106	104	104	105	106	103
15	109	108	108	106	106	106	105	105
19	106	105	106	105	107	104	105	103
22	108	106	109	105	109	107	102	102
26	106	106	106	105	110	106	101	102
29	103	103	105	102	102	104	102	102
33	104	102	102	102	107	106	103	102
36	102	100	102	103	101	101	99	99
40	105	104	109	105	104	104	104	99
43	98	96	100	102	100	101	102	100
47	89	91	97	99	92	94	99	95
50	95	95	102	100	101	99	101	95
54	93	94	99	99	99	98	100	95
57	94	96	103	102	96	97	99	95
61	90	90	101	100	93	91	95	91
63	91	90	102	97	95	94	95	93
Mean	**100.2**	**99.8**	**103.2**	**102.0**	**101.5**	**100.9**	**101.1**	**99.0**
SD	**6.2**	**5.7**	**3.4**	**2.5**	**5.0**	**4.6**	**3.0**	**3.9**
SD%	**6.2**	**5.7**	**3.3**	**2.5**	**4.9**	**4.5**	**3.0**	**3.9**

Table 18: pH value from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	Control				10			
Replicate	A	B	C	D	A	B	C	D
day	pH							
0	8.50	8.37	8.36	8.36	8.40	8.38	8.38	8.38
2	8.24	8.19	8.19	8.19	8.24	8.24	8.24	8.24
5	8.15	8.09	8.09	8.11	8.16	8.16	8.17	8.17
8	8.28	8.24	8.22	8.21	8.28	8.30	8.26	8.28
12	8.24	8.21	8.22	8.20	8.27	8.27	8.21	8.24
15	8.07	8.04	8.05	8.04	8.10	8.11	8.04	8.05
19	8.23	8.21	8.18	8.20	8.32	8.29	8.24	8.25
22	8.32	8.33	8.30	8.33	8.49	8.47	8.36	8.37
26	8.05	8.04	8.01	8.01	8.19	8.19	8.14	8.16
29	8.08	8.06	8.06	8.03	8.15	8.13	8.14	8.13
33	7.97	7.95	7.97	7.97	8.11	8.05	8.11	8.09
36	8.00	7.94	7.96	7.90	8.05	8.02	8.03	8.03
40	8.14	8.14	8.12	8.10	8.26	8.24	8.28	8.25
43	7.73	7.80	7.80	7.89	7.91	7.91	7.94	7.92
47	7.97	7.95	7.91	7.91	7.97	7.98	7.97	7.97
50	8.12	8.13	8.13	8.15	8.18	8.18	8.18	8.20
54	8.08	8.08	8.05	8.08	8.08	8.15	8.16	8.14
57	8.05	8.05	8.05	8.06	8.09	8.20	8.19	8.16
61	8.03	8.01	7.98	8.02	8.00	8.07	8.04	8.06
63	7.91	7.98	7.99	8.01	8.07	8.09	8.08	8.13
Mean	**8.11**	**8.09**	**8.08**	**8.09**	**8.17**	**8.17**	**8.16**	**8.16**
SD	**0.17**	**0.14**	**0.14**	**0.13**	**0.14**	**0.14**	**0.12**	**0.12**
SD%	**2.1**	**1.7**	**1.7**	**1.6**	**1.8**	**1.7**	**1.5**	**1.5**

Table 18 (continued): pH value from day 0 to day 63

	Nominal concentrations fadrozole [µg/L]							
	32				100			
Replicate	A	B	C	D	A	B	C	D
day	pH							
0	8.39	8.39	8.39	8.37	8.41	8.42	8.41	8.41
2	8.26	8.27	8.25	8.27	8.27	8.27	8.27	8.28
5	8.18	8.18	8.16	8.18	8.20	8.20	8.18	8.19
8	8.26	8.25	8.25	8.29	8.26	8.26	8.26	8.29
12	8.22	8.22	8.20	8.26	8.25	8.23	8.20	8.23
15	8.05	8.07	8.06	8.07	8.05	8.05	8.01	8.05
19	8.26	8.26	8.25	8.25	8.30	8.25	8.22	8.25
22	8.41	8.38	8.41	8.41	8.50	8.39	8.35	8.38
26	8.17	8.13	8.15	8.15	8.22	8.15	8.03	8.12
29	8.06	8.05	8.06	8.11	8.12	8.12	8.05	8.08
33	8.02	8.02	8.02	8.02	8.10	8.08	8.02	8.05
36	8.01	8.01	8.01	8.02	8.00	8.00	7.93	7.97
40	8.25	8.22	8.27	8.23	8.22	8.20	8.17	8.20
43	7.92	7.89	7.90	7.96	7.94	7.93	7.92	7.93
47	7.93	7.89	7.99	8.00	8.01	8.01	8.00	7.96
50	8.12	8.14	8.17	8.17	8.18	8.18	8.18	8.16
54	8.10	8.08	8.16	8.15	8.14	8.13	8.12	8.08
57	8.08	8.09	8.21	8.17	8.12	8.11	8.12	8.11
61	8.01	8.02	8.15	8.10	8.01	7.98	8.01	8.02
63	8.02	8.08	8.21	8.16	8.10	8.13	8.17	8.17
Mean	**8.14**	**8.13**	**8.16**	**8.17**	**8.17**	**8.15**	**8.13**	**8.15**
SD	**0.14**	**0.14**	**0.13**	**0.12**	**0.14**	**0.13**	**0.13**	**0.13**
SD%	**1.7**	**1.7**	**1.6**	**1.5**	**1.7**	**1.6**	**1.7**	**1.7**

8.1.2 Test item concentrations

Table 19: Test item concentrations (absolute; in µg/L) from day 0 to day 61

	Nominal concentrations fadrozole [µg/L]											
	10				32				100			
Replicate	A	B	C	D	A	B	C	D	A	B	C	D
day	Absolute fadrozole concentrations [µg/L]											
0	10.9	10.3	9.6	9.2	25.7	25.2	34.9	35.6	87.9	87.1	88.0	93.3
6	10.5	10.8	12.0	12.4	33.7	30.7	34.0	32.5	97.6	87.9	96.7	93.9
13	11.5	11.5	11.6	11.5	27.2	25.7	33.7	34.9	99.3	99.9	108.6	108.6
19	9.2	-	9.7	-	33.2	-	34.1	-	100.6	-	110.0	-
27	-	10.0	-	9.5	-	38.8	-	33.5	-	88.6	-	96.6
34	9.6	-	9.9	-	43.0	-	36.2	-	91.5	-	103.6	-
41	-	10.2	-	9.8	-	44.7	-	39.3	-	106.4	-	117.5
49	9.0	-	7.8	-	47.0	-	32.7	-	94.2	-	90.7	-
55	-	8.9	-	9.1	-	46.2	-	24.8	-	101.2	-	88.7
61	9.4	-	8.7	-	47.3	-	26.6	-	89.9	-	81.4	-
Mean	**10.0**	**10.3**	**9.9**	**10.3**	**36.7**	**35.2**	**33.2**	**33.4**	**94.4**	**95.2**	**97.0**	**99.8**
SD	**1.0**	**0.9**	**1.5**	**1.4**	**9.0**	**9.3**	**3.1**	**4.8**	**4.9**	**8.3**	**10.9**	**11.0**
SD%	**9.7**	**8.4**	**15.0**	**13.4**	**24.6**	**26.5**	**9.3**	**14.4**	**5.2**	**8.7**	**11.2**	**11.0**

Two adjacent vessels were served from one pump. After achievement of stable fadrozole concentrations, analysis was only performed in one of the two vessels, alternating from one week to the other.

Table 20: Test item concentrations (relative, in %) from day 0 to day 61

	Nominal concentrations fadrozole [µg/L]											
	10				32				100			
Replicate	A	B	C	D	A	B	C	D	A	B	C	D
day	Relative fadrozole concentrations [%]											
0	109.0	102.9	96.5	92.0	80.3	78.6	109.0	111.3	87.9	87.1	88.0	93.3
6	105.1	108.4	120.5	124.1	105.3	95.8	106.1	101.4	97.6	87.9	96.7	93.9
13	114.7	114.6	115.9	114.7	85.1	80.3	105.3	109.2	99.3	99.9	108.6	109.0
19	92.2	-	96.7	-	103.8	-	106.5	-	100.6	-	110.0	-
27	-	99.6	-	94.6	-	121.2	-	104.8	-	88.6	-	96.6
34	95.5	-	99.2	-	134.3	-	113.6	-	91.5	-	103.6	-
41	-	102.1	-	98.3	-	139.6	-	122.7	-	106.4	-	117.5
49	89.7	-	78.2	-	147.0	-	102.3	-	94.2	-	90.7	-
55	-	89.1	-	91.1	-	144.3	-	77.6	-	101.2	-	88.7
61	93.5	-	86.6	-	147.7	-	83.2	-	89.9	-	81.4	-
Mean	**100.0**	**102.8**	**99.1**	**102.5**	**114.8**	**110.0**	**103.7**	**104.5**	**94.4**	**95.2**	**97.0**	**99.8**
SD	**9.6**	**8.6**	**15.0**	**13.7**	**28.2**	**29.1**	**9.7**	**15.1**	**4.9**	**8.3**	**10.9**	**11.0**
SD%	**9.6**	**8.4**	**15.1**	**13.4**	**24.6**	**26.5**	**9.4**	**14.4**	**5.2**	**8.7**	**11.2**	**11.0**

Two adjacent vessels were served from one pump. After achievement of stable fadrozole concentrations, analysis was only performed in one of the two vessels, alternating from one week to the other.

8.1.3 Early life stage

8.1.3.1 Hatch

Table 21: Hatch between day 3 pf to day 6 pf

		Nominal concentration fadrozole [µg/L]			
		Control	10	32	100
Introduced eggs [n]	**A 1**	50	50	50	50
	A 2	35	35	35	35
	B 1	50	50	50	50
	B 2	35	35	35	35
	C 1	85	85	85	85
	C 2				
	D 1	50	50	50	50
	D 2	35	35	35	35
Hatch, day 3 pf [n]	**A 1**	1	0	0	1
	A 2	2	0	0	3
	B 1	0	0	0	2
	B 2	0	0	0	2
	C 1	32	2	1	4
	C 2				
	D 1	1	1	2	0
	D 2	2	0	0	0
Hatch, day 4 pf [n]	**A 1**	8	1	3	13
	A 2	6	3	2	15
	B 1	3	4	3	4
	B 2	4	1	1	11
	C 1	46	19	14	16
	C 2				
	D 1	5	6	3	4
	D 2	8	5	1	5
Hatch, day 5 pf [n]	Evaluation was not performed due to sampling procedure after 96 h				
Hatch, day 6 pf [n]	**A 1**	complete hatch in all test vessels			
	A 2				
	B 1				
	B 2				
	C 1				
	C 2				
	D 1				
	D 2				

Deviating from the original study plan, only one fry cage instead of two was used for replicate C of all treatment levels, as only a reduced number of fry cages were available. The re-placement fry cages were approx. twice the size of the other fry cages.

8.1.3.2 Survival

Table 22: Survival at day 28 pf in [n] and [%]

		Nominal concentration fadrozole [µg/L]			
		Control	10	32	100
Maximum number of embryos at day 21 [n]	**A**	45	45	45	45
	B	45	45	45	45
	C	45	45	45	45
	D	45	45	45	45
Survival, day 28 pf [n]	**A**	36	36	31	32
	B	35	33	31	33
	C	37	36	31	29
	D	37	35	34	28
Total survival, day 28 pf [%]	**A**	**80.0**	**80.0**	**68.9**	**71.1**
	B	**77.8**	**73.3**	**68.9**	**73.3**
	C	**82.2**	**80.0**	**68.9**	**64.4**
	D	**82.2**	**77.8**	**75.6**	**62.2**
	Mean	**80.6**	**77.8**	**70.6**	**67.8**
	SD	**2.1**	**3.1**	**3.3**	**5.3**
	SD%	**2.6**	**4.0**	**4.7**	**7.8**

8.1.3.3 Growth in terms of length

Table 23: Length [cm], day 28 pf

	Nominal concentration fadrozole [µg/L]							
	Control				10			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	36	35	37	38	36	33	36	35
1	0.90	0.90	0.80	0.82	0.88	0.83	1.40	1.55
2	1.10	1.03	1.06	0.84	0.85	1.06	0.91	0.90
3	1.54	0.95	1.17	0.92	0.76	0.97	0.95	0.90
4	1.46	1.19	1.47	1.19	0.80	0.85	1.04	1.26
5	0.99	0.83	0.81	0.98	1.17	0.90	0.99	1.18
6	1.51	0.59	1.18	0.78	1.17	0.94	1.17	1.07
7	0.85	1.19	1.05	1.53	1.03	0.85	1.47	1.16
8	1.13	0.90	1.23	0.87	1.45	0.82	1.23	1.22
9	1.02	0.65	1.18	0.85	1.06	1.11	1.40	1.19
10	1.02	0.81	1.01	0.95	0.86	1.55	1.33	1.13
11	1.16	1.01	1.13	1.44	1.09	0.90	0.79	1.34
12	0.89	1.27	0.94	0.81	1.03	1.02	1.14	1.02

Table 23 (continued): Length [cm], day 28 pf

	Nominal concentration fadrozole [µg/L]							
	Control				10			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	36	35	37	38	36	33	36	35
13	0.70	0.93	1.42	0.97	1.26	1.13	1.21	0.94
14	0.64	0.84	1.18	0.93	0.95	1.10	1.30	1.12
15	0.88	1.05	1.18	0.98	0.88	0.83	1.22	1.04
16	0.62	1.23	0.85	1.58	1.12	1.03	1.12	0.90
17	0.93	1.27	1.52	1.48	1.29	0.92	1.23	1.11
18	0.74	0.92	1.25	0.73	1.18	1.04	1.06	1.06
19	1.21	0.88	1.14	0.91	0.95	0.96	0.91	0.99
20	0.95	1.20	0.93	0.72	1.35	1.04	1.32	1.09
21	0.63	0.95	1.33	0.84	1.13	1.42	1.27	1.02
22	0.83	0.86	1.35	0.86	1.30	1.16	1.04	1.13
23	0.68	0.88	1.47	0.90	1.29	1.37	1.35	0.74
24	1.05	1.08	1.13	1.15	1.37	0.94	1.41	1.41
25	1.15	1.04	0.90	1.31	0.97	0.74	0.84	1.56
26	1.14	0.96	0.69	0.74	1.09	1.04	0.93	1.19
27	1.17	0.96	1.16	1.10	1.09	0.92	0.88	1.09
28	1.35	0.97	0.84	0.89	0.98	0.78	0.84	0.91
29	1.41	0.98	1.24	0.76	0.77	1.15	0.80	0.84
30	0.63	1.38	0.90	0.90	0.97	0.93	1.22	1.12
31	0.93	1.38	1.38	0.87	0.93	1.25	0.85	1.07
32	0.51	0.94	0.86	0.85	1.14	1.15	1.24	1.07
33	1.30	0.93	0.99	0.73	0.62	0.90	1.11	1.12
34	1.09	0.84	1.04	0.65	1.22	-	0.87	0.95
35	0.88	1.24	1.45	0.82	1.18	-	0.91	1.04
36	0.75	-	0.99	0.73	0.81	-	1.14	-
37	-	-	0.73	0.83	-	-	-	-
38	-	-	-	1.01	-	-	-	-
Mean	**0.99**	**1.00**	**1.11**	**0.95**	**1.06**	**1.02**	**1.11**	**1.10**
SD	**0.27**	**0.19**	**0.22**	**0.24**	**0.20**	**0.19**	**0.20**	**0.18**
SD%	**27.2**	**18.6**	**20.2**	**24.7**	**18.6**	**18.2**	**18.1**	**16.2**

Table 23 (continued): Length [cm], day 28 pf

	Nominal concentration fadrozole [µg/L]							
	32				100			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	31	31	31	34	32	33	29	28
1	1.20	0.84	1.17	0.82	1.33	1.32	0.77	1.33
2	1.19	1.11	1.29	0.99	1.27	1.14	1.38	1.37
3	1.37	1.02	1.16	0.83	1.46	0.84	1.31	0.94
4	0.88	0.93	0.96	0.92	0.87	0.85	0.80	0.85
5	1.35	1.18	0.87	1.32	1.13	1.14	0.88	0.80
6	1.05	1.18	1.17	1.23	1.30	1.18	1.06	0.98
7	1.33	0.97	1.04	1.20	1.52	0.93	0.89	1.11
8	1.24	0.75	0.98	1.33	1.32	1.32	1.14	1.04
9	0.89	1.37	1.37	1.02	0.89	1.27	0.84	0.92
10	1.15	1.22	0.85	0.74	0.96	1.40	0.91	0.92
11	0.88	1.35	0.90	0.85	1.49	1.26	1.06	1.23
12	1.07	0.91	1.20	1.34	1.37	0.81	1.34	1.39
13	0.86	0.90	1.11	1.06	1.05	1.16	1.25	0.93
14	1.02	1.26	1.24	1.07	1.06	0.83	1.08	1.23
15	1.47	1.07	0.92	0.81	0.95	1.06	0.83	0.95
16	0.89	1.18	1.10	0.97	1.32	1.17	1.22	1.18
17	1.12	0.98	0.92	0.93	1.13	0.74	0.90	1.56
18	1.03	0.92	0.89	0.91	1.00	0.84	1.00	0.82
19	1.33	0.89	0.99	1.17	1.31	0.87	0.93	1.09
20	1.38	0.80	1.28	1.34	1.44	0.68	1.07	1.45
21	1.37	1.25	1.07	0.87	1.46	0.80	0.91	1.70
22	1.30	1.35	1.32	0.81	1.45	1.22	0.86	1.12
23	1.34	1.00	1.30	1.16	0.82	1.08	0.73	1.13
24	1.19	1.18	1.30	1.36	1.02	0.84	1.28	1.15
25	0.90	1.03	1.26	1.02	0.93	1.23	0.90	0.97
26	0.94	0.82	0.92	0.83	1.32	0.79	0.87	0.60
27	0.81	0.92	1.33	0.89	0.93	0.79	0.84	0.71
28	0.99	0.87	0.94	1.02	0.87	0.84	0.99	1.36
29	1.34	1.11	0.71	0.77	0.79	0.72	0.61	-
30	0.80	1.25	0.74	0.75	1.04	0.95	-	-
31	1.29	1.01	0.75	1.03	0.85	0.73	-	-
32	-	-	-	1.14	0.90	1.13	-	-
33	-	-	-	1.11	-	0.82	-	-
34	-	-	-	0.87	-	-	-	-
Mean	**1.13**	**1.05**	**1.07**	**1.01**	**1.14**	**0.99**	**0.99**	**1.10**
SD	**0.20**	**0.18**	**0.19**	**0.19**	**0.23**	**0.21**	**0.20**	**0.26**
SD%	**18.1**	**16.7**	**18.2**	**18.7**	**20.5**	**21.5**	**19.9**	**23.6**

8.1.4 Juvenile Growth

8.1.4.1 Growth in terms of length and weight

Table 24: Length [cm], day 63 pf

	Nominal concentration fadrozole [µg/L]							
	Control				10			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	25	23	25	25	25	25	25	25
1	2.9	2.8	3.1	3.3	3.0	3.3	3.0	2.7
2	3.2	3.0	2.5	2.3	3.2	3.0	3.0	3.2
3	2.9	2.3	2.9	3.4	3.0	3.0	3.0	2.8
4	2.8	3.0	2.8	2.6	2.8	3.0	2.8	2.5
5	3.1	2.4	2.3	2.7	2.4	3.1	3.1	3.0
6	2.9	3.3	3.3	2.8	2.7	2.7	2.9	3.1
7	2.9	2.9	3.1	3.6	2.7	2.7	3.0	2.9
8	2.2	2.7	3.0	3.2	2.4	2.8	3.0	2.7
9	2.5	2.5	3.0	2.4	2.4	2.5	2.6	2.6
10	3.4	3.0	3.2	2.4	2.3	2.6	3.0	2.5
11	2.7	3.3	3.2	2.5	3.2	2.4	2.9	3.1
12	2.8	2.6	3.0	2.5	3.3	2.9	2.8	3.1
13	1.6	3.7	2.7	2.4	3.1	3.2	2.6	2.9
14	3.0	2.8	2.6	3.0	3.2	2.9	2.2	3.0
15	2.0	2.0	2.5	2.7	2.5	2.7	3.1	2.7
16	3.1	3.3	3.0	2.8	3.0	2.7	2.8	3.1
17	3.0	2.7	2.7	2.8	2.6	2.5	2.7	2.7
18	2.6	2.5	2.5	2.5	3.1	2.6	2.7	2.6
19	2.5	2.8	3.0	2.6	2.8	2.6	2.5	2.7
20	2.6	3.2	2.3	2.3	2.8	2.6	2.5	2.7
21	2.7	3.1	3.2	2.8	2.6	2.9	2.8	2.4
22	2.0	2.7	2.9	2.9	2.7	2.8	3.1	2.9
23	2.4	2.5	2.5	3.5	3.0	3.0	3.0	2.8
24	1.8	-	2.8	2.4	2.9	2.6	3.0	2.8
25	3.3	-	3.0	2.5	2.5	2.8	2.7	2.8
Mean	**2.7**	**2.8**	**2.8**	**2.8**	**2.8**	**2.8**	**2.8**	**2.8**
SD	**0.5**	**0.4**	**0.3**	**0.4**	**0.3**	**0.2**	**0.2**	**0.2**
SD%	**17.4**	**13.7**	**10.3**	**13.9**	**10.5**	**8.2**	**8.0**	**7.6**

Table 24 (continued): Length [cm], day 63 pf

	Nominal concentration fadrozole [µg/L]							
	32				100			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	25	25	25	25	25	24	25	25
1	3.0	3.0	3.1	3.0	2.8	2.4	3.1	3.0
2	3.3	3.3	2.9	3.0	2.4	2.7	3.0	3.0
3	2.0	3.1	2.9	3.0	2.6	3.1	3.0	3.0
4	3.4	2.9	2.8	3.0	2.8	3.1	2.6	3.1
5	2.8	2.7	2.8	3.0	2.9	3.0	3.1	2.7
6	2.7	3.0	2.9	2.8	2.8	2.8	2.5	2.9
7	2.5	3.2	2.4	2.5	3.2	3.0	3.2	2.3
8	2.7	2.7	2.4	3.0	2.6	3.1	2.7	3.4
9	2.9	2.8	3.2	2.5	2.9	3.0	2.7	2.5
10	2.4	2.3	3.0	2.6	3.0	2.9	2.9	2.8
11	2.5	3.2	3.2	2.7	3.3	2.7	2.4	2.6
12	2.6	3.0	3.0	2.8	3.1	3.1	2.6	2.6
13	3.1	3.1	2.9	2.5	3.1	2.8	3.1	2.7
14	3.2	2.6	3.0	2.5	2.5	3.0	2.5	3.1
15	2.8	2.8	2.8	3.1	2.6	3.0	2.9	3.1
16	3.0	2.5	2.7	2.6	2.9	2.8	2.6	3.1
17	3.0	2.4	2.6	2.5	3.0	3.2	3.0	3.1
18	3.1	2.6	3.0	2.4	3.1	3.2	2.8	3.0
19	3.0	2.8	3.1	2.4	2.3	2.7	2.7	3.2
20	2.8	2.5	2.6	1.8	3.0	2.7	2.5	2.5
21	2.7	3.1	2.7	2.9	2.6	2.6	2.3	2.7
22	3.2	2.7	2.6	2.8	2.9	2.8	3.0	2.6
23	3.2	2.7	2.9	3.1	3.2	2.4	2.9	2.8
24	3.1	2.9	3.0	3.0	3.1	2.6	2.4	2.9
25	2.6	3.0	2.7	2.8	3.2	-	3.0	2.4
Mean	**2.9**	**2.8**	**2.8**	**2.7**	**2.9**	**2.9**	**2.8**	**2.8**
SD	**0.3**	**0.3**	**0.2**	**0.3**	**0.3**	**0.2**	**0.3**	**0.3**
SD%	**11.3**	**9.4**	**7.7**	**11.1**	**9.4**	**8.1**	**9.4**	**9.7**

Table 25: Weight [g], day 63 pf

	Nominal concentration fadrozole [µg/L]							
	Control				10			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	25	23	25	25	25	25	25	25
1	0.196	0.193	0.232	0.253	0.241	0.263	0.194	0.157
2	0.243	0.230	0.111	0.098	0.264	0.220	0.225	0.253
3	0.237	0.084	0.190	0.354	0.238	0.226	0.204	0.171
4	0.168	0.243	0.210	0.170	0.167	0.209	0.179	0.178
5	0.247	0.127	0.108	0.159	0.175	0.245	0.230	0.238
6	0.186	0.295	0.277	0.166	0.194	0.146	0.182	0.221
7	0.211	0.215	0.257	0.354	0.173	0.162	0.209	0.220
8	0.078	0.150	0.231	0.255	0.122	0.180	0.226	0.154
9	0.136	0.132	0.218	0.121	0.122	0.132	0.144	0.152
10	0.298	0.235	0.252	0.144	0.121	0.143	0.209	0.148
11	0.154	0.274	0.290	0.116	0.242	0.128	0.200	0.260
12	0.169	0.130	0.230	0.131	0.265	0.219	0.207	0.246
13	0.167	0.180	0.182	0.102	0.265	0.289	0.131	0.183
14	0.216	0.174	0.137	0.210	0.265	0.219	0.093	0.224
15	0.087	0.081	0.120	0.157	0.099	0.170	0.238	0.166
16	0.218	0.251	0.216	0.228	0.232	0.191	0.200	0.234
17	0.204	0.166	0.179	0.192	0.155	0.129	0.164	0.180
18	0.160	0.140	0.156	0.158	0.217	0.152	0.185	0.178
19	0.132	0.166	0.214	0.136	0.178	0.157	0.127	0.179
20	0.154	0.241	0.102	0.095	0.190	0.135	0.121	0.161
21	0.153	0.236	0.241	0.142	0.120	0.160	0.170	0.096
22	0.054	0.151	0.190	0.205	0.150	0.155	0.201	0.198
23	0.106	0.117	0.210	0.380	0.206	0.223	0.205	0.169
24	0.033	-	0.159	0.117	0.194	0.127	0.222	0.161
25	0.257	-	0.207	0.108	0.126	0.176	0.161	0.188
Mean	**0.171**	**0.183**	**0.197**	**0.182**	**0.189**	**0.182**	**0.185**	**0.189**
SD	**0.066**	**0.060**	**0.053**	**0.082**	**0.053**	**0.046**	**0.038**	**0.039**
SD%	**38.4**	**32.7**	**26.7**	**45.0**	**28.2**	**25.1**	**20.5**	**20.9**

Table 25 (continued): Weight [g], day 63 pf

	Nominal concentration fadrozole [µg/L]							
	32				100			
Test vessel	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
n	25	25	25	25	25	24	25	25
1	0.224	0.201	0.230	0.233	0.151	0.103	0.241	0.230
2	0.275	0.262	0.207	0.221	0.121	0.153	0.206	0.212
3	0.059	0.223	0.226	0.232	0.137	0.237	0.210	0.239
4	0.284	0.212	0.183	0.233	0.174	0.250	0.133	0.264
5	0.221	0.176	0.174	0.225	0.206	0.260	0.260	0.187
6	0.158	0.214	0.194	0.195	0.196	0.198	0.129	0.245
7	0.122	0.268	0.102	0.157	0.264	0.222	0.241	0.112
8	0.149	0.168	0.097	0.230	0.157	0.247	0.161	0.337
9	0.185	0.181	0.235	0.151	0.210	0.243	0.151	0.146
10	0.110	0.103	0.216	0.149	0.215	0.240	0.209	0.197
11	0.103	0.245	0.237	0.158	0.294	0.177	0.118	0.145
12	0.123	0.223	0.198	0.184	0.259	0.251	0.156	0.134
13	0.247	0.265	0.170	0.128	0.259	0.174	0.237	0.156
14	0.234	0.168	0.236	0.122	0.134	0.227	0.143	0.246
15	0.208	0.185	0.142	0.247	0.162	0.223	0.190	0.262
16	0.223	0.138	0.147	0.144	0.188	0.202	0.157	0.242
17	0.210	0.119	0.199	0.137	0.230	0.261	0.220	0.226
18	0.239	0.175	0.238	0.097	0.252	0.273	0.187	0.229
19	0.235	0.199	0.223	0.108	0.112	0.164	0.138	0.251
20	0.210	0.155	0.147	0.042	0.245	0.171	0.121	0.123
21	0.143	0.240	0.153	0.182	0.143	0.120	0.084	0.162
22	0.257	0.164	0.137	0.187	0.208	0.151	0.215	0.142
23	0.276	0.167	0.173	0.244	0.255	0.127	0.200	0.190
24	0.249	0.199	0.217	0.216	0.273	0.141	0.103	0.212
25	0.158	0.217	0.131	0.151	0.273	-	0.234	0.088
Mean	**0.196**	**0.195**	**0.184**	**0.175**	**0.205**	**0.201**	**0.178**	**0.199**
SD	**0.061**	**0.043**	**0.043**	**0.053**	**0.054**	**0.051**	**0.049**	**0.059**
SD%	**31.3**	**22.3**	**23.4**	**30.4**	**26.6**	**25.3**	**27.7**	**29.5**

8.1.5 Histopathology

Table 26: Histopathology, females

Nominal conc. [µg/L]	Test vessel	Sample name	Sex	Ovary maturation stage	Increased oocyte atresia	Interstitial fibrosis	Egg debris	Granulomatous inflammation	Increased postovulatory follicles
control	**A**	0/1-2	f	1	0	0	0	0	0
		0/1-4	f	1	0	0	0	0	0
		0/1-10	f	2	0	0	0	0	0
		0/1-12	f	1	**0**	**0**	**0**	**0**	**0**
		0/1-13	f	1	0	0	0	0	0
		0/1-14	f	1	0	0	0	0	0
		0/1-15	f	1	0	0	0	0	0
		0/1-18	f	0	0	0	0	0	0
		0/1-19	f	0	0	0	0	0	0
control	**B**	0/2-3	f	0	0	0	0	0	0
		0/2-4	f	1	0	0	0	0	0
		0/2-5	f	0	0	0	0	0	0
		0/2-6	f	2	0	0	0	0	0
		0/2-7	f	0	0	0	0	0	0
		0/2-8	f	1	0	0	0	0	0
		0/2-9	f	0	0	0	0	0	0
		0/2-11	f	1	**0**	**0**	**0**	**0**	**0**
		0/2-13	f	2	0	0	0	0	0
		0/2-15	f	0	0	0	0	0	0
		0/2-16	f	0	0	0	0	0	0
control	**C**	0/3-2	f	0	0	0	0	0	0
		0/3-4	f	2	0	0	0	0	0
		0/3-6	f	2	0	0	0	0	0
		0/3-7	f	1	0	0	0	0	0
		0/3-9	f	1	0	0	0	0	0
		0/3-11	f	1	0	0	0	0	0
		0/3-13	f	1	0	0	0	0	0
		0/3-14	f	0	0	0	0	0	0
		0/3-16	f	1	0	0	0	0	0
		0/3-18	f	0	0	0	0	0	0

Table 26 (continued): Histopathology, females

Nominal conc. [µg/L]	Test vessel	Sample name	Sex	Ovary matura-tion stage	Increased oocyte atresia	Interstitial fibrosis	Egg debris	Granuloma-tomous in-flammation	Increased postovula-tory follicles
control	**D**	0/4-1	f	1	0	0	0	0	0
		0/4-3	f	1	0	0	0	0	0
		0/4-4	f	1	0	0	0	0	0
		0/4-7	f	1	0	0	0	0	0
		0/4-9	f	0	0	0	0	0	0
		0/4-13	f	0	0	0	0	0	0
		0/4-16	f	1	0	0	0	0	0
		0/4-17	f	1	0	0	0	0	0
10	**A**	1/1-10	f	0	0	0	0	0	0

Ovary maturation stages: 0 = undeveloped; 1 = early development; 2 = mid-development; 3 = late development; 4 = late development/hydrated; for calculation of the maturity index, stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc. (Baumann et al., 2013)
Severity grades: 0 = not observable; 1 = mild; 2 = medium; 3 = strong

Table 27: Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
control	A	0/1-1	m	1	0	0	0	0	0	0	0
		0/1-3	m	1	0	0	0	0	0	0	0
		0/1-6	m	1	0	0	0	0	0	0	0
		0/1-7	m	1	3	0	0	0	0	0	0
		0/1-8	m	1	0	0	0	0	0	0	0
		0/1-9	m	1	2	0	0	0	0	0	0
		0/1-11	m	1	0	0	0	0	0	0	0
		0/1-16	m	2	1	0	0	0	0	0	0
		0/1-17	m	2	0	0	0	0	0	0	0
		0/1-20	m	0	2	0	0	0	0	0	0
control	B	0/2-1	m	1	1	0	0	0	0	0	0
		0/2-2	m	1	0	0	0	0	0	0	0
		0/2-10	m	1	1	0	0	0	0	0	0
		0/2-12	m	1	3	0	0	0	0	0	0
		0/2-14	m	1	0	0	0	0	0	0	0
		0/2-17	m	1	0	0	0	0	0	0	0
		0/2-18	m	1	1	0	0	0	0	0	0
control	C	0/3-1	m	1	0	0	0	0	0	0	0
		0/3-3	m	1	0	0	0	0	0	0	0
		0/3-5	m	1	0	0	0	0	0	0	0
		0/3-8	m	1	0	0	0	0	0	0	0
		0/3-10	m	1	1	0	0	0	0	0	0
		0/3-12	m	2	0	0	0	0	0	0	0
		0/3-15	m	1	0	0	0	0	0	0	0
		0/3-17	m	1	0	0	0	0	0	0	0
		0/3-19	m	2	0	0	0	0	0	0	0
		0/3-20	m	1	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
control	D	0/4-2	m	1	0	0	0	0	0	0	0
		0/4-5	m	1	0	0	0	0	0	0	0
		0/4-8	m	2	0	0	0	0	0	0	0
		0/4-10	m	1	1	0	0	0	0	0	0
		0/4-11	m	1	1	0	0	0	0	0	0
		0/4-12	m	1	2	0	0	0	0	0	0
		0/4-14	m	1	1	0	0	0	0	0	0
		0/4-15	m	1	0	0	0	0	0	0	0
		0/4-18	m	1	0	0	0	0	0	0	0
		0/4-19	m	1	0	0	0	0	0	0	0
		0/4-20	m	1	2	0	0	0	0	0	0
10	A	1/1-1	m	2	0	0	0	0	0	0	0
		1/1-2	m	2	0	0	0	0	0	0	0
		1/1-3	m	1	0	0	0	0	0	0	0
		1/1-4	m	2	1	0	0	0	0	0	0
		1/1-5	m	1	0	0	0	0	0	0	0
		1/1-6	m	2	1	0	0	0	0	0	0
		1/1-7	m	1	0	0	0	0	0	0	0
		1/1-8	m	1	0	0	0	0	0	0	0
		1/1-9	m	1	0	0	0	0	0	0	0
		1/1-11	m	2	0	0	0	0	0	0	0
		1/1-12	m	1	0	0	0	0	0	0	0
		1/1-13	m	2	0	0	0	0	0	0	0
		1/1-14	m	2	0	0	0	0	0	0	0
		1/1-15	m	1	0	0	0	0	0	0	0
		1/1-16	m	1	0	0	0	0	0	0	0
		1/1-17	m	1	1	0	0	0	0	0	0
		1/1-18	m	1	3	0	0	0	0	0	0
		1/1-19	m	1	0	0	0	0	0	0	0
		1/1-20	m	1	1	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
10	B	1/2-1	m	2	0	0	0	0	0	0	0
		1/2-2	m	1	0	0	0	0	0	0	0
		1/2-3	m	1	0	0	0	0	0	0	0
		1/2-4	m	2	0	0	0	0	0	0	0
		1/2-6	m	1	0	0	0	0	0	0	0
		1/2-7	m	1	0	0	0	0	0	0	0
		1/2-8	m	2	0	0	0	0	0	0	0
		1/2-9	m	1	0	0	0	0	0	0	0
		1/2-10	m	1	0	0	0	0	0	0	0
		1/2-11	m	1	2	0	0	0	0	0	0
		1/2-13	m	2	1	0	0	0	0	0	0
		1/2-14	m	1	0	0	0	0	0	0	0
		1/2-15	m	1	0	0	0	0	0	0	0
		1/2-16	m	1	0	0	0	0	0	0	0
		1/2-17	m	1	0	0	0	0	0	0	0
		1/2-18	m	2	0	0	0	0	0	0	0
		1/2-19	m	2	0	0	0	0	0	0	0
		1/2-20	m	1	0	0	0	0	0	0	0
10	C	1/3-1	m	2	0	0	0	0	0	0	0
		1/3-3	m	2	1	0	0	0	0	0	0
		1/3-4	m	1	0	0	0	0	0	0	0
		1/3-5	m	2	0	0	0	0	0	0	0
		1/3-6	m	1	0	0	0	0	0	0	0
		1/3-7	m	2	0	0	0	0	0	0	0
		1/3-8	m	2	0	0	0	0	0	0	0
		1/3-9	m	1	0	0	0	0	0	0	0
		1/3-10	m	2	0	0	0	0	0	0	0
		1/3-11	m	2	0	0	0	0	0	0	0
		1/3-13	m	1	0	0	0	0	0	0	0
		1/3-14	m	1	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
10	C	1/3-15	m	2	0	0	0	0	0	0	0
		1/3-16	m	1	0	0	0	0	0	0	0
		1/3-17	m	1	0	0	0	0	0	0	0
		1/3-18	m	2	0	0	0	0	0	0	0
		1/3-19	m	2	0	0	0	0	0	0	0
		1/3-20	m	1	0	0	0	0	0	0	0
10	D	1/4-1	m	1	0	0	0	0	0	0	0
		1/4-2	m	2	0	0	0	0	0	0	0
		1/4-3	m	1	0	0	0	0	0	0	0
		1/4-4	m	1	0	0	0	0	0	0	0
		1/4-5	m	2	0	0	0	0	0	0	0
		1/4-6	m	1	0	0	0	0	0	0	0
		1/4-7	m	1	0	0	0	0	0	0	0
		1/4-8	m	1	1	0	0	0	0	0	0
		1/4-9	m	1	0	0	0	0	0	0	0
		1/4-10	m	1	1	0	0	0	0	0	0
		1/4-11	m	2	0	0	0	0	0	0	0
		1/4-12	m	2	0	0	0	0	0	0	0
		1/4-13	m	1	0	0	0	0	0	0	0
		1/4-14	m	2	0	0	0	0	0	0	0
		1/4-15	m	1	2	0	0	0	0	0	0
		1/4-16	m	2	0	0	0	0	0	0	0
		1/4-17	m	1	0	0	0	0	0	0	0
		1/4-18	m	1	0	0	0	0	0	0	0
		1/4-19	m	1	0	0	0	0	0	0	0
		1/4-20	m	1	1	0	0	0	0	0	0
32	A	2/1-1	m	2	1	0	0	0	0	0	0
		2/1-2	m	2	1	0	0	0	0	0	0
		2/1-3	m	1	0	0	0	0	0	0	0
		2/1-4	m	2	0	0	0	0	0	0	0
		2/1-5	m	2	0	0	0	0	0	0	0
		2/1-6	m	1	1	0	0	0	0	0	0
		2/1-7	m	1	0	0	0	0	0	0	0
		2/1-8	m	1	0	0	0	0	0	0	0
		2/1-9	m	1	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
32	A	2/1-10	m	1	0	0	0	0	0	0	0
		2/1-11	m	1	0	0	0	0	0	0	0
		2/1-12	m	1	0	0	0	0	0	0	0
		2/1-13	m	1	0	0	0	0	0	0	0
		2/1-14	m	2	2	0	0	0	0	0	0
		2/1-15	m	2	0	0	0	0	0	0	0
		2/1-17	m	1	0	0	0	0	0	0	0
		2/1-18	m	2	0	0	0	0	0	0	0
		2/1-19	m	1	0	0	0	0	0	0	0
		2/1-20	m	2	0	0	0	0	0	0	0
32	B	2/2-1	m	2	2	0	0	0	0	0	0
		2/2-2	m	2	0	0	0	0	0	0	0
		2/2-3	m	1	0	0	0	0	0	0	0
		2/2-4	m	1	0	0	0	0	0	0	0
		2/2-5	m	2	0	0	0	0	0	0	0
		2/2-6	m	2	0	0	0	0	0	0	0
		2/2-7	m	2	0	0	0	0	0	0	0
		2/2-8	m	1	1	0	0	0	0	0	0
		2/2-9	m	1	0	0	0	0	0	0	0
		2/2-10	m	1	0	0	0	0	0	0	0
		2/2-11	m	2	0	0	0	0	0	0	0
		2/2-12	m	2	0	0	0	0	0	0	0
		2/2-13	m	2	0	0	0	0	0	0	0
		2/2-14	m	1	0	0	0	0	0	0	0
		2/2-15	m	2	0	0	0	0	0	0	0
		2/2-16	m	1	1	0	0	0	0	0	0
		2/2-17	m	1	0	0	0	0	0	0	0
		2/2-18	m	1	1	0	0	0	0	0	0
		2/2-19	m	1	0	0	0	0	0	0	0
		2/2-20	m	1	0	0	0	0	0	0	0
32	C	2/3-1	m	1	3	0	0	0	0	0	0
		2/3-2	m	1	0	0	0	0	0	0	0
		2/3-3	m	2	0	0	0	0	0	0	0
		2/3-4	m	2	0	0	0	0	0	0	0
		2/3-5	m	2	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
32	C	2/3-6	m	2	0	0	0	0	0	0	0
		2/3-7	m	1	0	0	0	0	0	0	0
		2/3-8	m	1	0	0	0	0	0	0	0
		2/3-9	m	1	0	0	0	0	0	0	0
		2/3-10	m	2	0	0	0	0	0	0	0
		2/3-11	m	2	0	0	0	0	0	0	0
		2/3-12	m	1	0	0	0	0	0	0	0
		2/3-13	m	2	0	0	0	0	0	0	0
		2/3-14	m	2	1	0	0	0	0	0	0
		2/3-15	m	1	0	0	0	0	0	0	0
		2/3-16	m	1	1	0	0	0	0	0	0
		2/3-17	m	1	0	0	0	0	0	0	0
		2/3-18	m	2	0	0	0	0	0	0	0
		2/3-19	m	2	0	0	0	0	0	0	0
		2/3-20	m	2	0	0	0	0	0	0	0
32	D	2/4-1	m	2	0	0	0	0	0	0	0
		2/4-2	m	2	0	0	0	0	0	0	0
		2/4-3	m	2	0	0	0	0	0	0	0
		2/4-4	m	1	0	0	0	0	0	0	0
		2/4-5	m	2	0	0	0	0	0	0	0
		2/4-7	m	1	1	0	0	0	0	0	0
		2/4-9	m	1	0	0	0	0	0	0	0
		2/4-10	m	1	0	0	0	0	0	0	0
		2/4-11	m	2	0	0	0	0	0	0	0
		2/4-12	m	2	0	0	0	0	0	0	0
		2/4-13	m	1	0	0	0	0	0	0	0
		2/4-14	m	1	0	0	0	0	0	0	0
		2/4-15	m	1	0	0	0	0	0	0	0
		2/4-16	m	2	0	0	0	0	0	0	0
		2/4-17	m	2	0	0	0	0	0	0	0
		2/4-18	m	2	0	0	0	0	0	0	0
		2/4-19	m	1	0	0	0	0	0	0	0
		2/4-20	m	1	2	0	0	0	0	0	0
100	A	3/1-1	m	2	0	0	0	0	0	0	0
		3/1-2	m	2	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
100	A	3/1-3	m	1	1	0	0	0	0	0	0
		3/1-4	m	2	0	0	0	0	0	0	0
		3/1-5	m	1	0	0	0	0	0	0	0
		3/1-6	m	2	0	0	0	0	0	0	0
		3/1-7	m	2	0	0	0	0	0	0	0
		3/1-8	m	1	0	0	0	0	0	0	0
		3/1-9	m	2	0	0	0	0	0	0	0
		3/1-10	m	2	0	0	0	0	0	0	0
		3/1-11	m	2	0	0	0	0	0	0	0
		3/1-12	m	2	0	0	0	0	0	0	0
		3/1-13	m	1	0	0	0	0	0	0	0
		3/1-14	m	2	1	0	0	0	0	0	0
		3/1-15	m	1	0	0	0	0	0	0	0
		3/1-16	m	2	0	0	0	0	0	0	0
		3/1-17	m	2	0	0	0	0	0	0	0
		3/1-18	m	2	0	0	0	0	0	0	0
		3/1-19	m	1	0	0	0	0	0	0	0
		3/1-20	m	2	1	0	0	0	0	0	0
100	B	3/2-1	m	1	2	0	0	0	0	0	0
		3/2-2	m	2	0	0	0	0	0	0	0
		3/2-3	m	2	0	0	0	0	0	0	0
		3/2-4	m	1	0	0	0	0	0	0	0
		3/2-5	m	2	1	0	0	0	0	0	0
		3/2-6	m	2	3	0	0	0	0	0	0
		3/2-8	m	2	0	0	0	0	0	0	0
		3/2-9	m	2	0	0	0	0	0	0	0
		3/2-10	m	2	2	0	0	0	0	0	0
		3/2-11	m	2	0	0	0	0	0	0	0
		3/2-12	m	2	0	0	0	0	0	0	0
		3/2-13	m	2	0	0	0	0	0	0	0
		3/2-14	m	1	0	0	0	0	0	0	0
		3/2-15	m	2	0	0	0	0	0	0	0
		3/2-16	m	2	0	0	0	0	0	0	0
		3/2-17	m	2	0	0	0	0	0	0	0
		3/2-18	m	2	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
100	B	3/2-19	m	2	0	0	0	0	0	0	0
100	C	3/3-1	m	2	0	0	0	0	0	0	0
		3/3-2	m	2	0	0	0	0	0	0	0
		3/3-3	m	2	1	0	0	0	0	0	0
		3/3-4	m	2	0	0	0	0	0	0	0
		3/3-5	m	2	0	0	0	0	0	0	0
		3/3-6	m	2	0	0	0	0	0	0	0
		3/3-7	m	2	0	0	0	0	0	0	0
		3/3-8	m	2	0	0	0	0	0	0	0
		3/3-9	m	2	0	0	0	0	0	0	0
		3/3-10	m	2	1	0	0	0	0	0	0
		3/3-11	m	1	0	0	0	0	0	0	0
		3/3-12	m	2	0	0	0	0	0	0	0
		3/3-13	m	2	0	0	0	0	0	0	0
		3/3-14	m	2	0	0	0	0	0	0	0
		3/3-15	m	2	0	0	0	0	0	0	0
		3/3-16	m	1	0	0	0	0	0	0	0
		3/3-17	m	1	0	0	0	0	0	0	0
		3/3-18	m	1	0	0	0	0	0	0	0
		3/3-19	m	1	0	0	0	0	0	0	0
		3/3-20	m	1	0	0	0	0	0	0	0
100	D	3/4-1	m	2	0	0	0	0	0	0	0
		3/4-2	m	2	0	0	0	0	0	0	0
		3/4-3	m	2	2	0	0	0	0	0	0
		3/4-4	m	2	0	0	0	0	0	0	0
		3/4-5	m	2	0	0	0	0	0	0	0
		3/4-6	m	2	1	0	0	0	0	0	0
		3/4-7	m	1	1	0	0	0	0	0	0
		3/4-8	m	2	0	0	0	0	0	0	0
		3/4-9	m	1	0	0	0	0	0	0	0
		3/4-10	m	2	0	0	0	0	0	0	0
		3/4-11	m	2	0	0	0	0	0	0	0
		3/4-12	m	1	0	0	0	0	0	0	0
		3/4-13	m	1	0	0	0	0	0	0	0
		3/4-14	m	2	0	0	0	0	0	0	0

Table 27 (continued): Histopathology, males

Nominal conc. [ng/L]	Test vessel	Sample name	Sex	Testis maturation stage	Testis ova	Increased testicular degeneration	Interstitial cell hyper-trophy/hyperplasia	Increased proportion of spermatogonia	Increased interstitial proteinaceous fluid	Asynchronous germ cell development	Granulomatomous inflammation
100	D	3/4-15	m	2	0	0	0	0	0	0	0
		3/4-16	m	2	1	0	0	0	0	0	0
		3/4-17	m	2	0	0	0	0	0	0	0
		3/4-18	m	1	0	0	0	0	0	0	0
		3/4-19	m	2	0	0	0	0	0	0	0
		3/4-20	m	1	1	0	0	0	0	0	0

Testis maturation stages: 0 = undeveloped; 1 = early spermatogenic; 2 = mid-spermatogenic; 3 = late spermatogenic; for calculation of the maturity index, stage 0 corresponds to value 1; stage 1 corresponds to value 2; etc. (Baumann et al., 2013); Severity grades: 0 = not observable; 1 = mild; 2 = medium; 3 = strong

8.1.6 Vitellogenin measurements

Table 28: Calibration data of VTG assays, equation parameters, regression coefficient and absorbance of blank (NSB)

Assay			Equation ($y=a*x^b$)			Absorbance E		
No.	Date	Plate	a	b	R^2	Standard (high)	Blank (NSB)	% of SD
1	07.04.2014	A	0.1299	0.8795	0.9908	2.170	0.092	4.2
2	08.04.2014	A	0.1957	0.8569	0.9949	2.520	0.097	3.8
3	08.04.2014	B	0.1694	0.8015	0.9963	2.301	0.094	4.1
4	10.04.2014	A	0.1574	0.8351	0.9926	2.263	0.106	4.7
5	10.04.2014	B	0.1449	0.8413	0.9939	2.150	0.096	4.5
6	11.04.2014	A	0.1414	0.8401	0.9925	2.097	0.117	5.6
7	11.04.2014	B	0.1228	0.8828	0.9964	1.929	0.113	5.9
8	14.04.2014	A	0.1201	0.8769	0.9969	1.942	0.107	5.5
9	14.04.2014	B	0.1384	0.8239	0.9940	2.006	0.107	5.3
10	15.04.2014	A	0.1253	0.8646	0.9983	1.807	0.101	5.6
11	15.04.2014	B	0.1196	0.8809	0.9964	1.826	0.101	5.5
12	16.04.2014	A	0.1620	0.7851	0.9980	2.174	0.111	5.1
13	16.04.2014	B	0.1611	0.8060	0.9949	2.191	0.111	5.1
14	22.04.2014	A	0.1192	0.8492	0.9974	1.173	0.102	8.7
15	22.04.2014	B	0.1020	0.9402	0.9975	1.214	0.103	8.5
16	23.04.2014	A	0.1476	0.8617	0.9982	1.444	0.104	7.2

Table 28 (continued): Calibration data of VTG assays, equation parameters, regression coefficient and absorbance of blank (NSB)

Assay			Equation ($y=a*x^b$)			Absorbance E		
No.	Date	Plate	a	b	R^2	Standard (high)	Blank (NSB)	% of SD
17	23.04.2014	B	0.1316	0.8875	0.9979	1.382	0.107	7.7
18	28.04.2014	A	0.1201	0.8478	0.9951	1.141	0.099	8.7
19	28.04.2014	B	0.1024	0.8871	0.9987	1.104	0.102	9.2
20	29.04.2014	A	0.1394	0.8523	0.9986	1.349	0.102	7.6
21	29.04.2014	B	0.1731	0.7599	0.9995	1.411	0.102	7.2

Table 29: LOD and LOQ of all VTG assays

No.	Date	Plate	LOD [ng/mL]	Lowest dilution factor	LOQ [ng/mL]	Sex of analysed fish
1	07.04.2014	A	0.44	5000	2200	female
2	08.04.2014	A	0.44	500	220	female
3	08.04.2014	B	0.44	500	220	female
4	10.04.2014	A	0.44	50	22	male
5	10.04.2014	B	0.44	50	22	male
6	11.04.2014	A	0.44	50	22	male
7	11.04.2014	B	0.44	50	22	male
8	14.04.2014	A	0.44	50	22	male
9	14.04.2014	B	0.44	50	22	male
10	15.04.2014	A	0.44	50	22	male
11	15.04.2014	B	0.44	50	22	male
12	16.04.2014	A	0.44	50	22	male
13	16.04.2014	B	0.44	50	22	male
14	22.04.2014	A	0.22	50	11	male
15	22.04.2014	B	0.22	50	11	male
16	23.04.2014	A	0.22	50	11	male
17	23.04.2014	B	0.22	50	11	male
18	28.04.2014	A	0.22	50	11	male
19	28.04.2014	B	0.22	50	11	male
20	29.04.2014	A	0.22	50	11	male
21	29.04.2014	B	0.22	50	11	male

Table 30: Vitellogenin content in the control treatment

Control							
females				males			
A							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
0/1 2	2.24E+02	755.2	0.30	0/1 1	2.29E+00	1875.51	0.001
0/1 4	2.90E+02	611.65	0.47	0/1 3	1.32E+01	889.38	0.015
0/1 10	-	876.9	-	0/1 6	1.69E+01	652.22	0.026
0/1 12	2.44E+02	508.67	0.48	0/1 7	3.68E+00	864.42	0.004
0/1 13	5.50E+00	904.99	0.01	0/1 8	1.58E+00	308.94	0.005
0/1 14	5.09E+04	664.7	76.51	0/1 9	1.47E+00	1020.45	0.001
0/1 15	5.50E+00	723.99	0.01	0/1 11	3.88E+00	1048.54	0.004
0/1 18	5.50E+00	642.85	0.01	0/1 16	2.90E+00	805.13	0.004
0/1 19	5.05E+01	714.63	0.07	0/1 17	3.18E+00	730.23	0.004
-	-	-	-	0/1 20	5.17E+00	471.22	0.011
Mean			**9.73**	**Mean**			**0.008**
SD			**26.98**	**SD**			**0.008**
SD%			**277.3**	**SD%**			**102.4**
n			**8**	**n**			**10**
B							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
0/2 3	1.38E+01	421.29	0.03	0/2 1	2.18E+00	833.21	0.003
0/2 4	7.21E+03	1110.95	6.49	0/2 2	4.79E+00	695.9	0.007
0/2 5	5.50E+00	636.61	0.01	0/2 10	4.87E+00	483.7	0.010
0/2 6	7.65E+04	917.47	83.42	0/2 12	4.29E+00	337.03	0.013
0/2 7	3.65E+02	708.39	0.52	0/2 14	2.88E+00	549.23	0.005
0/2 8	4.13E+03	730.23	5.66	0/2 17	0.00E+00	689.66	0.000
0/2 9	4.35E+01	470.76	0.09	0/2 18	2.34E+00	861.3	0.003
0/2 11	1.58E+04	739.59	21.40	-	-	-	-
0/2 13	4.74E+03	755.2	6.28	-	-	-	-
0/2 15	1.94E+01	461.86	0.04	-	-	-	-
0/2 16	3.21E+03	833.21	3.85	-	-	-	-
Mean			**11.62**	**Mean**			**0.006**
SD			**24.62**	**SD**			**0.004**
SD%			**211.9**	**SD%**			**78.1**
n			**11**	**n**			**7**

Table 30 (continued): Vitellogenin content in the control treatment

C							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
0/3 2	5.81E+01	808.25	0.07	0/3 1	5.07E+00	1563.44	0.003
0/3 4	5.05E+03	701.14	7.20	0/3 3	4.18E+00	780.09	0.005
0/3 6	6.32E+03	1320.16	4.78	0/3 5	6.93E+00	691.66	0.010
0/3 7	4.00E+04	941.16	42.54	0/3 8	4.00E+00	631.65	0.006
0/3 9	7.71E+02	622.18	1.24	0/3 10	3.78E+00	792.73	0.005
0/3 11	1.01E+04	770.62	13.11	0/3 12	2.77E+00	647.45	0.004
0/3 13	1.02E+05	988.54	103.66	0/3 15	-	600.07	-
0/3 14	1.14E+02	540.06	0.21	0/3 17	6.67E+00	745.35	0.009
0/3 16	6.16E+03	792.73	7.77	0/3 19	7.01E+00	903.27	0.008
0/3 18	1.05E+02	663.24	0.16	0/3 20	1.94E+00	454.79	0.004
Mean			**18.07**	**Mean**			**0.006**
SD			**32.65**	**SD**			**0.002**
SD%			**180.7**	**SD%**			**38.2**
n			**10**	**n**			**9**
D							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
0/4 1	8.39E+01	1256.99	0.07	0/4 2	-	701.14	-
0/4 3	3.50E+04	1225.41	28.58	0/4 5	3.63E+00	808.52	0.004
0/4 4	4.78E+03	843.26	5.66	0/4 8	3.74E+01	1035.91	0.036
0/4 7	3.68E+04	1121.19	32.82	0/4 10	-	549.54	-
0/4 9	4.87E+01	533.75	0.09	0/4 11	-	521.11	-
0/4 13	7.94E+01	663.24	0.12	0/4 12	4.15E+00	524.27	0.008
0/4 16	5.27E+03	859.05	6.13	0/4 14	5.51E+00	1035.91	0.005
0/4 17	7.05E+02	773.78	0.91	0/4 15	6.53E+00	739.04	0.009
-	-	-	-	0/4 18	5.37E+00	900.11	0.006
-	-	-	-	0/4 19	2.53E+00	530.59	0.005
-	-	-	-	0/4 20	9.90E-01	685.34	0.001
Mean			**9.30**	**Mean**			**0.009**
SD			**13.48**	**SD**			**0.011**
SD%			**145.0**	**SD%**			**118.1**
n			**8**	**n**			**8**
Total mean females			**12.18**	**Total mean males**			**0.007**
SD			**4.06**	**SD**			**0.002**
SD%			**33.3**	**SD%**			**22.8**
n			**4**	**n**			**4**

Table 31: Vitellogenin content in the 10 µg fadrozole/L treatment

10 µg fadrozole/L							
females				males			
A							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
1/1 10	5.50E+00	502.45	0.011	1/1 1	1.14E+01	1326.47	0.009
	-	-	-	1/1 2	6.67E+00	789.57	0.008
	-	-	-	1/1 3	2.07E+00	900.21	0.002
	-	-	-	1/1 4	-	544.32	-
	-	-	-	1/1 5	3.09E+00	678.9	0.005
	-	-	-	1/1 6	2.23E+00	628.06	0.004
	-	-	-	1/1 7	3.83E+00	610.11	0.006
	-	-	-	1/1 8	1.06E+00	535.34	0.002
	-	-	-	1/1 9	2.25E+00	487.49	0.005
	-	-	-	1/1 11	5.03E+00	858.34	0.006
	-	-	-	1/1 12	1.56E+00	888.25	0.002
	-	-	-	1/1 13	1.56E+00	801.52	0.002
	-	-	-	1/1 14	-	559.27	-
	-	-	-	1/1 15	2.41E+00	622.08	0.004
	-	-	-	1/1 16	4.14E+00	801.52	0.005
	-	-	-	1/1 17	1.78E+00	574.22	0.003
	-	-	-	1/1 18	-	741.7	-
	-	-	-	1/1 19	4.35E+00	604.13	0.007
	-	-	-	1/1 20	2.63E+00	505.44	0.005
Mean			**0.011**	**Mean**			**0.005**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**47.6**
n			**1**	**n**			**16**
B							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
No females present				1/2 1	3.18E+00	601.14	0.005
				1/2 2	4.08E+00	610.11	0.007
				1/2 3	4.55E+00	660.95	0.007
				1/2 4	2.32E+00	565.25	0.004
				1/2 6	-	448.61	-
				1/2 7	3.36E+00	463.57	0.007
				1/2 8	3.68E+00	1028.82	0.004
				1/2 9	-	481.51	-
				1/2 10	-	532.35	-

Table 31 (continued): Vitellogenin content in the 10 µg fadrozole/L treatment

B							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				1/2 11	8.40E-01	753.67	0.001
				1/2 12	6.94E+00	499.45	0.014
				1/2 13	5.40E+00	780.58	0.007
				1/2 14	6.58E+00	526.37	0.013
				1/2 15	2.01E+00	619.08	0.003
				1/2 16	2.02E+00	589.18	0.003
				1/2 17	2.88E+00	565.25	0.005
				1/2 18	2.32E+00	825.45	0.003
				1/2 19	-	804.51	-
				1/2 20	2.94E+00	646	0.005
Mean			**na**	**Mean**			**0.006**
SD			**na**	**SD**			**0.003**
SD%			**na**	**SD%**			**59.6**
n			**0**	**n**			**15**
C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				1/3 1	3.68E+00	604.13	0.006
				1/3 3	-	836.06	-
				1/3 4	1.11E+01	890.31	0.012
				1/3 5	4.87E+00	692.46	0.007
				1/3 6	8.02E+00	931.79	0.009
				1/3 7	6.19E+00	848.82	0.007
				1/3 8	-	772.24	-
				1/3 9	1.92E+00	877.5	0.002
				1/3 10	1.39E+00	676.5	0.002
	No females present			1/3 11	1.14E+00	612.68	0.002
				1/3 13	4.74E+00	532.91	0.009
				1/3 14	-	462.7	-
				1/3 15	6.07E+00	1072.2	0.006
				1/3 16	4.54E+00	682.89	0.007
				1/3 17	5.35E+00	686.08	0.008
				1/3 18	3.43E+00	568.01	0.006
				1/3 19	-	670.12	-
				1/3 20	4.33E+00	593.54	0.007

Table 31 (continued): Vitellogenin content in the 10 µg fadrozole/L treatment

C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
Mean			**na**	**Mean**			**0.006**
SD			**na**	**SD**			**0.003**
SD%			**na**	**SD%**			**45.2**
n			**0**	**n**			**14**
D							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				1/4 1	1.67E+00	698.84	0.002
				1/4 2	2.12E+00	1145.59	0.002
				1/4 3	8.53E+00	740.33	0.012
				1/4 4	6.58E+00	743.52	0.009
				1/4 5	3.62E+00	617.45	0.006
				1/4 6	9.20E+00	1037.09	0.009
				1/4 7	4.36E+00	982.85	0.004
				1/4 8	5.99E+00	612.68	0.010
				1/4 9	2.70E+00	832.87	0.003
				1/4 10	3.81E+00	539.29	0.007
				1/4 11	6.81E+00	1362.58	0.005
				1/4 12	6.82E+00	1008.38	0.007
				1/4 13	4.14E+00	689.27	0.006
				1/4 14	2.97E+00	903.07	0.003
				1/4 15	-	839.25	-
				1/4 16	1.97E+00	625.45	0.003
				1/4 17	1.56E+00	689.27	0.002
				1/4 18	2.46E+00	545.67	0.005
				1/4 19	-	666.93	-
				1/4 20	2.19E+00	631.83	0.003
No females present							
Mean			**na**	**Mean**			**0.005**
SD			**na**	**SD**			**0.003**
SD%			**na**	**SD%**			**52.0**
n			**0**	**n**			**18**
Total mean			**na**	**Total mean**			**0.006**
SD			**na**	**SD**			**0.001**
SD%			**na**	**SD%**			**13.3**
n			**1**	**n**			**4**

Table 32: Vitellogenin content in the 32 µg fadrozole/L treatment

32 µg fadrozole/L							
females				males			
A							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
No females present				2/1 1	2.33E+00	1330.67	0.002
				2/1 2	2.79E+00	2760.27	0.001
				2/1 3	-	489.13	-
				2/1 4	1.63E+00	666.44	0.002
				2/1 5	3.70E-01	657.27	0.001
				2/1 6	5.32E+00	620.58	0.009
				2/1 7	3.53E+00	706.18	0.005
				2/1 8	1.30E+00	675.61	0.002
				2/1 9	2.45E+00	816.24	0.003
				2/1 10	3.27E+00	758.15	0.004
				2/1 11	2.60E+00	452.45	0.006
				2/1 12	1.15E+00	776.49	0.001
				2/1 13	-	892.66	-
				2/1 14	3.99E+00	797.89	0.005
				2/1 15	4.85E+00	904.89	0.005
				2/1 17	1.15E+00	663.38	0.002
				2/1 18	2.53E+00	892.66	0.003
				2/1 19	1.30E+00	748.98	0.002
				2/1 20	8.60E-01	1883.15	0.000
Mean			**na**	**Mean**			**0.003**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**71.2**
n			**0**	**n**			**17**
B							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
No females present				2/2 1	2.31E+00	727.58	0.003
				2/2 2	2.24E+00	1002.72	0.002
				2/2 3	2.89E+00	889.61	0.003
				2/2 4	2.64E+00	1030.23	0.003
				2/2 5	1.32E+00	1030.23	0.001
				2/2 6	3.71E+00	843.58	0.004
				2/2 7	2.08E+00	950.75	0.002
				2/2 8	1.73E+00	715.35	0.002
				2/2 9	1.55E+00	972.15	0.002

Table 32 (continued): Vitellogenin content in the 32 µg fadrozole/L treatment

B							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				2/2 10	4.35E+00	443.27	0.010
				2/2 11	9.16E+00	1311.48	0.007
				2/2 12	6.90E-01	1262.57	0.001
				2/2 13	2.80E+00	752.04	0.004
				2/2 14	-	709.24	-
				2/2 15	3.83E+00	620.58	0.006
				2/2 16	-	843.75	-
				2/2 17	1.75E+00	706.18	0.002
				2/2 18	1.37E+00	752.04	0.002
				2/2 19	4.03E+00	1088.32	0.004
				2/2 20	1.60E+00	862.09	0.002
Mean			**na**	**Mean**			**0.003**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**68.4**
n			**0**	**n**			**18**
C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				2/3 1	2.68E+00	1021.06	0.003
				2/3 2	1.37E+00	1424.59	0.001
				2/3 3	2.53E+00	1200.56	0.002
				2/3 4	1.82E+00	850.26	0.002
				2/3 5	1.45E+00	808.87	0.002
				2/3 6	9.90E-01	1066.81	0.001
				2/3 7	1.07E+00	706.96	0.002
				2/3 8	-	573.21	-
				2/3 9	1.37E+00	898.03	0.002
	No females present			2/3 10	3.16E+00	936.25	0.003
				2/3 11	3.89E+00	1060.44	0.004
				2/3 12	2.75E+00	741.99	0.004
				2/3 13	4.55E+00	821.6	0.006
				2/3 14	2.57E+00	1155.98	0.002
				2/3 15	2.28E+00	566.84	0.004
				2/3 16	4.29E+00	920.32	0.005
				2/3 17	8.80E-01	636.9	0.001
				2/3 18	7.65E+00	1955.26	0.004
				2/3 19	-	901.22	-

Table 32 (continued): Vitellogenin content in the 32 µg fadrozole/L treatment

C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				2/3 20	5.87E+00	1025.41	0.006
Mean			**na**	**Mean**			**0.003**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**52.3**
n			**0**	**n**			**18**
D							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
No females present				2/4 1	2.47E+00	799.1	0.003
				2/4 2	3.00E+00	1019.99	0.003
				2/4 3	5.38E+00	1149.61	0.005
				2/4 4	-	850.26	-
				2/4 5	8.85E+00	999.94	0.009
				2/4 7	4.60E+00	706.96	0.007
				2/4 9	3.48E+00	716.52	0.005
				2/4 10	1.13E+00	888.48	0.001
				2/4 11	-	815.24	-
				2/4 12	5.73E+00	850.26	0.007
				2/4 13	4.24E+00	1296.1	0.003
				2/4 14	2.26E+00	741.99	0.003
				2/4 15	5.31E+00	1130.5	0.005
				2/4 16	1.37E+00	818.42	0.002
				2/4 17	2.04E+00	726.07	0.003
				2/4 18	4.45E+00	796.13	0.006
				2/4 19	-	620.98	-
				2/4 20	-	503.15	-
Mean			**na**	**Mean**			**0.004**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**49.3**
n			**0**	**n**			**14**
Total mean			**na**	**Total mean**			**0.003**
SD			**na**	**SD**			**0.001**
SD%			**na**	**SD%**			**18.1**
n			**0**	**n**			**4**

Table 33: Vitellogenin content in the 100 µg fadrozole/L treatment

100 µg fadrozole/L							
females				males			
A							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
No females present				3/1 1	1.16E+00	850.26	0.001
				3/1 2	-	713.33	-
				3/1 3	-	542.48	-
				3/1 4	2.73E+00	503.5	0.005
				3/1 5	2.87E+00	698.4	0.004
				3/1 6	9.51E+00	509.99	0.019
				3/1 7	3.62E+00	1146.67	0.003
				3/1 8	4.18E+00	617.19	0.007
				3/1 9	-	532.73	-
				3/1 10	3.24E+00	805.6	0.004
				3/1 11	6.34E+00	1497.5	0.004
				3/1 12	-	630.18	-
				3/1 13	5.63E+00	979.05	0.006
				3/1 14	-	386.56	-
				3/1 15	2.89E+00	938.78	0.003
				3/1 16	-	773.11	-
				3/1 17	3.58E+00	760.12	0.005
				3/1 18	-	977.76	-
				3/1 19	-	360.57	-
				3/1 20	-	626.93	-
Mean			**na**	**Mean**			**0.006**
SD			**na**	**SD**			**0.005**
SD%			**na**	**SD%**			**82.1**
n			**0**	**n**			**11**
B							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
No females present				3/2 1	3.65E+00	289.1	0.013
				3/2 2	4.08E+00	328.09	0.012
				3/2 3	2.42E+00	909.54	0.003
				3/2 4	-	555.47	-
				3/2 5	4.19E+00	945.27	0.004
				3/2 6	3.09E+00	701.65	0.004
				3/2 7	-	1026	-

Table 33 (continued): Vitellogenin content in the 100 µg fadrozole/L treatment

B							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				3/2 8	7.47E+00	1107.69	0.007
				3/2 9	-	756.87	-
				3/2 10	5.77E+00	581.46	0.010
				3/2 11	-	990.75	-
				3/2 12	5.80E+00	1416.29	0.004
				3/2 13	3.07E+00	691.9	0.004
				3/2 14	1.54E+00	623.69	0.002
				3/2 15	2.71E+00	1692.4	0.002
				3/2 16	7.90E-01	535.98	0.001
				3/2 17	-	769.86	-
				3/2 18	-	847.82	-
				3/2 19	-	1136.93	-
Mean			**na**	**Mean**			**0.006**
SD			**na**	**SD**			**0.004**
SD%			**na**	**SD%**			**70.9**
n			**0**	**n**			**12**
C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				3/3 1	2.47E+00	724.39	0.003
				3/3 2	2.40E+00	1107.69	0.002
				3/3 3	2.51E+00	873.81	0.003
				3/3 4	-	628.18	-
				3/3 5	5.00E+00	948.55	0.005
				3/3 6	2.63E+00	584.21	0.005
				3/3 7	4.71E+00	807.21	0.006
				3/3 8	-	706.7	-
	No females present			3/3 9	-	653.31	-
				3/3 10	7.19E+00	1482.5	0.005
				3/3 11	1.34E+00	615.62	0.002
				3/3 12	4.50E-01	590.49	0.001
				3/3 13	8.70E-01	945.41	0.001
				3/3 14	-	750.67	-
				3/3 15	-	744.39	-
				3/3 16	-	552.8	-
				3/3 17	9.10E-01	725.55	0.001

Table 33 (continued): Vitellogenin content in the 100 µg fadrozole/L treatment

C							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
				3/3 18	-	734.97	-
				3/3 19	1.01E+00	669.01	0.002
				3/3 20	6.60E-01	835.48	0.001
Mean			**na**	**Mean**			**0.003**
SD			**na**	**SD**			**0.002**
SD%			**na**	**SD%**			**64.8**
n			**0**	**n**			**13**
D							
Sample name	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**	**Sample name**	**VTG [ng/mL]**	**Total protein [µg/mL]**	**VTG/Total protein [ng/µg]**
No females present				3/4 1	-	1171.56	-
				3/4 2	-	983.1	-
				3/4 3	2.84E+00	1237.51	0.002
				3/4 4	2.59E+00	932.85	0.003
				3/4 5	8.70E-01	917.14	0.001
				3/4 6	2.52E+00	904.58	0.003
				3/4 7	-	515.11	-
				3/4 8	2.42E+00	1579.87	0.002
				3/4 9	-	609.33	-
				3/4 10	-	873.17	-
				3/4 11	-	640.74	-
				3/4 12	-	807.21	-
				3/4 13	-	932.85	-
				3/4 14	-	939.13	-
				3/4 15	-	1724.35	-
				3/4 16	-	929.71	-
				3/4 17	-	860.61	-
				3/4 18	-	967.4	-
				3/4 19	-	565.36	-
				3/4 20	-	474.28	-

Table 33 (continued): Vitellogenin content in the 100 µg fadrozole/L treatment

D							
Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]	Sample name	VTG [ng/mL]	Total protein [µg/mL]	VTG/Total protein [ng/µg]
Mean			na	Mean			0.002
SD			na	SD			0.001
SD%			na	SD%			39.1
n			0	n			5
Total mean			na	Total mean			0.004
SD			na	SD			0.002
SD%			na	SD%			46.1
n			0	n			4

8.1.7 Gene expression data (mean 2'-ΔΔ Ct and log2 fold-change (FC) values plus standard errors (SEM))

Table 34: Gene expression at 48 hpf

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
mvd	0.010923	0.002212	0.031680	0.006954	1.536	0.286	0.04299	0.005444	1.977	0.172	0.051880	0.024640	2.248	0.560
lss	0.000389	0.000067	0.000271	0.000026	-0,519	0.129	0.000259	0.000005	-0.588	0.024	0.000251	0.000022	-0.632	0.118
sc4mol	0.001683	0.000418	0.001089	0.000025	-0.624	0.032	0.001277	0.000147	-0.396	0.157	0.001075	0.000089	-0.646	0.112
vtg1	0.000128	0.000012	0.000086	0.000008	-0.582	0.126	0.000100	0.000009	-0.369	0.127	0.000081	0.000002	-0.657	0.031
star	0.014660	0.001775	0.015538	0.002565	0.083	0.220	0.015758	0.002147	0.104	0.184	0.018802	0.003242	0.358	0.229
apoA1	0.357202	0.029999	0.415923	0.045154	0.219	0.148	0.420393	0.053252	0.235	0.171	0.341319	0.018812	-0.065	0.049
apoEb	0.057532	0.015184	0.071578	0.033944	0.315	0.559	0.060152	0.028116	0.064	0.553	0.116601	0.022335	1.019	0.252
esr1	0.000267	0.000033	0.000233	0.000089	-0.192	0.192	0.000233	0.000088	-0.192	0.462	0.000233	0.000033	-0.192	0.192
esr2a	0.000057	0.000001	0.000050	0.000005	-0.161	0.141	0.000052	0.000007	-0.133	0.181	0.000045	0.000004	-0.322	0.136
esr2b	0.000001	0.000000	0.000001	0.000000	-0.530	0.253	0.000001	0.000000	0.428	0.512	0.000001	0.000000	-0.176	0.120
ar	0.001138	0.000133	0.000896	0.000107	-0.337	0.161	0.001111	0.000110	-0.034	0.134	0.000852	0.000044	-0.419	0.074
kiss1rb	0.000006	0.000001	0.000007	0.000000	0.079	0.045	0.000007	0.000001	0.038	0.266	0.000007	0.000001	0.157	0.205
kiss1ra	0.000005	0.000001	0.000004	0.000000	-0.312	0.137	0.000003	0.000001	-0.861	0.252	0.000003	0.000001	-0.604	0.319
npy1r														
npy8br	0.001025	0.000158	0.001038	0.000171	0.013	0.220	0.000965	0.000062	-0.087	0.088	0.000869	0.000052	-0.244	0.086
gnrhr1														
gnrhr2														
gnrhr3														
gnrhr4	0.000061	0.000008	0.000047	0.000006	-0.376	0.169	0.000041	0.000003	-0.584	0.101	0.000045	0.000001	-0.438	0.031
igfbp5a														
cyr61	0.000197	0.000019	0.000216	0.000018	0.136	0.116	0.000218	0.000025	0.149	0.155	0.000177	0.000011	-0.154	0.085
igf1	0.000006	0.000003	0.000014	0.000005	1.338	0.467	0.000021	0.000002	1.912	0.149	0.000023	0.000006	2.020	0.366

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
sox9b	0.000748	0.000036	0.000907	0.000051	0.279	0.078	0.001085	0.000130	0.537	0.163	0.000996	0.000233	0.413	0.302
prox1														
cyp19a1a														
cyp19a1b	0.000063	0.000016	0.000052	0.000007	-0.271	0.184	0.000075	0.000012	0.245	0.217	0.000055	0.000002	-0.197	0.039
fshr	0.000001	0.000000	0.000001	0.000000	0.222	0.318	0.000001	0.000000	0.078	0.250	0.000001	0.000000	0.473	0.547
zgc:64022	0.000038	0.000002	0.000045	0.000011	0.254	0.308	0.000045	0.000009	0.254	0.254	0.000040	0.000013	0.061	0.406
lhcgr														

Table 35: Gene expression at 96 hpf

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
mvd	0.013280	0.003234	0.01008	0.001566	-0.397	0.208	0.01381	0.003987	0.056	0.365	0.02553	0.015190	0.942	0.673
lss	0.000805	0.000008	0.000696	0.000078	-0.212	0.156	0.000635	0.000034	-0.342	0.072	0.000737	0.000027	-0.124	0.054
sc4mol	0.006540	0.000588	0.005777	0.000425	-0.179	0.102	0.005893	0.001004	-0.150	0.227	0.005837	0.000279	-0.164	0.067
vtg1	0.000128	0.000015	0.000083	0.000013	-0.616	0.204	0.000110	0.000007	-0.212	0.088	0.000116	0.000005	-0.137	0.064
star	0.001132	0.000099	0.000954	0.000104	-0.244	0.146	0.001015	0.000111	-0.156	0.148	0.001088	0.000167	-0.060	0.206
apoA1	0.333378	0.042070	0.265204	0.011328	-0.330	0.060	0.315839	0.019226	-0.078	0.085	0.343145	0.026752	0.041	0.108
apoEb	0.007177	0.002007	0.008564	0.000638	0.254	0.103	0.007183	0.000597	0.001	0.115	0.010233	0.000575	0.511	0.078
esr1	0.000381	0.000075	0.000303	0.000026	-0.309	0.117	0.000369	0.000063	-0.038	0.219	0.000385	0.000058	0.025	0.205
esr2a	0.001623	0.000226	0.001825	0.000438	0.170	0.309	0.000833	0.000086	-0.961	0.145	0.001278	0.000260	-0.346	0.268
esr2b	0.000217	0.000119	0.000033	0.000009	-2.700	0.338	0.000070	0.000049	-1.630	0.726	0.000123	0.000113	-0.813	0.941
ar	0.002352	0.000097	0.001834	0.000246	-0.360	0.182	0.002145	0.000130	-0.132	0.083	0.001984	0.000231	-0.246	0.159
kiss1rb	0.000006	0.000002	0.000005	0.000001	-0.398	0.365	0.000007	0.000001	0.129	0.105	0.000008	0.000002	0.227	0.316
kiss1ra	0.000090	0.000019	0.000071	0.000004	-0.347	0.064	0.000053	0.000011	-0.807	0.293	0.000056	0.000005	-0.719	0.160
npy1r	0.000208	0.000071	0.000173	0.000025	-0.253	0.201	0.000252	0.000028	0.274	0.145	0.000222	0.000030	0.089	0.184
npy8br	0.003483	0.000272	0.002459	0.000277	-0.502	0.153	0.003206	0.000541	-0.119	0.225	0.003020	0.000094	-0.205	0.044
gnrhr1														
gnrhr2	0.000346	0.000023	0.000240	0.000029	-0.516	0.172	0.000286	0.000024	-0.276	0.117	0.000264	0.000022	-0.382	0.107
gnrhr3	0.000169	0.000015	0.000130	0.000012	-0.372	0.133	0.000173	0.000014	0.036	0.109	0.000121	0.000005	-0.471	0.064
gnrhr4	0.000540	0.000027	0.000422	0.000049	-0.355	0.159	0.000465	0.000066	-0.216	0.190	0.000522	0.000046	-0.049	0.121
igfbp5a	0.008667	0.006333	0.006667	0.003756	-0.378	0.644	0.006667	0.003756	-0.378	0.644	0.005667	0.003480	-0.613	0.690
cyr61	0.000226	0.000056	0.000238	0.000034	0.104	0.197	0.000244	0.000056	0.123	0.303	0.000196	0.000057	-0.207	0.372
igf1	0.000026	0.000004	0.000060	0.0000003	1.221	0.006	0.000075	0.000001	1.591	0.022	0.000078	0.000008	1.606	0.139
sox9b	0.000158	0.000025	0.000231	0.000029	0.574	0.169	0.000671	0.000389	1.960	0.291	0.000187	0.000077	0.252	0.508
prox1	0.001922	0.000340	0.001975	0.000519	0.000	0.365	0.002007	0.000165	0.000	0.000	0.002000	0.000000	0.000	0.000
cyp19a1a	0.000023	0.000007	0.000019	0.000005	-0.485	0.485	0.000022	0.000011	-0.222	0.657	0.000056	0.000016	1.192	0.347

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
cyp19a1b	0.000240	0.000032	0.000147	0.000024	-0.710	0.212	0.000233	0.000018	-0.040	0.105	0.000198	0.000042	-0.287	0.291
fshr														
zgc:64022	0.000046	0.000006	0.000052	0.000003	0.299	0.087	0.000071	0.000010	0.691	0.192	0.000045	0.000004	0.000	0.106
lhcgr														

Table 36: Gene expression at 28 dpf

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
mvd	0.002733	0.000326	0.001993	0.000508	-0.455	0.768	0.002557	0.000139	-0.096	0.076	0.003450	0.000320	0.336	0.128
lss	0.001005	0.000297	0.000584	0.000069	-0.783	0.398	0.000600	0.000119	-0.744	0.261	0.000846	0.000155	-0.247	0.243
sc4mol	0.083123	0.020012	0.049281	0.012798	-0.754	0.542	0.066873	0.012927	-0.313	0.254	0.099810	0.028469	0.263	0.362
vtg1	0.000045	0.000010	0.000041	0.000013	-0.137	0.431	0.000054	0.000025	0.258	0.555	0.000039	0.000004	-0.213	0.136
star	0.001903	0.000374	0.001933	0.000753	0.022	0.557	0.001900	0.000307	-0.002	0.216	0.002317	0.000394	0.283	0.226
apoA1	0.243931	0.118783	0.173950	0.122297	-0.487	0.289	0.225410	0.094903	-0.113	0.506	0.220253	0.098156	-0.147	0.531
apoEb	0.025183	0.008759	0.022150	0.007042	-0.184	0.552	0.019867	0.006686	-0.341	0.418	0.026467	0.003786	0.071	0.192
esr1	0.000290	0.000140	0.000193	0.000088	-0.584	0.115	0.000300	0.000126	0.048	0.505	0.000313	0.000159	0.111	0.591
esr2a	0.001660	0.000619	0.000995	0.000347	-0.738	0.637	0.001328	0.000475	-0.322	0.441	0.001574	0.000495	-0.077	0.394
esr2b	0.000028	0.000013	0.000023	0.000011	-0.303	0.456	0.000026	0.000012	-0.114	0.524	0.000038	0.000020	0.418	0.605
ar	0.016923	0.005392	0.010300	0.002289	-0.716	0.152	0.011683	0.003596	-0.534	0.387	0.015430	0.005500	-0.132	0.439
kiss1rb	0.000031	0.000016	0.000011	0.000005	-1.503	0.353	0.000023	0.000010	-0.440	0.545	0.000056	0.000029	0.868	0.608
kiss1ra	0.000028	0.000009	0.000033	0.000003	0.263	0.444	0.000042	0.000019	0.591	0.532	0.000037	0.000013	0.406	0.428
npy1r	0.000120	0.000051	0.000077	0.000043	-0.646	0.343	0.000138	0.000061	0.198	0.525	0.000143	0.000068	0.256	0.557
npy8br	0.002698	0.000452	0.002166	0.000807	-0.316	0.476	0.002789	0.001001	0.047	0.442	0.002932	0.000565	0.120	0.254
gnrhr1	0.000064	0.000016	0.000091	0.000010	0.509	0.603	0.000218	0.000169	1.766	0.827	0.000115	0.000005	0.836	0.067
gnrhr2	0.000090	0.000025	0.000110	0.000031	0.289	0.262	0.000090	0.000021	0.000	0.300	0.000120	0.000015	0.415	0.172
gnrhr3	0.000249	0.000020	0.000204	0.000074	-0.286	0.589	0.000389	0.000120	0.640	0.388	0.000230	0.000017	-0.118	0.102
gnrhr4	0.000179	0.000014	0.000204	0.000055	0.188	0.734	0.000257	0.000033	0.523	0.175	0.000185	0.000037	0.044	0.265
igfbp5a	0.025786	0.008939	0.018014	0.007055	-0.517	0.481	0.025439	0.008381	-0.019	0.410	0.022896	0.009728	-0.171	0.510
cyr61	0.000160	0.000065	0.000138	0.000072	-0.210	0.418	0.000151	0.000082	-0.087	0.629	0.000126	0.000071	-0.340	0.643
igf1	0.000309	0.000087	0.000358	0.000072	0.214	0.000	0.000500	0.000180	0.693	0.443	0.000535	0.000113	0.792	0.275
sox9b	0.000703	0.000405	0.000333	0.000168	-1.077	0.768	0.000563	0.000380	-0.320	0.743	0.000547	0.000247	-0.363	0.537
prox1	0.000913	0.000506	0.000603	0.000401	-0.598	0.398	0.001123	0.000512	0.298	0.541	0.000847	0.000394	-0.109	0.551

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
cyp19a1a	0.000044	0.000005	0.000108	0.000043	1.285	0.542	0.000080	0.000033	0.856	0.498	0.000103	0.000023	1.222	0.293
cyp19a1b	0.000866	0.000113	0.000734	0.000247	-0.238	0.431	0.000812	0.000204	-0.091	0.323	0.000722	0.000076	-0.261	0.144
fshr	0.000003	0.000001	0.000003	0.000000	0.234	0.557	0.000007	0.000003	1.349	0.469	0.000005	0.000003	0.819	0.637
zgc:64022	0.000045	0.000020	0.000051	0.000028	0.200	0.619	0.000079	0.000029	0.816	0.452	0.000064	0.000029	0.511	0.544
lhcgr	0.000005	0.000005	0.000000	0.000000	-	-	0.000000	0.000000	-	-	0.000000	0.000000	-	-

Table 37: Gene expression at 63 dpf (compared to low *vtg1* control group)

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
mvd	0.079660	0.048170	0.028070	0.001622	-1.504	0.081	0.047740	0.012000	-0.738	0.323	0.044250	0.019840	-0.848	0.534
lss	0.013120	0.003639	0.023790	0.003372	0.858	0.191	0.038770	0.001499	1.562	0.054	0.036890	0.006810	1.491	0.244
sc4mol	0.707400	0.065640	1.245000	0.278000	0.815	0.290	1.502000	0.603000	1.085	0.487	1.355000	0.240900	0.937	0.236
vtg1	0.000575	0.000274	0.000548	0.000084	-0.068	0.205	0.000367	0.000057	-0.645	0.208	0.000487	0.000145	-0.238	0.376
star	0.009216	0.000396	0.016650	0.001987	0.853	0.162	0.018180	0.005399	0.980	0.375	0.027700	0.001639	1.587	0.082
apoA1	4.217000	0.734900	2.703000	0.275500	-0.641	0.140	3.186000	0.484100	-0.404	0.204	4.145000	0.398300	-0.024	0.132
apoEb	0.093640	0.036990	0.045050	0.008007	-1.055	0.236	0.045690	0.003661	-1.035	0.111	0.072370	0.011380	-0.371	0.210
esr1	0.005908	0.000669	0.004443	0.000124	-0.411	0.039	0.004437	0.000881	-0.413	0.261	0.005004	0.000836	-0.239	0.222
esr2a	0.024710	0.003064	0.017340	0.000809	-0.511	0.065	0.015230	0.000786	-0.698	0.072	0.017360	0.001992	-0.509	0.156
esr2b	0.000025	0.000015	0.000133	0.000027	2.399	0.263	0.000035	0.000014	0.492	0.484	0.000013	0.000005	-0.984	0.514
ar	0.510500	0.174800	0.602800	0.137200	0.239	0.295	0.680600	0.087640	0.414	0.174	0.792300	0.108600	0.634	0.185
kiss1rb	0.000043	0.000009	0.000029	0.000006	-0.577	0.286	0.000027	0.000013	-0.648	0.578	0.000023	0.000011	-0.861	0.547
kiss1ra														
npy1r	0.000425	0.000185	0.000277	0.000013	-0.618	0.067	0.000353	0.000026	-0.266	0.103	0.000487	0.000010	0.197	0.030
npy8br	0.009663	0.001189	0.010150	0.001013	0.071	0.137	0.009644	0.000186	-0.002	0.027	0.010850	0.001759	0.167	0.216
gnrhr1														
gnrhr2														
gnrhr3	0.001058	0.000348	0.001246	0.000196	0.236	0.210	0.001582	0.000156	0.580	0.135	0.001682	0.000155	0.668	0.126
gnrhr4	0.000060	0.000019	0.000083	0.000021	0.475	0.318	0.000058	0.000005	-0.051	0.114	0.000047	0.000012	-0.346	0.327
igfbp5a	0.131200	0.011670	0.102400	0.007627	-0.358	0.103	0.109500	0.008137	-0.261	0.103	0.150000	0.020860	0.192	0.187
cyr61	0.000728	0.000019	0.000622	0.000014	-0.225	0.032	0.000851	0.000059	0.224	0.097	0.001221	0.000294	0.746	0.310
igf1	0.000734	0.000242	0.001883	0.000443	1.358	0.304	0.003191	0.001067	2.119	0.416	0.003229	0.000225	2.136	0.097
sox9b	0.010410	0.008156	0.001130	0.000361	-3.204	0.399	0.006115	0.004267	-0.768	0.763	0.018100	0.005203	0.797	0.364
prox1	0.000612	0.000233	0.000615	0.000187	0.007	0.383	0.000852	0.000271	0.478	0.398	0.001886	0.000063	1.623	0.047

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
cyp19a1a	0.012340	0.010820	0.002092	0.001377	-2.560	0.729	0.009121	0.009099	-0.436	0.998	0.004098	0.003798	-1.590	0.946
cyp19a1b	0.000431	0.000132	0.000549	0.000035	0.349	0.090	0.000664	0.000060	0.624	0.125	0.000604	0.000052	0.486	0.118
fshr	0.000074	0.000026	0.000068	0.000007	-0.112	0.144	0.000068	0.000014	-0.123	0.268	0.000075	0.000019	0.018	0.324
zgc:64022	0.000292	0.000044	0.000213	0.000074	-0.460	0.430	0.000154	0.000039	-0.922	0.323	0.000151	0.000030	-0.952	0.263
lhcgr														

Table 38: Gene expression at 63 dpf (compared to high *vtg1* control group)

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
mvd	0.171700	0.101000	0.028070	0.001622	-2.612	0.081	0.047740	0.012000	-1.846	0.323	0.044250	0.019840	-1.955	0.534
lss	0.007087	0.001957	0.023790	0.003372	1.747	0.191	0.038770	0.001499	2.451	0.054	0.036890	0.006810	2.380	0.244
sc4mol	0.697400	0.276800	1.245000	0.278000	0.836	0.290	1.502000	0.603000	1.106	0.487	1.355000	0.240900	0.957	0.236
vtg1	0.246500	0.045200	0.000548	0.000084	-8.813	0.205	0.000367	0.000057	-9.390	0.208	0.000487	0.000145	-8.983	0.376
star	0.009925	0.003135	0.016650	0.001987	0.746	0.162	0.018180	0.005399	0.873	0.375	0.027700	0.001639	1.480	0.082
apoA1	4.802000	1.552000	2.703000	0.275500	-0.828	0.140	3.186000	0.484100	-0.591	0.204	4.145000	0.398300	-0.212	0.132
apoEb	0.122200	0.056350	0.045050	0.008007	-1.439	0.236	0.045690	0.003661	-1.418	0.111	0.072370	0.011380	-0.755	0.210
esr1	0.006804	0.003797	0.004443	0.000124	-0.614	0.039	0.004437	0.000881	-0.616	0.261	0.005004	0.000836	-0.443	0.222
esr2a	0.042550	0.008210	0.017340	0.000809	-1.295	0.065	0.015230	0.000786	-1.481	0.072	0.017360	0.001992	-1.293	0.156
esr2b	0.000060	0.000000	0.000133	0.000027	1.152	0.263	0.000035	0.000014	-0.754	0.484	0.000013	0.000005	-2.231	0.514
ar	0.898900	0.393100	0.602800	0.137200	-0.576	0.295	0.680600	0.087640	-0.401	0.174	0.792300	0.108600	-0.182	0.185
kiss1rb	0.000041	0.000011	0.000029	0.000006	-0.510	0.286	0.000027	0.000013	-0.580	0.578	0.000023	0.000011	-0.793	0.547
kiss1ra														
npy1r	0.000342	0.000024	0.000277	0.000013	-0.305	0.067	0.000353	0.000026	0.046	0.103	0.000487	0.000010	0.510	0.030
npy8br	0.012080	0.002648	0.010150	0.001013	-0.250	0.137	0.009644	0.000186	-0.325	0.027	0.010850	0.001759	-0.155	0.216
gnrhr1														
gnrhr2														
gnrhr3	0.001729	0.000966	0.001246	0.000196	-0.472	0.210	0.001582	0.000156	-0.128	0.135	0.001682	0.000155	-0.040	0.126
gnrhr4	0.003452	0.003078	0.000083	0.000021	-5.369	0.318	0.000058	0.000005	-5.896	0.114	0.000047	0.000012	-6.191	0.327
igfbp5a	0.086220	0.009440	0.102400	0.007627	0.247	0.103	0.109500	0.008137	0.344	0.103	0.150000	0.020860	0.798	0.187
cyr61	0.000753	0.000061	0.000622	0.000014	-0.274	0.032	0.000851	0.000059	0.176	0.097	0.001221	0.000294	0.698	0.310
igf1	0.001068	0.000899	0.001883	0.000443	0.818	0.304	0.003191	0.001067	1.579	0.416	0.003229	0.000225	1.596	0.097
sox9b	0.003827	0.003727	0.001130	0.000361	-1.760	0.399	0.006115	0.004267	0.676	0.763	0.018100	0.005203	2.241	0.364
prox1	0.000753	0.000245	0.000615	0.000187	-0.292	0.383	0.000852	0.000271	0.178	0.398	0.001886	0.000063	1.323	0.047

	Nominal concentration fadrozole [µg/L]													
	0.0 (control)		10				32				100			
ZF gene	Mean 2'-ΔΔCt	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM	Mean 2'-ΔΔCt	SEM	Log_2 FC	SEM
cyp19a1a	0.000399	0.000018	0.002092	0.001377	2.388	0.729	0.009121	0.009099	4.512	0.998	0.004098	0.003798	3.358	0.946
cyp19a1b	0.004530	0.002721	0.000549	0.000035	-3.044	0.090	0.000664	0.000060	-2.769	0.125	0.000604	0.000052	-2.907	0.118
fshr	0.000079	0.000030	0.000068	0.000007	-0.217	0.144	0.000068	0.000014	-0.228	0.268	0.000075	0.000019	-0.086	0.324
zgc:64022	0.000804	0.000413	0.000213	0.000074	-1.918	0,430	0.000154	0.000039	-2.380	0.323	0.000151	0.000030	-2.410	0.263
lhcgr														

8.2 Statistics

In this section of the report, the statistical evaluations of the endocrine-relevant apical endpoints sex ratio and vitellogenin content in males were presented. Further, statistical evaluation of other apical endpoints displaying significant differences in treatments compared to the control, i.e. survival during the early life stages, were presented. Relative data were arcsine-transformed prior to evaluation.

8.2.1 Survival during the early life stages

Statistical Characteristics of the Samples

Statistical characteristics with survival at 28 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	1,11	1,12	1,08	1,14	4	0,027	2,4	0,013	1,2	1,07	1,16
10,0	1,08	1,09	1,03	1,11	4	0,037	3,4	0,019	1,7	1,02	1,14
32,0	1,00	0,98	0,98	1,05	4	0,037	3,7	0,019	1,9	0,94	1,06
100,0	0,97	0,97	0,91	1,03	4	0,057	5,9	0,028	2,9	0,88	1,06

One-way Analysis of Variance

One-way Analysis of Variance with survival at 28 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	0,0567	3	0,0189	11,258	< 0.001
Residuals	0,02	12	0,0017		
Total	0,077	15			

p(F) is smaller than or equal to the selected significance level of 0,05; therefore, treatments are significantly different.

Shapiro-Wilk´s Test on Normal Distribution

Shapiro-Wilk´s Test on Normal Distribution with survival at 28 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	1,11	0,027	4
10,0	1,08	0,037	4
32,0	1,00	0,037	4
100,0	0,97	0,057	4

Results:

Number of residuals = 12; Shapiro-Wilk´s W = 0,9484; p(W) = 0,614; p(W) is greater than the selected significance level of 0,01; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0,01).

Levene´s Test on Variance Homogeneity (with Residuals)

Levene´s Test on Variance Homogeneity (with Residuals) with survival at 28 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	0,0016	3	0,0005	1,957	0,174
Residuals	0,00	12	0,0003		
Total	0,005	15			

The Levene test indicates variance homogeneity (p > 0,01).

Variance homogeneity check was passed (p > 0,01).

Williams Multiple Sequential t-test Procedure

Comparison of treatments with "Control" by the t test procedure after Williams with survival at 28 d: Significance was Alpha = 0,05, one-sided smaller; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; 't*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case |t| > |t*| (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	1,11	0,0410						
10,0	1,08	0,0410	12	1,08	-4,6	-1,17	-1,78	-
32,0	1,00	0,0410	12	1,00	-4,9	-4,03	-1,87	+
100,0	0,97	0,0410	12	0,97	-4,9	-5,06	-1,90	+

+: significant; -: non-significant

A NOEC of 10,0 µg/L is suggested by the program.

8.2.2 Sex ratio at test termination

Statistical Characteristics of the Samples

Statistical characteristics with sex ratio at 63 d: Mean: arithmetic mean (X); Med: median; Min: minimum value, Max: maximum value; n: sample size; s: standard deviation; s%: coefficient of variation; s(X): standard error; %s(X): %standard error; 95%l, 95%u: lower, upper 95%-confidence limits.

Treatm. [µg/L]	Mean	Med	Min	Max	n	s	%s	s(X)	%s(X)	95%l	95%u
Control	0,77	0,79	0,67	0,84	4	0,069	8,9	0,034	4,5	0,66	0,88
10,0	1,33	1,25	1,25	1,57	4	0,161	12,1	0,080	6,1	1,07	1,59
32,0	1,43	1,46	1,25	1,57	4	0,163	11,4	0,081	5,7	1,17	1,69
100,0	1,43	1,46	1,25	1,57	4	0,163	11,4	0,081	5,7	1,17	1,69

One-way Analysis of Variance

One-way Analysis of Variance with sex ratio at 28 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	1,2170	3	0,4057	19,412	< 0.001
Residuals	0,25	12	0,0209		
Total	1,468	15			

p(F) is smaller than or equal to the selected significance level of 0,05; therefore, treatments are significantly different.

Shapiro-Wilk´s Test on Normal Distribution

Shapiro-Wilk´s Test on Normal Distribution with sex ratio at 28 d: Mean: arithmetic mean; n: sample size; p(ShapiroWilk´s W): probability of the W statistic (i.e. that the observed deviations from the normal distributions are dues to chance). In case p(ShapiroWilk´s W) is greater than the chosen significance level, the normality hypothesis(Ho) is accepted.

Treatm. [µg/L]	Mean	s	n
Control	0,77	0,069	4
10,0	1,33	0,161	4
32,0	1,43	0,163	4
100,0	1,43	0,163	4

Results:

Number of residuals = 8; Shapiro-Wilk´s W = 0,9517; p(W) = 0,636; p(W) is greater than the selected significance level of 0,01; thus treatment data do not significantly deviate from normal distribution.

Normality check was passed (p > 0,01).

Levene´s Test on Variance Homogeneity (with Residuals)

Levene´s Test on Variance Homogeneity (with Residuals) with sex ratio at 28 d: Source: source of variance; SS: sum of squares; df: degrees of freedom; MSS: mean sum of squares; F: test statistic: p: probability that the variance explained by the treatment is due to chance

Source	SS	df	MSS	F	p(F)
Treatment	0,0214	3	0,0071	2,564	0,104
Residuals	0,03	12	0,0028		
Total	0,055	15			

The Levene test indicates variance homogeneity (p > 0,01).

Williams Multiple Sequential t-test Procedure

Comparison of treatments with "Control" by the t test procedure after Williams with sex ratio at 28 d: Significance was Alpha = 0,05, one-sided greater; Mean: arithmetic mean; n: sample size; s: standard deviation; LhM: max. likelihood mean; MDD: minimum detectable difference to Control (in percent of Control); t: sample t; 't*: critical t for Ho: µ1 = µ2 = ... = µk; the differences are significant in case t > t* (The residual variance of an ANOVA was applied; df = N - k; N: sum of treatment replicates n(i); k: number of treatments).

Treatm. [µg/L]	Mean	s	df	LhM	%MDD	t	t*	Sign.
Control	0,77	0,1446						
10,0	1,33	0,1446	12	1,33	23,7	5,47	1,78	+
32,0	1,43	0,1446	12	1,43	24,9	6,50	1,87	+
100,0	1,43	0,1446	12	1,43	25,3	6,50	1,90	+

+: significant; -: non-significant

The NOEC is lower than 10,0 µg/L.

8.3 List of genes and primers

Table 39: List of tested genes and primer sequences

Generic biological function	Gene name	Accession	Protein name	Sense primer	Antisense primer	Source	Annealing temp. for PCR (°C)	Melting temp. of PCR product (°C)
Steroid and terpenoid synthesis	*mvd*	NM_001007422.1	mevalonate (diphospho) decarboxylase	GCATCAAGATCACCTGAGAAC	TATGGACCTTGTTGCTGACA	NCBI PrimerBlast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)	60	78.5
	lss	NM_001083567.1	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	AGCACACGGGACTGCGGTTGG	TCCAGCAGTTTCCCGCCACGT	NCBI PrimerBlast (Dissertation Schiller 2013)	60	82
	sc4mol (new:*msmo1*)	NM_213353.1	methylsterol monooxygenase 1	CCACCGAGCTCTACATCATC	AAGAGGGTGGGCATATTCTG	NCBI PrimerBlast	60	80.5
Lipid transport/ lipid metabolic process	*vtg1*	NM_001044897.2	vitellogenin 1	GCAGCCTTGCCATCTCAGAGGTCC	TGGATTGATGGGAACAGCGACAGGA	NCBI PrimerBlast (Dissertation Schiller 2013)	60	74.5
	star	NM_131663.1	steroidogenic acute regulatory protein	ACCTGTTTTCTGGCTGGGATG	GGGTCCATTCTCAGCCCTTAC	Ings and Van Der Kraak (2006)	54	80
	apoA1a	NM_131128	apolipoprotein A-1a	CCTTGGTGTACCTGAAC	GAGATCCTCAACGTCAGT	NCBI PrimerBlast	54	84
	apoEb	NM_131098.1	apolipoprotein Eb	ATTCTGTGAGAGGTTGATAA	CTCAGACATGGGTTAATACT	NCBI PrimerBlast	54	76.5
Steroid receptors	*esr1*	NM_152959	estrogen receptor 2a	ACAAAGGAATGGAGCACTTA	GTGTAGATGGAGGGTTTTTC	NCBI PrimerBlast (Dissertation Schiller 2013)	54	83.5
	esr2a	NM_180966.2	estrogen receptor 1	AGAGTCGACTTCAACAGAAC	TCTCCTCTGTATCTGCTACC	NCBI PrimerBlast	54.5	79
	esr2b	NM_174862.3	estrogen receptor 2b	TATTCTGATGTTAGGATTGA	CGAACAGTTATTAGAGTTGA	NCBI PrimerBlast	54	81.3

	ar	NM_001083123.1	androgen receptor	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	Hossain et al. (2008)	60	83
Neuropeptide receptor activity	*kiss1rb*	NM_001110531	kiss1 receptor b	GATATTTGGGGATTTTATGT	AGACTCAGTGTGCTGATACA	NCBI PrimerBlast	54.5	84.5
	kiss1ra	NM_001105679	kiss1 receptor a	TCTCTTTCTGTTATTCCTTC	ACTATGACCACTACCATTTT		54.5	79
	npy1r	NM_001102391	neuropeptide Y1 receptor)	GAAATCAAACTCACCTACAC	GTTTATCCGTTTAGACTCAC	NCBI PrimerBlast	54	79.5
	npy8br	NM_131436.1	neuropeptide Y receptor Y8b	CCTCTCATGCTCCGACATCC	CTGCTACGGCCAGGTATGAG	NCBI PrimerBlast	60	76
	gnrhr1	NM_001144980	gonadotropin-releasing hormone receptor1	GTAATGAGCTTCTGCTATAC	AGCAGATAATACGGTGTC	NCBI PrimerBlast	60	83
	gnrhr2	NM_001144979	gonadotropin-releasing hormone receptor2	CGATGAGAAGAAATAAAGTT	ATGATGAACAGTGGTAAGAG	NCBI PrimerBlast	60	82.5
	gnrhr3	NM_001177450.1	gonadotropin-releasing hormone receptor3	ACATGTTCCACTTTGTGAC	TGTGTAGTTGTCTGTTGATCT	NCBI PrimerBlast	54.5	80.5
	gnrhr4	NM_001098193.1	gonadotropin-releasing hormone receptor4	GATGAGTGTTGTTCTCTCC	AGTGGTACACTGAGTGAAAT	NCBI PrimerBlast	54.5	78.3
Cell growth	*igfbp5a*	NM_001126463	insulin-like growth factor binding protein 5a	TCTCTCTACCTGCCTAACT	GTTGATTCTCACTCGTTG	NCBI PrimerBlast	54.5	85
	cyr61	NM_001080987	cysteine-rich, angiogenic inducer, 61	ATGACTGTAGGTTTGTCTTT	GTATACAAGCGAGAGTCTA	NCBI PrimerBlast	54.5	78
	igf 1	NM_131825	insulin-like growth factor 1	ACCAAAGAAACCTATATCTG	TCTTGTCTCTCTCAGTTCAT	NCBI PrimerBlast	54.5	80
Transcription	*sox9b*	AY029578	SRY (sex determining region Y)-box 9b	CATCTATATTCTTAGCCACA	ACATTAGGTAATGAACTTCC	NCBI PrimerBlast	54.5	79.5
Haematopoiesis	*prox1a* (previous: *prox1*)	NM_131405	prospero homeobox 1	ACATAGGAGTGAAGAGGACT	TTTCAGAAGCTGAGATATGA	NCBI PrimerBlast	54	83.5
Others	*cyp19a1a*	NM_131154.2	aromatase a	TCTGCTTCAGAAGATTCATAAATACTTT	CCTGCAACTCCTGAGCATCTC	Trickler et al. 2013	60	78.5
	cyp19a1b	NM_131642.1	aromatase b	GCTCCAGACACGCTCTCCAT	CATCCTCCAGAGACTGCCTCA	NCBI PrimerBlast	60	81.5

						(Dissertation Schiller 2013)		
	fshr	NM_001001812.1	follicle stimulating hormone receptor	TACACACTCATCTACTTGACC	TTTAGTGAAGAAGGCATAG	NCBI PrimerBlast	54.5	(73)
	lhcgr (previous: *lhr*)	NM_205625	luteinizing hormone/choriogonadotropin receptor	TGGATTTTAGCAGTTTATTA	ATGGTCTTCACTGAGATGTA	NCBI PrimerBlast	54	82
	zgc:64022	NM_200365	unknown transcript	GTTCATAGGAGAATATGGAG	TGCTGTAAACAAGGATATAG	NCBI PrimerBlast	54	81.5
Reference genes	*18s* (new: *zgc:158463*)	FJ915075.1	18S rRNA	TCGCTAGTTGGCATCGTTTATG	CGGAGGTTCGAAGACGATCA	Cooper et al. 2006		77.5
	rpl8	NM_200713.1	ribosomal protein L8	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	NCBI PrimerBlast		78,5

8.4 Analytical report - Details of method and results

8.4.1 Preface and Scope

The analytical method for the quantitative determination of fadrozole hydrochloride ("Fadrozole", CAS RN 102676-31-3) in 'holding- and dilution-water of the flow through system' using LC-MS/MS was developed in experiments which were completed prior to the validation of the analytical method. The validation of the method was performed according to the guideline SANCO/3029/99[1] and is part of this report.

The quantitative measurements of fadrozole were done by liquid chromatography (LC) coupled to a triple quadrupole mass spectrometer (MS); the MS was operated in the tandem mass spectrometry mode (MS/MS).

The non-steroidal aromatase inhibitor Letrozole (CAS RN 112809-51-5) was used as internal standard (IS); the method is applicable for matrix charged water samples in concentrations above the validated LOQ of 5.0 µg/L.

8.4.2 Chemicals, reagents and analytical equipment

- Analytical standard fadrozole hydrochloride; Sigma-Aldrich product no. F3806; Lot no.: 121M4604V; quality release date: 12. Dec. 2011
- Internal standard (IS) Letrozole[2]; Sigma-Aldrich product no. L6545; Lot no.: 032M4706V; quality release date: 09. Mar. 2012
- Working solution of the IS Letrozole in methanol, conc.: 10.0 µg/mL
- Purified water, produced with purification system PURELAB® Ultra (ELGA LabWater)
- Methanol, 'Baker HPLC analysed', Article No. 8402 (J.T. Baker)
- Acetonitrile (ACN), 'Baker HPLC analysed', Article No. 9017 (J.T. Baker)
- Ammonium acetate (NH_4Ac), 'Optima™ LC-MS Grade', Article No. 11317490 (Fisher Scientific)
- Formic acid (99.5%), 'Optima™ LC-MS Grade', Article No. 10596814 (Fisher Scientific)
- Piston operated pipette, 'research 5000', variable volume selection (Eppendorf)
- Sample vials with 15 mm Ø screw thread, 12 mL capacity, clear glass (Wicom)
- Screw caps with Teflon®/silicon septa, 15 mm Ø (Wicom)
- 'Microman' pipettes, M25, M50, M250 and M1000 (Gilson Medical Electronics)
- 1.8mL glass (HPLC) vials with screw caps and Teflon coated sealing disks (WiCom)
- Volumetric flasks with different volumes (Brand)

8.4.3 Sample preparation and LC-MS/MS measurement

8.4.3.1 Sampling

The samplings were done by the staff of the department ecotoxicology or the analytical laboratory; the samples were prepared and measured directly after sampling without further storage.

To prevent degradation of the analytes or adsorption on the glass wall of the sample vials the water samples were stabilised by diluting with methanol in the ratio of 5+1 (v/v). To this end at the start of the sampling procedure aliquots of accurately 1.0 mL methanol were filled into each 12mL sample vial. Afterwards the water samples were taken out of the test aquaria using a 'research 5000' pipette. Pooled samples of exactly 5.0 mL were taken by moving the pipette tips through the aquarium; the taken samples were then pipetted into the prepared sample vials. The vials were closed with screw caps and the samples were mixed by hand.

[1] European commission, Directorate General Health and Consumer Protection, SANCO/3029/99 rev.4 (11/07/00), Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. Working document.

[2] Chemical name (IUPAC): 4,4'-((1*H*-1,2,4-triazol-1-yl)methylene)dibenzonitrile.

Following the samples were transported to the analytical laboratory under protection against sunlight and were analysed directly.

8.4.3.2 Sample preparation

Aliquots of exact 50 µL of the IS working solution (containing the IS Letrozole in methanol, conc.: 10 µg/mL) were pipetted into 1.8 mL HPLC vials; afterwards analytical sub-samples of 1.2 mL of the pre-diluted water samples were added. After tightly closing and manual shaking, aliquots of 5.0 µL of the mixtures were analysed by LC-MS/MS.

The remaining water samples were stored deep-frozen in a freezer as retain samples.

8.4.3.3 LC-MS/MS measurement

The determination of the analyte fadrozole was carried out by liquid chromatography (LC) and tandem mass spectrometry detection (LC-MS/MS) using electrospray ionization (ESI). The measurements were done by direct injection of the pre-treated samples into the LC-MS/MS system, the measurement conditions and instrument settings are listed below.

LC-MS/MS system

HPLC system:	Waters 2695
Mass spectrometer:	Waters / Micromass LC/MS/MS Quattro Micro (triple quadrupole system)
Software:	Waters / Micromass MassLynx Ver. 4.0
Quantitation software:	Waters / Micromass QuanLynx Ver. 4.0

LC parameter

Column:	Phenomenex Gemini 5 µm, C18, 150 mm x 3 mm
Guard column:	Phenomenex Gemini 5 µm, C18, 4 mm x 3 mm
Column temperature:	30°C
Injection volume:	5.0 µL
Flow rate:	0.5 mL/min
Mobile phase A:	Methanol containing 2 mmol NH4Ac
Mobile phase B:	Purified water containing 0.2vol-% formic acid

Gradient program

Time [min]	Mobile phase A [%]	Mobile phase B [%]
0.0	0	100
0.1	0	100
3.5	100	0
4.5	100	0
4.6	0	100
7.0	0	100

MS method

Type:	**MRM**	Ion mode:	**ESI+**
Span [Da]:	0.1	Solvent delay [min]:	0.1 – 3.7
End Time [min]:	7.0	Collision gas:	Argon

Compound table 1 (mass transitions)

Analyte	Retention time [min]	Precursor ion [m/z]	Product ion [m/z]	Dwell time [sec]	Cone voltage [V]	Collision energy [eV]
Fadrozole, quantification ion	4.2	224.15	81.15	0.12	40	22
Fadrozole, qualifier ion	4.2	224.15	82.15	0.12	40	24
Letrozol (IS), quantification ion	5.8	286.15	217.30	0.12	21	15

MS parameter

Source settings (ESI+)		Analyser settings	
Capillary [kV]:	2.5	LM 1 Resolution:	13.0
Extractor [V]:	2.0	HM 1Resolution:	13.0
RF Lens [V]:	0.1	Ion Energy 1:	0.5
Source temperature [°C]:	120	Entrance:	-1
Desolvation temperature [°C]:	350	Exit:	1
Cone Gas Flow [L/h]:	80	LM 2 Resolution:	15.0
Desolvation Gas Flow [L/h]:	500	HM 2 Resolution:	15.0
		Ion Energy 2:	1.0
		Multiplier voltage [V]:	650

8.4.4 Matrix calibration, Quantification and Calculation of the analytical results

8.4.4.1 Solutions of the analyte and the internal standard

Stock solutions of the analyte fadrozole and the IS letrozole were prepared in acetonitrile at nominal concentrations of 1 mg/mL. The stock solutions were prepared by precisely weighing and solving the analytical standards in volumetric flasks.

Afterwards an intermediate solution of the analyte and the working solution of the IS were prepared by pipetting aliquots of the respective stock solutions solution into separate volumetric flasks and filling up to the ring mark with methanol; the concentration of both solutions were exact 10.0 µg/mL.

8.4.4.2 Preparation of the calibration standards and the calibration samples

Due to the expected higher stability of the analyte fadrozole in an organic solvent the 'calibration standards' were prepared and stored in methanol; seven 'calibration standards' were prepared in a volumetric flask on October 01, 2013. The concentration range was 2.0 to 200 µg/L (see pipetting plan in Table 40).

The matrix matched 'calibration samples' were prepared afterwards in 1.8mL HPLC vials by mixing 200 µL of the respective calibration standards with 1000 µL 'holding- and dilution-water' and aliquots of exact 50 µL of the IS solutions containing Letrozole in a conc. of 10 µg/mL.

Table 40: Preparation of the 'calibration standards' for fadrozole

No. of the calibration standard	Volume intermediate solution I	Nominal capacity of the volumetric flask	Fadrozole concentration, volumetric flask	Fadrozole concentration, HPLC vials *)
[-]	[µL]	[mL]	[µg/L]	[µg/L]
1	10.0	10	10.0	2.00
2	25.0	10	25.0	5.00
3	50.0	10	50.0	10.0
4	125	10	125	25.0
5	250	10	250	50.0
6	500	10	500	100
7	1000	10	1000	200

*) Remark: After dilution of 200 µL of the calibration standards with 1000 µL 'holding- and dilution-water' and addition of the IS working solution, respective after preparation for LC/MS measurement, the actual concentrations are lower than specified in column 5 of the aforementioned table. However, the given concentrations refer on exact 1.0 mL water contained in the 'ready to measure' calibration samples. The aqueous test samples to be measured were pre-treated equally and therefore contained water and the further compounds (e.g. methanol and the IS) at same concentrations. This procedure allows the concentration of the analyte in the aqueous samples to be calculated directly from the calibration function.[3]

8.4.4.3 Creating of the matrix matched calibration function

The LC-MS/MS system was calibrated by measuring the prepared 'calibration samples' according to the instructions given in section 8.4.3.3. The calibration function (type: internal standard) was set up afterwards by plotting the calculated peak area ratio (peak area analyte / peak area IS × conc. IS) against the injected analyte concentrations.

With the calibration data quadratic regression analyses were carried out. On every 'measuring day' new calibrations were recorded.

8.4.4.4 Quantification and calculation of the analytical results

The quantification data were generated by processing (integration) the chromatographic raw data and by subsequent calculation of the quantification results using the currently valid basic calibration function.

As the aqueous test medium samples and the calibration solutions were analysed by direct injection into the LC-MS/MS system, the concentrations of the analyte fadrozole in the aqueous test medium was quantified directly (in units of µg/L).

[3] As already mentioned the calibration standards were prepared in solvent mixture I. Immediately prior to measurement these calibration standards were diluted with 'water' for proper liquid chromatography. The arising contraction in volume is not precisely ascertainable. To eliminate this potential source of error the aqueous test samples and the calibration samples were prepared with exactly the same volume ratios of water and organic solvent.

8.4.5 Validation of the analytical method

8.4.5.1 Preliminary remarks

The validation of the analytical method for the 'Quantitative determination of fadrozole in holding- and dilution-water of the flow through system using LC-MS/MS' was performed following the guideline SANCO/3029/99 rev.4 (11/07/00) on the proposed limits of quantification (LOQ) of 5.0 µg/L.

For validation of the analytical method matrix charged fortification samples were prepared and analysed; the obtained quantification results were processed statistically and were compared afterwards to facts listed in the EU guidance documents.

8.4.5.2 Fortification procedure

The fortification experiments were carried out on the respective LOQ level, on the '10 × LOQ' level and additionally to the SANCO requirements on a residue level of 150 µg/L.

The required 'fortification solutions' were prepared on October 11, 2013 by diluting the analyte intermediate solutions (fadrozole conc. = 10.0 µg/mL) with 'holding- and dilution-water' in volumetric flasks. The preparation of the fortification solutions is illustrated in Table 41.

Afterwards the matrix matched 'fortification samples' were made analogous to the water samples in 12mL sample vials by mixing 1.0 mL methanol with 5.0 mL of the respective aqueous 'fortification solutions'. For each fortification level five separate fortification samples were prepared (replicates A to E); in addition two untreated blank samples (only 'holding- and dilution-water') were prepared.

Table 41: Preparation of the fortification solutions for method validation of fadrozole

Fortification level (F) /fadrozole concentration	Fortification type	Number of replications	Dilution schema	
			Spiked matrix	Used volume of the intermediate solution
-	Blank	2 samples	20 mL 'holding- and dilution-water'	-
Level F1 / 5.00 µg/L	LOQ	5 samples	50 mL 'holding- and dilution-water'	25.0 µL
Level F2 / 50.0 µg/L	10 × LOQ	5 samples	50 mL 'holding- and dilution-water'	250 µL
Level F3 / 150 µg/L	Residue level	5 samples	50 mL 'holding- and dilution-water'	750 µL

The fortification samples were prepared for LC-MS/MS measurement analogous to the water matrix by pipetting aliquots of 50.0 µL of the IS working solution into 1.8 mL HPLC vials and adding 1.2 mL of the respective fortification samples. After closing and manual shaking, aliquots of 5.0 µL of the mixtures were analysed as described in section 8.4.3.3.

8.4.6 Results

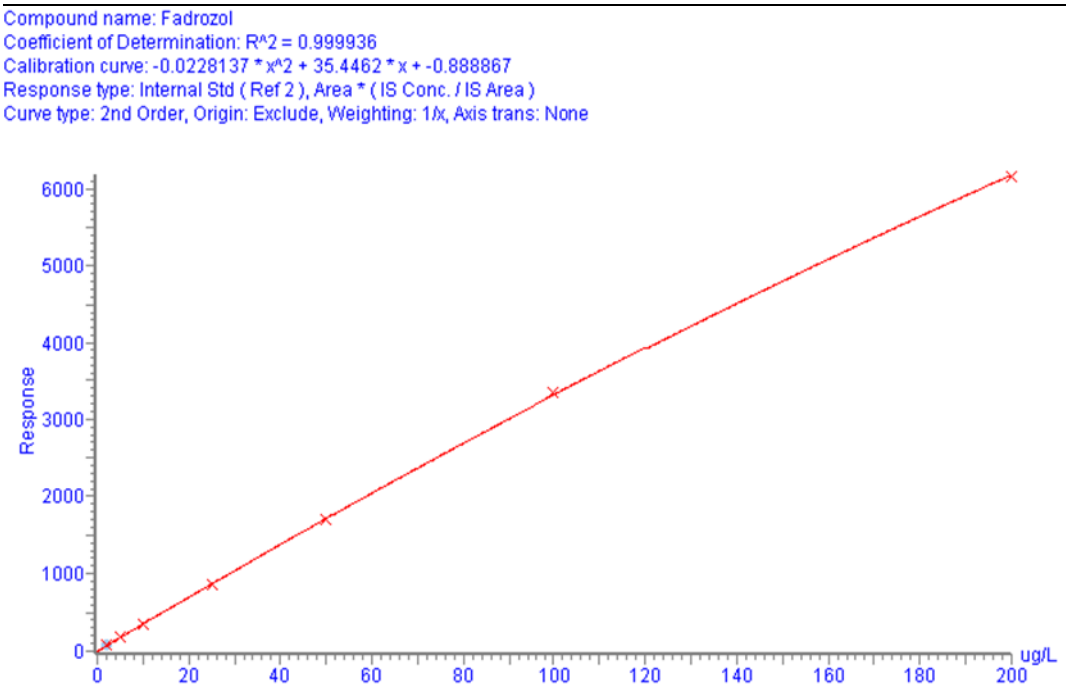
8.4.6.1 Matrix matched calibration

As already mentioned new matrix matched calibration functions were recorded on every measuring day (matrix: holding- and dilution-water, response type: internal standard).

The calibration function for fadrozole measured on October 11, 2013 is shown in Figure 18 as a typical example. The calibration function was calculated by quadratic regression analysis using the Waters QuanLynx software to:

$PAR = -0.02281 \times (CCal)2 + 35.45 \times CCal - 0.8889; \quad r2 = 0.9999$

Figure 18: Basic calibration function of fadrozole



The regression type 2nd order was chosen for the analyte fadrozole. As the measured coefficient of determination (r^2) was very close to 1 the correctness of the chosen quadratic curve fitting was approved.

In reverse, the correctness of this curve fitting to the present analytical task was verified during method validation by the determined recovery rates on three fortification levels in the concentration range from 5.0 to 150 µg/L (Ct Table 42: overall mean recovery rate of 95.8%).

8.4.6.2 Method validation

The analytical method for the 'Quantitative determination of fadrozole in holding- and dilution-water of the flow through system using LC-MS/MS' has been successfully validated in accordance with the EU guidance document SANCO/3029/99 rev.4 (11/07/00) on three fortification levels. The analytical results (measured analyte concentrations) and the calculated statistical values of the performed fortification experiments are summarised in Table 42.

Accuracy (Recovery)

The accuracy of the validated method is reported as the mean recovery ± relative standard deviation (RSD). The recovery rates of the analyte (%R) were calculated using the following equation:

$$\%R = \frac{C_{W,F} * 100\%}{F}$$

%R = Recovery rate of the analyte [%]

CW,F = Analyte concentration in the aqueous fortification samples (water) [µg/L]

F = Fortification level, analyte concentration [µg/L]

Example for sample F1a, analyte fadrozole (see Table A 2- 3):

$$\%R = \frac{4.93\ \mu g/L * 100\%}{5.0\ \mu g/L}$$

The accuracy of the method is given as the mean recovery on each fortification level and as the overall mean recovery of the entire validation experiment.

Table 42: Analytical and statistical results of the fortification experiments for fadrozole (mass transition used for quantification: m/z 224.15 to m/z 81.15)

Fortification level (F)		Analytical results			Statistical results			
No. (label) and level	Analyte conc.	LC-MS/MS quantific. data	Quantific. data, blank corrected	Measured analyte conc.	Recovery %R	Mean recovery	Standard deviation SD	Relative standard deviation, RSD
-	[µg/L]	[µg/L]	[µg/L]	[µg/L]	[%]	[%]	[%]	[%]
F0a - F0b, Blanks	-	0.190	Mean blank = 0.166		-	-	-	-
		0.142						
F1a – F1e, LOQ level	5.00	4.928	4.762	**4.76**	**95.2**	89.7	3.54	3.95
		4.506	4.340	**4.34**	**86.8**			
		4.707	4.541	**4.54**	**90.8**			
		4.500	4.334	**4.33**	**86.7**			
		4.611	4.445	**4.45**	**88.9**			
F2a – F2e, 10 × LOQ level	50.0	49.242	49.076	**49.1**	**98.2**	98.4	0.30	0.30
		49.434	49.268	**49.3**	**98.5**			
		49.595	49.429	**49.4**	**98.9**			
		49.263	49.097	**49.1**	**98.2**			
		49.295	49.129	**49.1**	**98.3**			
F3a – F3e, residue level	150	150.928	150.762	**150.8**	**100.5**	99.2	0.88	0.88
		148.839	148.673	**148.7**	**99.1**			
		147.358	147.192	**147.2**	**98.1**			
		148.312	148.146	**148.1**	**98.8**			
		149.135	148.969	**149.0**	**99.3**			
Overall mean (n = 15):						**95.8**	**4.86**	**5.08**

The mean recoveries of 89.7%, 98.4 and 99.2% as well as the overall mean recovery of 95.8% measured for fadrozole comply with the requirements of the guideline and prove the accuracy of the analytical method. [4]

Precision (Repeatability)

The precision of the validated method is reported in Table 42 as the relative standard deviation (RSD) of the repeatability for each fortification level. The repeatability depending on 15 determinations is reported as the overall relative standard deviation.

The mean RSDs of 3.95%, 0.30% and 0.88% as well as the overall mean RSD of 5.08% prove the repeatability of the described analytical method for the analyte fadrozole.[5]

The measured recovery rate and precision data prove that there was no matrix effect for the described analytical method.

[4] SANCO/3029/99 rev.4, chapter 3.1 (iii): Mean recoveries for each level should be in the range 70-110%, ideally with the mean in the range 80-100%.

[5] SANCO/3029/99 rev.4, chapter 3.1 (iv): Five determinations should be made at each fortification level. In general the RSD should be ≤ 20% per level.

Specificity and Blanks

The LC tandem mass spectrometry system used was capable to determine the analytes fadrozole in the worked-up injections solution with a slight interference of 'matrix compounds'.

The specificity of the analytical method for fadrozole (mass transition m/z 224.15 to m/z 81.15) is shown by LC-MS/MS chromatograms of untreated fortification samples (blanks) and control samples of the investigated matrix, see chromatograms in Figure 21, Figure 22, Figure 23, and Figure 24.

Marginal blank peaks for fadrozole were detected on both measured ion traces in the fortification blanks as well as in the control samples. Nevertheless the “blank requirement” of SANCO/3029/00[6] was fulfilled. The chromatogram of the ‘LOQ level’ fortification sample for fadrozole showed an abundance of 2.107e+004 at the ion trace m/z 81.15 (top chromatogram in Figure 22); however the abundance of the blank fortification sample was 5.035e+002 (Ct. Figure 21). This means that the abundance of the fortification blank was equivalent to only 2.4% of the LOQ level.

[6] According to SANCO/3029/00 blank values must reported. Blank values (procedural blanks and untreated samples) should not exceed 30% of the LOQ.

8.4.6.3 Results of the analysed samples

As already mentioned, the concentrations of the analyte fadrozole in the test medium were quantified directly using the currently valid calibration function.

At test start (t_0) and afterwards weekly water samples of the test aquaria were taken for analysis (controls and 3 treatment levels, each control/treatment in 4 replicates/test aquaria).

The results of the analysed samples are listed in Table 43 together with the calculated 'Percent of nominal' values.

Table 43: Analysed fadrozole concentrations in µg/L and corresponding 'Percent of nominal' values (mass transition used for quantification of fadrozole: m/z 224.15 to m/z 81.15)

Treatment level	Replicate	LC/MS/MS quantification data fadrozole [ng/mL]	Measured analyte concentration Fadrozole [µg/L]	Nominal test conc. [µg/L]	Percent of nominal fadrozole [%]
Sampling at test start on October 16, 2013					
Control	1	0.126	<LOQ	-	-
	2	0.138	<LOQ		-
	3	0.132	<LOQ		-
	4	0.128	<LOQ		-
Test conc. 1	1	10.897	**10.9**	**10**	**109.0**
	2	10.289	**10.3**		**102.9**
	3	9.645	**9.65**		**96.5**
	4	9.204	**9.20**		**92.0**
Test conc. 2	1	25.680	**25.7**	**32**	**80.3**
	2	25.166	**25.2**		**78.6**
	3	34.895	**34.9**		**109.0**
	4	35.601	**35.6**		**111.3**
Test conc. 3	1	87.859	**87.9**	**100**	**87.9**
	2	87.121	**87.1**		**87.1**
	3	88.031	**88.0**		**88.0**
	4	93.295	**93.3**		**93.3**

Table 43 (continued): Analysed Fadrozole concentrations in µg/L and corresponding 'Percent of nominal' values

Treatment level	Replicate	LC/MS/MS quantification data fadrozole [ng/mL]	Measured analyte concentration fadrozole [µg/L]	Nominal test conc. [µg/L]	Percent of nominal fadrozole [%]
Sampling at test start on October 22, 2013 (day 6)					
Control	1	0.213	<LOQ	-	-
	2	0.175	<LOQ		-
	3	0.157	<LOQ		-
	4	0.138	<LOQ		-
Test conc. 1	1	10.506	**10.5**	**10**	**105.1**
	2	10.842	**10.8**		**108.4**
	3	12.045	**12.0**		**120.5**
	4	12.413	**12.4**		**124.1**
Test conc. 2	1	33.696	**33.7**	**32**	**105.3**
	2	30.668	**30.7**		**95.8**
	3	33.951	**34.0**		**106.1**
	4	32.453	**32.5**		**101.4**
Test conc. 3	1	97.592	**97.6**	**100**	**97.6**
	2	87.947	**87.9**		**87.9**
	3	96.689	**96.7**		**96.7**
	4	93.858	**93.9**		**93.9**
Sampling at test start on October 29, 2013 (day 13)					
Control	1	n.d. *)	<LOQ	-	-
	2	n.d.	<LOQ		-
	3	n.d.	<LOQ		-
	4	n.d.	<LOQ		-
Test conc. 1	1	11.468	**11.5**	**10**	**114.7**
	2	11.455	**11.5**		**114.6**
	3	11.585	**11.6**		**115.9**
	4	11.470	**11.5**		**114.7**
Test conc. 2	1	27.231	**27.2**	**32**	**85.1**
	2	25.682	**25.7**		**80.3**
	3	33.697	**33.7**		**105.3**
	4	34.934	**34.9**		**109.2**
Test conc. 3	1	99.347	**99.3**	**100**	**99.3**
	2	99.906	**99.9**		**99.9**
	3	108.640	**108.6**		**108.6**
	4	108.969	**109.0**		**109.0**

Table 43 (continued): Analysed fadrozole concentrations in µg/L and corresponding 'Percent of nominal' values

Treatment level	Replicate	LC/MS/MS quantification data fadrozole [ng/mL]	Measured analyte concentration fadrozole [µg/L]	Nominal test conc. [µg/L]	Percent of nominal fadrozole [%]
Sampling on November 04, 2013 (day 19)					
Control	1	0.296	<LOQ	-	-
	3	0.283	<LOQ		-
Test conc. 1	1	9.223	**9.22**	**10**	**92.2**
	3	9.667	**9.67**		**96.7**
Test conc. 2	1	33.220	**33.2**	**32**	**103.8**
	3	34.069	**34.1**		**106.5**
Test conc. 3	1	100.642	**100.6**	**100**	**100.6**
	3	109.986	**110.0**		**110.0**
Sampling on November 12, 2013 (day 27)					
Control	2	n.d. *)	<LOQ	-	-
	4	n.d.	<LOQ		-
Test conc. 1	2	9.958	**9.96**	**10**	**99.6**
	4	9.461	**9.46**		**94.6**
Test conc. 2	2	38.784	**38.8**	**32**	**121.2**
	4	33.535	**33.5**		**104.8**
Test conc. 3	2	88.648	**88.6**	**100**	**88.6**
	4	96.623	**96.6**		**96.6**
Sampling on November 19, 2013 (day 34)					
Control	1	0.070	<LOQ	-	-
	3	0.069	<LOQ		-
Test conc. 1	1	9.545	**9.55**	**10**	**95.5**
	3	9.915	**9.92**		**99.2**
Test conc. 2	1	42.968	**43.0**	**32**	**134.3**
	3	36.221	**36.2**		**113.2**
Test conc. 3	1	91.521	**91.5**	**100**	**91.5**
	3	103.589	**103.6**		**103.6**

*) n.d.: not detected

Table 43 (continued): Analysed fadrozole concentrations in µg/L and corresponding 'Percent of nominal' values

Treatment level	Replicate	LC/MS/MS quantification data fadrozole [ng/mL]	Measured analyte concentration fadrozole [µg/L]	Nominal test conc. [µg/L]	Percent of nominal fadrozole [%]
Sampling on November 26, 2013 (day 41)					
Control	2	0.549	<LOQ	-	-
	4	0.546	<LOQ		-
Test conc. 1	2	10.212	**10.2**	**10**	**102.1**
	4	9.833	**9.83**		**98.3**
Test conc. 2	2	44.675	**44.7**	**32**	**139.6**
	4	39.266	**39.3**		**122.7**
Test conc. 3	2	106.413	**106.4**	**100**	**106.4**
	4	117.509	**117.5**		**117.5**
Sampling on December 04, 2013 (day 49)					
Control	1	0.111	<LOQ	-	-
	3	0.080	<LOQ		-
Test conc. 1	1	8.968	**8.97**	**10**	**89.7**
	3	7.822	**7.82**		**78.2**
Test conc. 2	1	47.027	**47.0**	**32**	**147.0**
	3	32.727	**32.7**		**102.3**
Test conc. 3	1	94.166	**94.2**	**100**	**94.2**
	3	90.660	**90.7**		**90.7**
Sampling on December 10, 2013 (day 55)					
Control	2	0.602	<LOQ	-	-
	4	0.580	<LOQ		-
Test conc. 1	2	8.912	**8.91**	**10**	**89.1**
	4	9.108	**9.11**		**91.1**
Test conc. 2	2	46.183	**46.2**	**32**	**144.3**
	4	24.817	**24.8**		**77.6**
Test conc. 3	2	101.158	**101.2**	**100**	**101.2**
	4	88.669	**88.7**		**88.7**
Sampling on December 16, 2013 (day 61)					
Control	1	0.116	<LOQ	-	-
	3	0.116	<LOQ		-
Test conc. 1	1	9.348	**9.35**	**10**	**93.5**
	3	8.656	**8.66**		**86.6**
Test conc. 2	1	47.279	**47.3**	**32**	**147.7**
	3	26.624	**26.6**		**83.2**
Test conc. 3	1	89.892	**89.9**	**100**	**89.9**
	3	81.434	**81.4**		**81.4**

8.4.7 Representative LC-MS/MS chromatograms

Typical LC-MS/MS chromatograms of calibration samples, matrix matched blanks, fortification samples and samples measured during the study are shown in Figure 19 to Figure 32 for the analyte fadrozole.

The figures are divided into 3 parts; the ion chromatogram of the quantification ion of the analyte is shown at the top (m/z 224.15 to 81.15), the qualifier ion of the analyte is shown in the middle (m/z 224.15 to 82.15) and the ion chromatogram of the quantification ion of the internal standard letrozole is shown at the bottom (m/z 286.15 to 217.3).

The retention time (t_R) of the analyte fadrozole was approximately 4.2 min and the t_R of the IS was approximately 5.8 min.

Figure 19: Calibration sample level 1, fadrozole conc.: 2.0 µg/L

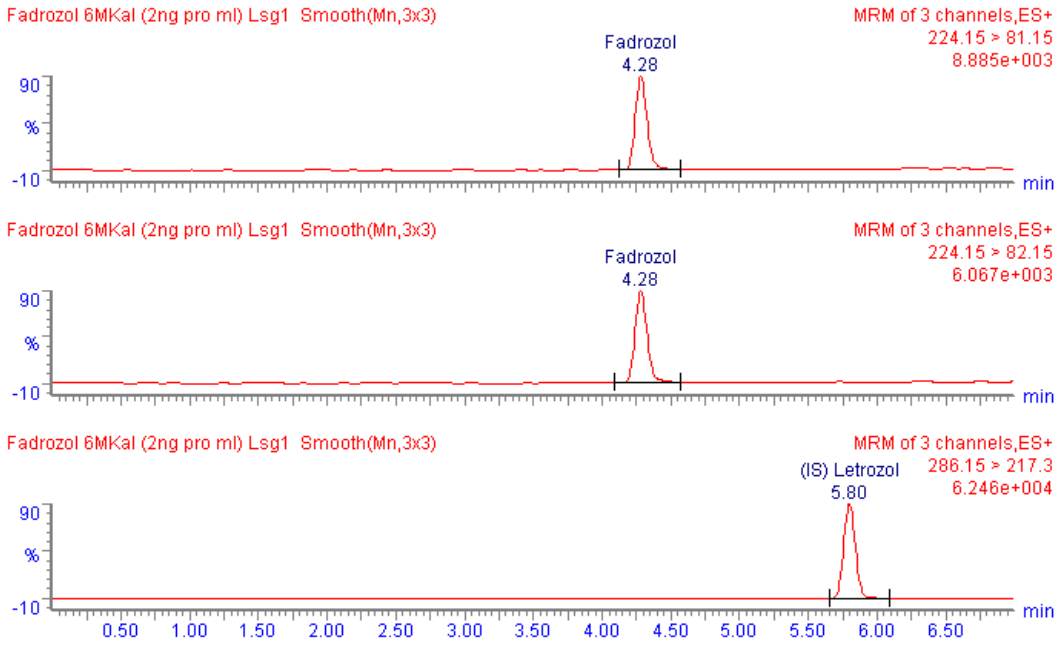


Figure 20: Calibration sample level 7, fadrozole conc.: 200 µg/L

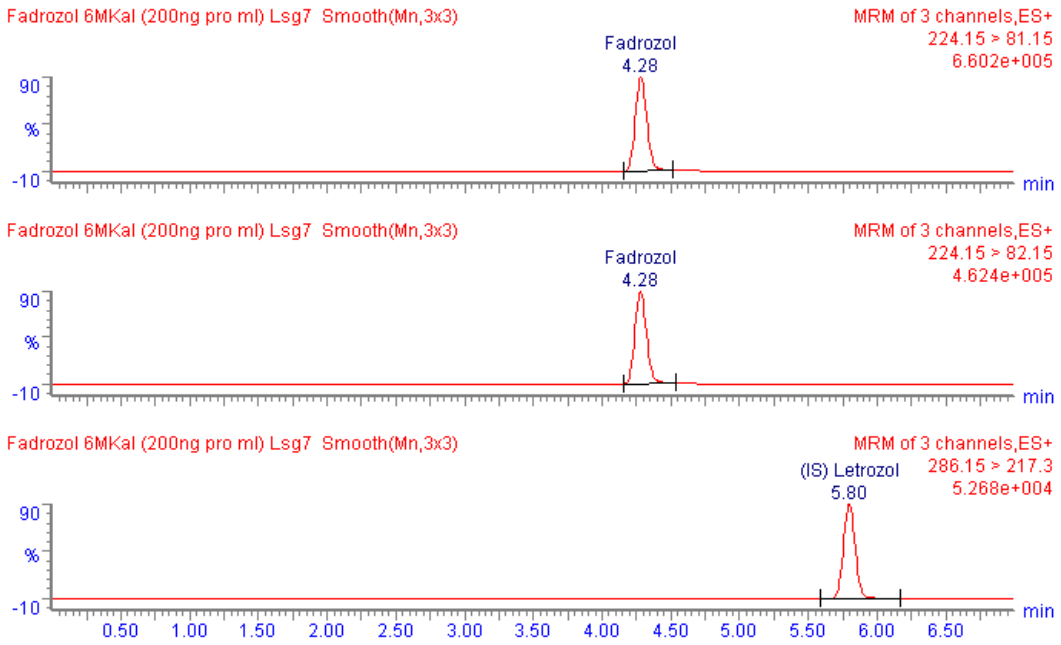


Figure 21: 'Blank' fortification sample (fadrozole, replicate b)

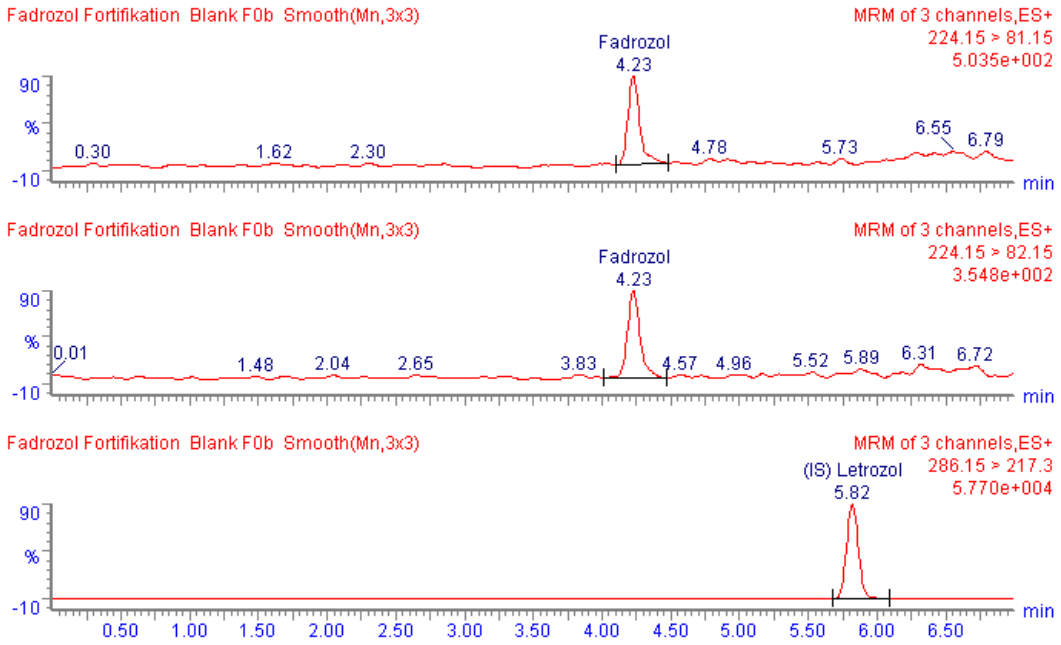


Figure 22: Fortification sample level 1 (LOQ level, replicate a), nominal fadrozole conc.: 5.0 µg/L

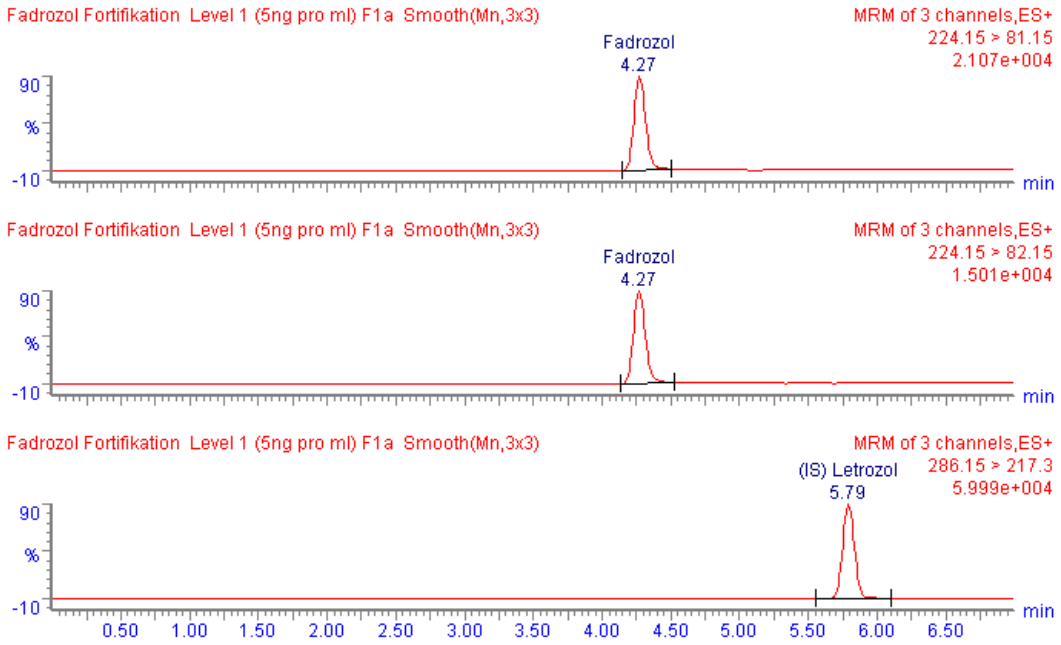


Figure 23: Fortification sample level 2 (10xLOQ level, replicate c), nominal fadrozole conc.: 50 µg/L

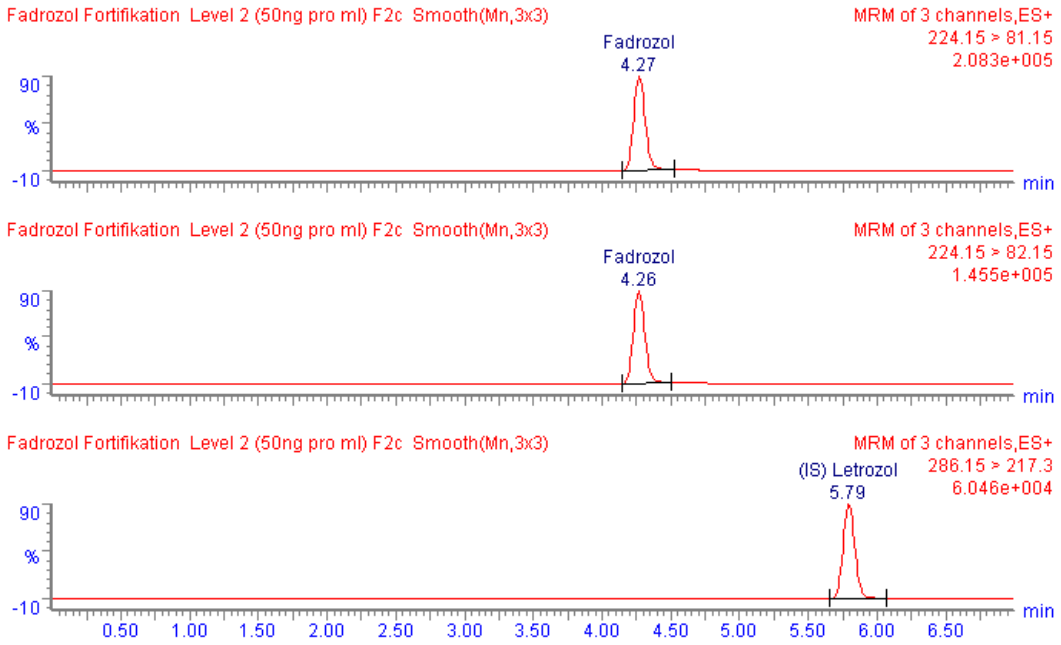


Figure 24: Fortification sample level 3 (residue level, replicate d), nominal fadrozole conc.: 150 µg/L

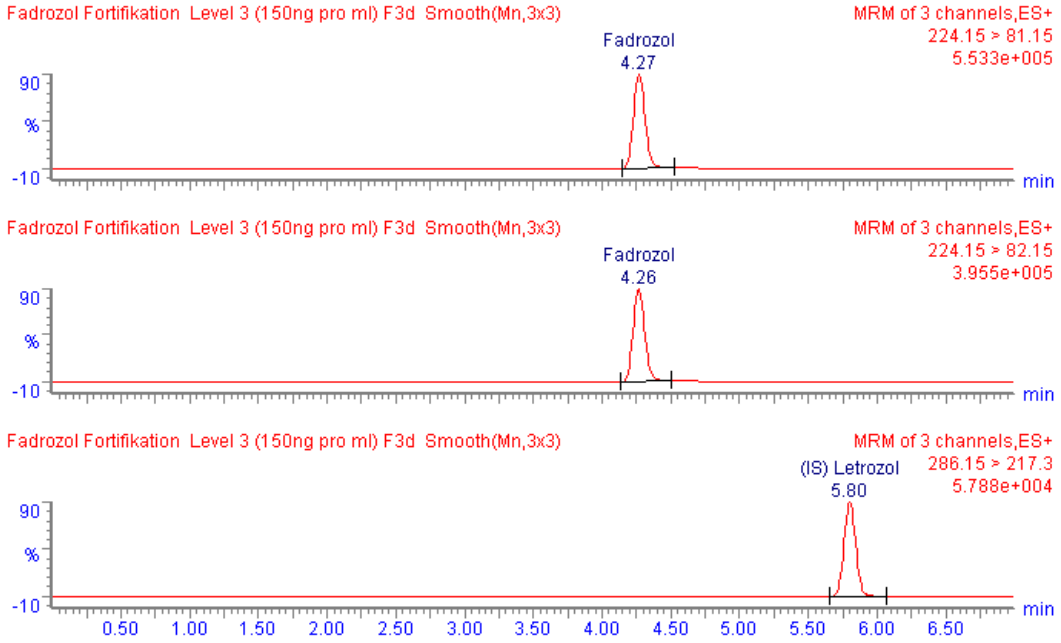


Figure 25: Control sample, measured at test start (repl. 1; October 16, 2013)

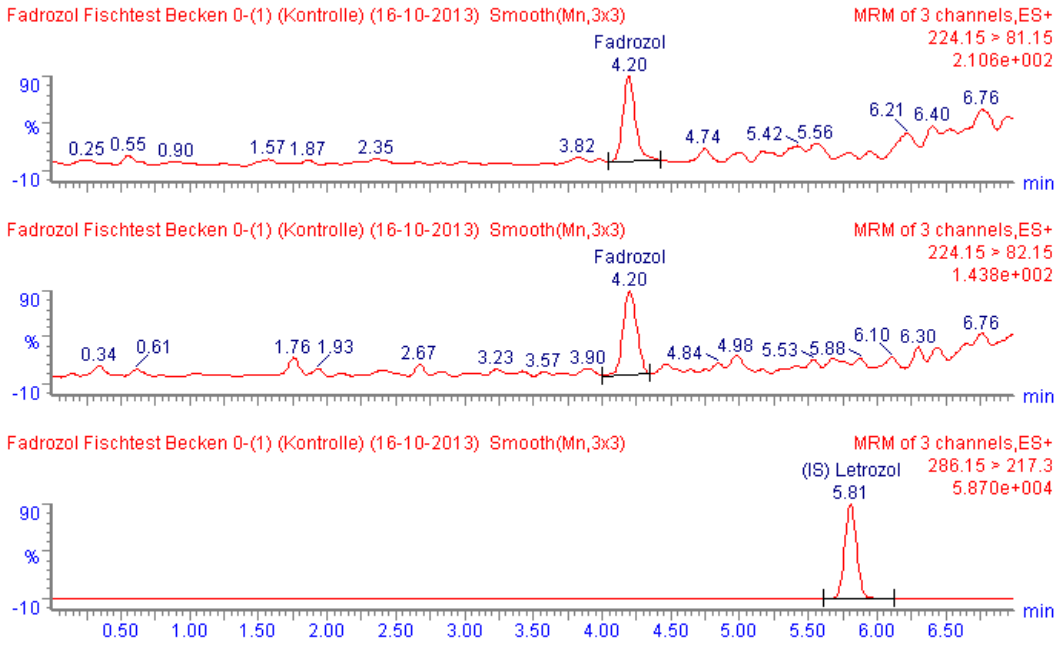


Figure 26: Control sample, measured at test end (repl. 1; December 16, 2013).

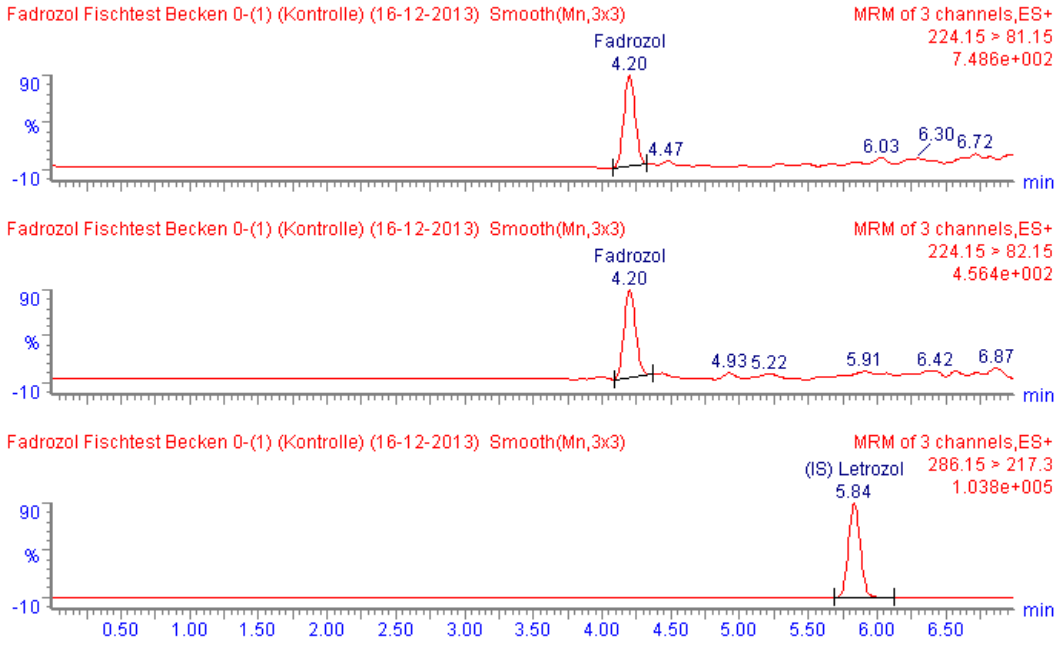


Figure 27: Test sample, test conc.: 10 µg/L, measured at test start (repl. 1)

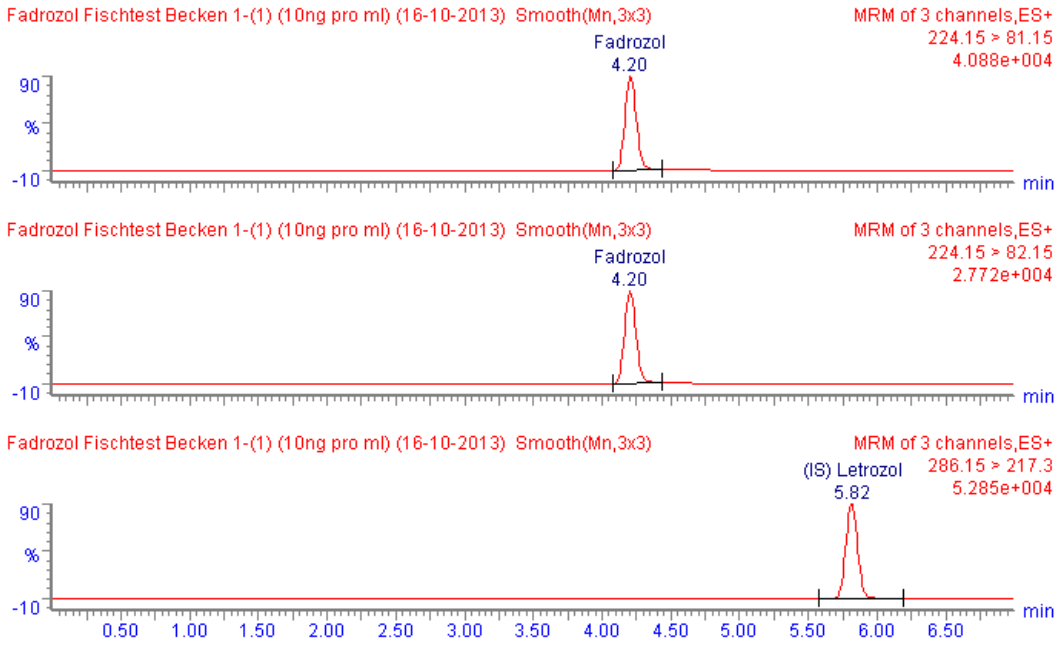


Figure 28: Test sample, test conc.: 10 µg/L, measured at test end (repl. 1)

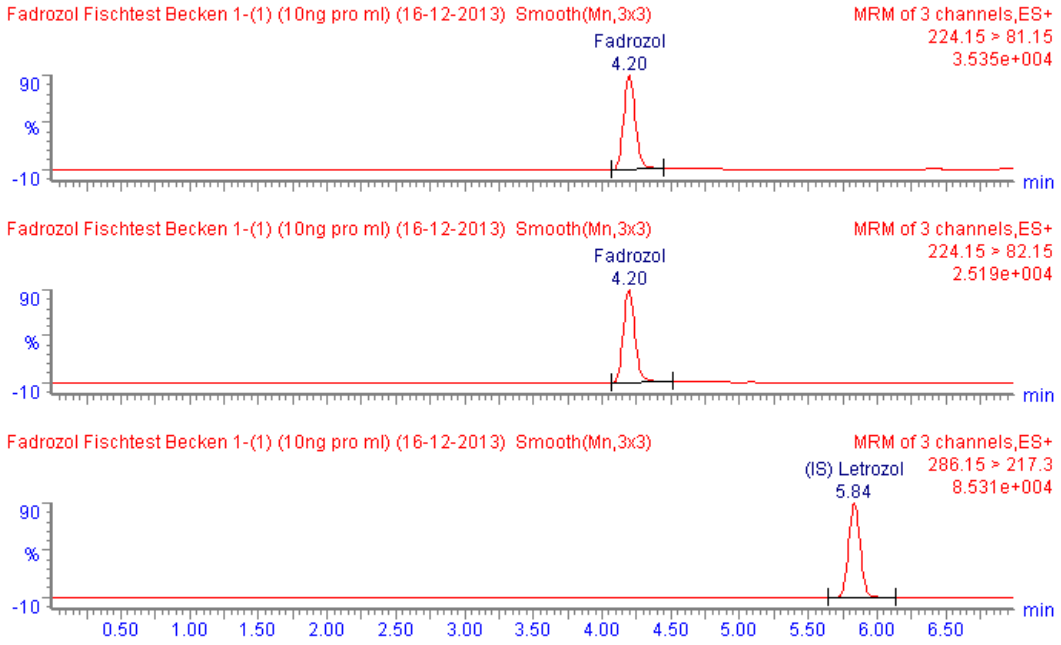


Figure 29: Test sample, test conc.: 32 µg/L, measured at test start (repl. 1)

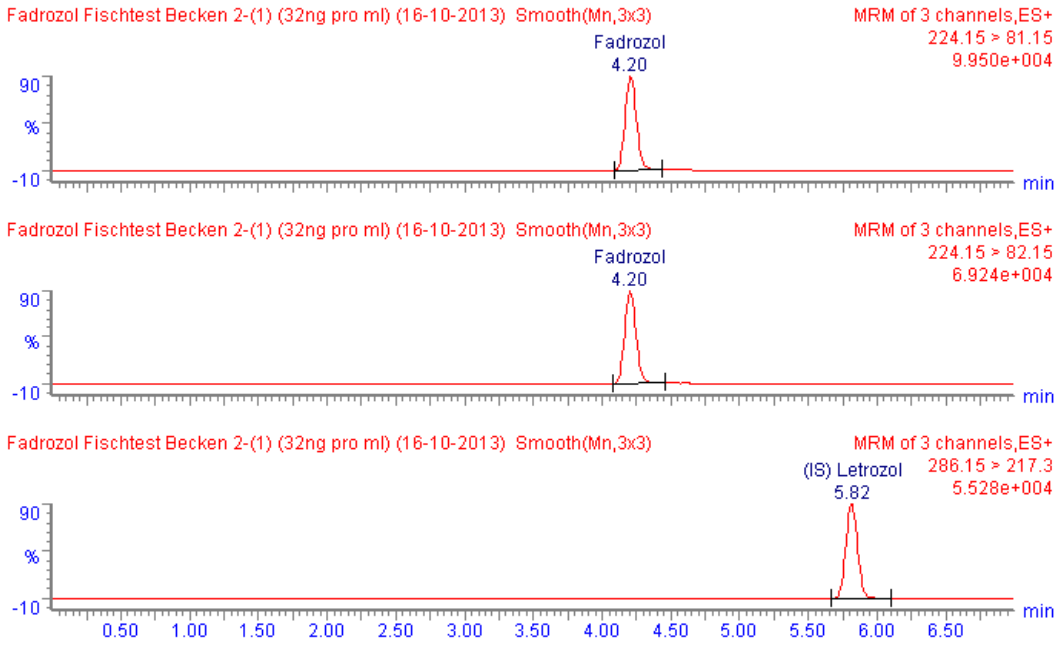


Figure 30: Test sample, test conc.: 32 µg/L, measured at test end (repl. 1)

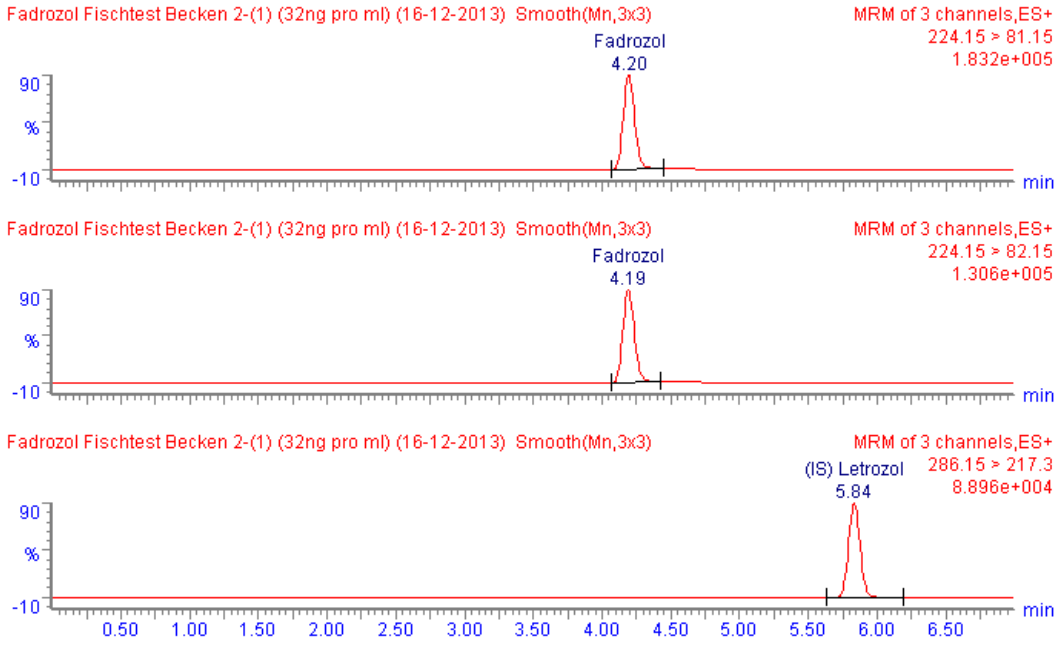


Figure 31: Test sample, test conc.: 100 µg/L, measured at test start (repl. 3)

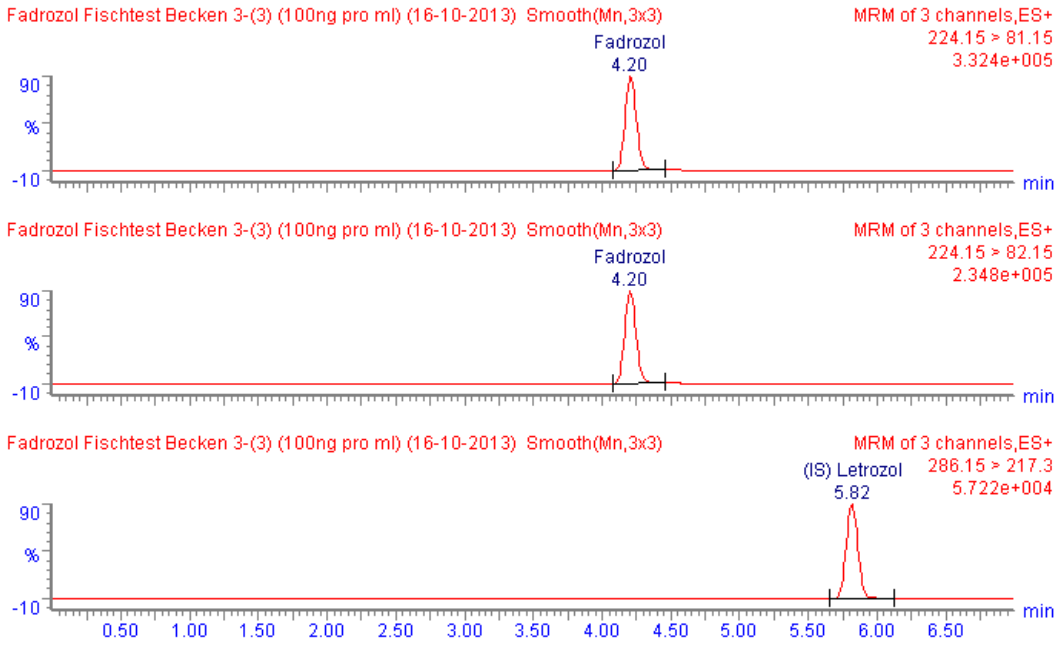


Figure 32: Test sample, test conc.: 100 µg/L, measured at test end (repl. 3)