TEXTE
57/2024

Final report

New-Generation Steroid Hormones

Tailored Assessment Strategies for Environmental Protection

by:

Dr. Matthias Teigeler, Dr. Kornelija Franzen,
Dr. Elke Eilebrecht, Prof. Dr. Christoph Schäfers

Fraunhofer-Institut für Molekularbiologie und Angewandte Ökologie IME, Schmallenberg

Dr. Dirk Schaudien

Fraunhofer-Institut für Toxikologie und Experimentelle Medizin ITEM, Hannover

publisher:
German Environment Agency

German Environment Agency

Umwelt Bundesamt

TEXTE 57/2024

Ressortforschungsplan of the Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection

Project No. (FKZ) 3717 67 405 0

FB001183/ENG

Final report

New-Generation Steroid Hormones

Tailored Assessment Strategies for Environmental Protection

by

Dr. Matthias Teigeler, Dr. Kornelija Franzen,
Dr. Elke Eilebrecht, Prof. Dr. Christoph Schäfers
Fraunhofer-Institut für Molekularbiologie und Angewandte Ökologie IME, Schmallenberg

Dr. Dirk Schaudien
Fraunhofer-Institut für Toxikologie und Experimentelle Medizin ITEM, Hannover

On behalf of the German Environment Agency

Imprint

Publisher
Umweltbundesamt
Wörlitzer Platz 1
06844 Dessau-Roßlau
Tel: +49 340-2103-0
Fax: +49 340-2103-2285
buergerservice@uba.de
Internet: www.umweltbundesamt.de

Report performed by:
Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie IME
Auf dem Aberg 1
57392 Schmallenberg
Germany

Report completed in:
May 2023

Edited by:
Section IV 2.2 “Pharmaceuticals”
Jean Bachmann

Publication as pdf:
http://www.umweltbundesamt.de/publikationen

ISSN 1862-4804

Dessau-Roßlau, April 2024

Abstract: New-Generation Steroid Hormones– Tailored Assessment Strategies for Environmental Protection

The entry of pharmaceuticals into the aquatic environment has become a major concern in environmental research and has gained increasing public interest. Pharmaceuticals, both human and veterinary, find their way into water bodies through various routes such as wastewater treatment plants and agricultural activities, posing risks to aquatic organisms. In particular, hormone-active substances have been identified as potential threats, even at low concentrations in water. These pharmaceutical residues are known to persist in the environment and may cause adverse effects on non-target organisms.

The objective of this project was to develop a tailored testing strategy for assessing the environmental risk of novel hormonal active substances, focusing on synthetic progestins and glucocorticoids, to aquatic organisms. In the initial phase of the project, a comprehensive literature review was conducted to gather and evaluate existing findings on the effects of these substances. Based on the literature review, two candidate substances representing both substance classes were selected for further study. Two long-term laboratory experiments were conducted using aquatic vertebrates, and an additional study with an aquatic invertebrate was carried out for the progestin. Dienogest and Dexamethasone were selected to represent progestins and glucocorticoids, respectively.

For Dienogest, a Zebrafish one generation reproduction test (ZEOGRT) was performed, and a Chironomid Life Cycle Test was conducted for the invertebrates. For Dexamethasone, only the Zebrafish study was conducted. The experiments involved exposing the organisms to different concentrations of the test substances and measuring various endpoints related to growth, reproduction, and survival. The physical water parameters were monitored to ensure stable test conditions.

For Dienogest, the results showed that it had no significant effects on the parental generation (F_0) of zebrafish but adversely affected the fertility and early larval survival in the first filial generation (F_1). Hatching success of the second filial generation (F_2) was also reduced. Based on the endpoint hatching success of the second filial generation (F_2), which was the most sensitive endpoint throughout the study, the overall NOEC of the ZEOGRT was determined to be 3.51 ng Dienogest/L and the LOEC was 10.3 ng Dienogest/L. In the Chironomid study, no effects were observed, indicating a lack of biological impact.

Regarding Dexamethasone, it caused reduced growth in both F_0 and F_1 generations of zebrafish, with males being more affected. However, reproductive capability and other endpoints were not negatively impacted. Based on the endpoint growth in terms of wet weight and total length the NOEC was determined to be 10.5 µg Dexamethasone/L. The LOEC was set at 34.7 µg Dexamethasone/L.

The results indicate that synthetic progestins, such as Dienogest, can have similar effects to potent endocrine substances like estrogens and androgens. However, the underlying mechanism remain unclear. On the other hand, glucocorticoid exposure, specifically Dexamethasone, had effects on the growth of fish across different life stages, but did not significantly affect reproductive performance or sex ratios. The studies suggest that fish are more sensitive to endocrine impacts compared to other aquatic organisms, however, in order to identify the underlying mode of action, additional methodological approaches, such as innovative Omics methods or the immune challenge, could provide valuable information on the molecular effects of the substances. Thus, further research is necessary to improve the identification of underlying mechanisms and their acceptance in the regulatory context.

Kurzbeschreibung: Steroidhormone der neuen Generation – Angepasste Bewertungsstrategie für den Umweltschutz

Der Eintrag von Arzneimitteln in die aquatische Umwelt ist zu einem wichtigen Thema in der Umweltforschung geworden und stößt auf zunehmendes öffentliches Interesse. Human- und Tierarzneimittel gelangen auf verschiedenen Wegen in die Gewässer und stellen eine Gefahr für Wasserorganismen dar. Es ist bekannt, dass Arzneimittelrückstände in der Umwelt verbleiben und schädliche Auswirkungen auf Nicht-Zielorganismen haben können. Insbesondere hormonaktive Substanzen wurden als potenzielle Bedrohung identifiziert, da diese selbst in geringen Konzentrationen im Wasser Schädigungen hervorrufen können.

Ziel dieses Projekts war die Entwicklung einer maßgeschneiderten Prüfstrategie zur Bewertung des Umweltrisikos neuartiger hormoneller Wirkstoffe, insbesondere synthetischer Gestagene und Glukokortikoide. In der ersten Phase des Projekts wurde eine umfassende Literatur-recherche durchgeführt, um vorhandene Erkenntnisse über die Auswirkungen dieser Stoffe zu sammeln und zu bewerten. Auf der Grundlage wurden zwei repräsentative Substanzen für die Wirkstoffklassen für weitere Untersuchungen identifiziert. Dienogest und Dexamethason wurden als Gestagene bzw. Glukokortikoide ausgewählt. Es wurden zwei Langzeit-Labor-experimente mit Fischen und eine zusätzliche Studie mit einem aquatischen Wirbellosen durchgeführt.

Für Dienogest wurde ein Zebrafisch-Ein-Generationen-Reproduktionstest (ZEOGRT) und für die Wirbellosen ein Chironomiden-Lebenszyklustest durchgeführt. Für Dexamethason wurde nur die Zebrabärblingstudie durchgeführt. Bei den Experimenten wurden die Organismen verschiedenen Konzentrationen der Prüfsubstanzen ausgesetzt und verschiedene Endpunkte in Bezug auf Wachstum, Fortpflanzung und Überleben gemessen.

Für Dienogest zeigten die Ergebnisse keine signifikanten Auswirkungen auf die Elterngeneration (F_0) der Zebrabärblinge. Die Befruchtungsfähigkeit und das Überleben der frühen Larven in der ersten Filialgeneration (F_1) waren beeinträchtigt, der Schlupferfolg der zweiten Filialgeneration (F_2) war ebenfalls verringert. Auf Basis dieses Endpunkts wurde die Gesamt-NOEC des ZEOGRT auf 3,51 ng Dienogest/L und die LOEC auf 10,3 ng Dienogest/L festgelegt. In der Chironomiden-Studie wurden keine Auswirkungen als Folge der Substanzexposition beobachtet.

Dexamethason verursachte sowohl in der F_0- als auch in der F_1-Generation der Zebrabärblinge ein verringertes Wachstum, wobei die Männchen stärker betroffen waren. Die Fortpflanzungs-fähigkeit und andere Endpunkte wurden jedoch nicht negativ beeinflusst. Auf der Grundlage des Endpunkts Wachstum in Bezug auf Nassgewicht und Gesamtlänge wurde der NOEC-Wert auf 10,5 µg Dexamethason/L festgelegt. Die LOEC wurde auf 34,7 µg Dexamethason/L festgelegt.

Die Ergebnisse deuten darauf hin, dass synthetische Gestagene wie Dienogest ähnliche Wirkungen wie potente endokrine Substanzen wie Östrogene und Androgene haben können. Der zugrunde liegende Mechanismus bleibt jedoch unklar. Andererseits hatte die Exposition gegenüber Glukokortikoiden, insbesondere Dexamethason, Auswirkungen auf das Wachstum der Fische in verschiedenen Lebensstadien, wirkte sich jedoch nicht signifikant auf die Fort-pflanzungsleistung oder das Geschlechterverhältnis aus. Die Studien deuten darauf hin, dass Fische im Vergleich zu anderen Wasserorganismen empfindlicher auf endokrine Wirkungen reagieren. Um die zugrunde liegende Wirkungsweise zu ermitteln, könnten jedoch zusätzliche methodische Ansätze, wie innovative Omics-Methoden oder Untersuchungen zur immunsuppressiven Wirkung, wertvolle Informationen über die molekularen Wirkungen der Substanzen liefern. Daher sind weitere Forschungsarbeiten erforderlich, um die Identifizierung der zugrundeliegenden Mechanismen zu verbessern und damit die Akzeptanz im regulatorischen Kontext zu gewährleisten.

Table of content

List of figures

List of tables

List of abbreviations

Anova	Analysis of Variance
(d)pf	(days) post fertilisation
ELISA	Enzyme linked immunosorbent assay
ELS	Early life stage
F_0 generation	Parental generation (F_0)
F_1 generation	First filial generation (F_1)
F_2 generation	Second filial generation (F_2)
FLCT	Fish life cycle test
FSDT	Fish sexual development test
FSTRA	Fish short term reproduction assay
IME	(Fraunhofer) Institute for Molecular Biology and Applied Ecology
LC MS	Liquid Chromatography - Mass Spectrometry
LOEC	Lowest observed effect concentration
LPS	Lipopolysaccharides
MEOGRT	Medaka extended one generation reproduction test
MoA	Mode of action
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PaMP	Pathogen-associated molecular pattern
pf	Post fertilisation
RSD	Relative standard deviation
SD	Standard deviation
SOP	Standard operation procedure
TIU	Trypsin Inhibitor Unit
UBA	Umweltbundesamt (German Environment Agency)
US-EPA	US Environmental Protection Agency
VTG	Vitellogenin
ZEOGRT	Zebrafish extended one generation reproduction test

Summary

The entry of pharmaceuticals into the aquatic environment is a central topic in environmental research with growing interest of the public.

Human pharmaceuticals enter the municipal wastewater system primarily through human excretions and as a result of improper disposal. Wastewater treatment plants can only inadequately remove the drug residues that are washed in. As a result, a large proportion of drug residues enters the water cycle almost unchanged. In comparison, veterinary drugs and pharmaceuticals are mainly discharged into the environment through the application of manure and slurry.

Due to advances in medical research, it is it possible to design and produce active ingredients with specific targets. These drugs are often based on natural molecules in their structure. By modifying the molecular structure, these active ingredients often become more stable, specific and potent in their mode of action. There are numerous examples, in particular of hormone-active substances, which can trigger adverse effects in aquatic environmental organisms even at a very low water concentration.

Pharmaceutical residues often exhibit a high degree of stability. Consequently, they persist and can therefore harm non-target organisms over a long period of time. In addition, accumulation and mutually reinforcing effects must also be considered.

The aim of the project was to develop an adapted strategy for assessing the environmental risk of novel hormonal active substances to aquatic organisms, in particular synthetic progestins and glucocorticoids. During a detailed literature research, the current findings on the effects of both substance classes were collected and evaluated. Based on the results, two candidate substances were identified, representing both classes. In a next step, two long-term effect studies with aquatic vertebrates with the two identified test substances were carried out, for the progestin a study with an aquatic invertebrate was also conducted.

The results of the laboratory tests were collected and compared with the results from the literature and discussed. From this, an evaluation strategy was developed on the basis of the two example active ingredients, with the aim to develop a tailored risk assessment.

Based on the results of literature research, but also considering practical aspects, it was decided to conduct the ecotoxicological study with Dienogest, representing the progestins, and Dexamethasone representing the glucocorticoids. Both substances were identified to be stable in aqueous media and thus considered as applicable for a long-term study under flow though conditions. For both substances, a Zebrafish one generation reproduction test (ZEOGRT) was conducted. For Dienogest, additionally a Chironomid Life Cycle Test according to OECD guideline 233 was performed. Both study protocols aim to detect potential endocrine effects related to population relevant endpoints.

The ZEOGRT protocol covers a multi-generation approach, including parental adult spawners, a full filial 1 (F_1) generation, starting with embryos and including juvenile growth as well as reproduction of adults, and furthermore a filial 2 (F_2) generation including embryo development and hatch. The procedure is described in a draft protocol which is currently validated at OECD.

The setup of the Chironomid Life Cycle Test with Dienogest covers two midge generations, starting with first instar larvae for the filial 1 (F_1) generation, emergence of F_1 adults, transfer of F_1 egg ropes to prepare a second filial generation (F_2) and keep it until emergence of the adult F_2 animals. This study is conducted in a water sediment system.

For Dexamethasone, only a ZEOGRT was conducted.

As part of the project, analytical measurements of aqueous samples were conducted collected from two *Daphnia magna* reproduction tests (conducted outside the project) with the same test substances.

Studies with Dienogest

Zebrafish, Extended one generation reproduction test

The nominal test concentrations applied were at 3.2, 10, 32, 100, and 320 ng Dienogest/L. Based on a regular measurement of test substance concentrations once per week, the mean measured concentrations were calculated to be 3.51, 10.3, 31.7, 105, and 335 ng Dienogest/L. The measurement of the physical water parameters, i.e. water temperature, oxygen concentration and pH, confirmed stable test conditions throughout the study. However, during a limited time period of eight days of the initial part of the study, the water temperature was higher than the recommended limit of 27.5 °C, but did not exceed 28 °C. Thus, the impact of this higher temperature was considered as minor. No impact on hatching and survival of the larvae was observed.

For the **parental generation (F_0)**, no statistically significant effect of Dienogest on any population-relevant endpoint was determined. Fish growth in terms of lengths and weights was not affected. The inspection of the fish gonads for phenotypic sex characteristics confirmed the correct setting of the spawning groups, i.e. 5 males and 5 females, for most of the fish groups applied. Furthermore, Dienogest did not alter the Vitellogenin concentration in blood plasma of both males and females. The histopathological analysis of maturation stages and further lesions did not reveal an effect linked to test substance exposure.

In contrast to that, reproduction in terms of fertility and early larval survival was affected by Dienogest in the **first filial generation (F_1)**. More precisely, fertility was significantly reduced compared to control at the highest treatment level of 335 ng Dienogest/L, the resulting NOEC for this endpoint was thus 105 ng Dienogest/L. A significant reduction of post hatch survival at day 21 pf and 35 pf was observed at concentrations of ≥105 ng Dienogest /L, the corresponding NOEC was thus 31.7 ng Dienogest/L. Notably, mortality of larvae occurred mainly before day 21 pf, during the phase of feed transition from yolk sac feeding to external feeding. Thereafter, i.e. during the phase of juvenile growth and the adult phase, no further mortality was observed until test end.

Hatching success of the **second filial generation (F_2)** was significantly reduced compared to control in all treatment levels except the lowest concentration at 3.51 ng Dienogest/L. It can be assumed that a low quality of F_1 eggs was the reason for a reduced hatching success of the F_2 generation larvae. The observed reduction of F_1 fertility underlines this assumption. However, a reduced VTG concentration in the blood plasma of F_1 females was not measured, suggesting that the yolk amount in the eggs was not impacted.

Based on the endpoint hatching success of the second filial generation (F_2), which was the most sensitive endpoint throughout the study, the overall NOEC of the ZEOGRT was determined to be 3.51 ng Dienogest/L and the LOEC was 10.3 ng Dienogest/L. The observed effect on the early life stage survival of F_1 underlines the overall observation that especially embryonic and larval stages were directly impacted by this substance.

Chironomid Life Cycle Test, according to OECD 233

A Chironomid life cycle test was conducted to detect the effects of a Dienogest exposure to two generations of *Chironomus riparius* under static conditions. The nominal test concentrations applied were at 6.25, 12.5, 25, 50, and 100 mg/kg sediment dw Dienogest. The test substance was applied by spiking the sediment.

The water parameters recorded confirmed stable test conditions during the study. The measured initial concentrations of the test substance in sediment were at 6.33, 12.0, 20.1, 49.6, and 93.3 mg/kg sediment dw Dienogest for the F_1 generation and 5.41, 11.9, 23.6, 46.7, and 94.7 mg/kg sediment dw Dienogest for the F_2 generation. The water concentrations of Dienogest were low at test start and also after 4 weeks, suggesting a stable binding of the substance at the sediment matrix. Thus, the major exposure was during the larval development of the midges.

No effect on the developmental stages of the exposed midges was observed. The time of emergence of adult F_1 stages was without statistically significance compared to the control. The evaluation of the sex ratio of the emerged midges did not identify a substance related effect.

The number of F_1 egg clutches and the calculated number of egg clutches per female did not reveal a substance related effect. No effect on F_2 emergence and sex ratio was detected.

Thus, due to the absence of biological effects in this study, the overall NOEC was determined to be ≥ 100 mg Dienogest/kg sed. dw (nominal concentration).

Study with Dexamethasone

Zebrafish, Extended one generation reproduction test

The nominal test concentrations applied were at 0.32, 1.0, 3.2, 10, 32, and 100 µg Dexamethasone/L. Based on a regular measurement of test substance concentrations once per week, the mean measured concentrations were calculated to be 0.33, 0.91, 3.2, 10.5, 34.7, and 100 µg Dexamethasone/L. The measurement of the physical water parameters, i.e. water temperature, oxygen concentration and pH, confirmed stable test conditions throughout the study.

In the **parental generation (F_0)**, no effects on reproduction in terms of fecundity and fertility were detected. The determination of the phenotypic sex confirmed the correct setting of spawning groups for most of the test vessels, i.e. 5 males and 5 females. Effects related to test substance exposure were mainly found for the growth parameters. Male wet weight was significantly reduced compared to control starting at a concentration of 34.7 µg Dexamethasone/L. Thus, the NOEC was set at 10.5 µg Dexamethasone/L. Female wet weight as well as total length were also reduced in the presence of Dexamethasone, but only at the highest treatment level of 100 µg Dexamethasone/L. Accordingly, the NOEC was set as 34.7 µg Dexamethasone/L. Vitellogenin concentration in male and female blood plasma and the histopathological pattern of the examined gonads of both males and females were not affected by the test substance.

Also, in the **first filial generation (F_1)**, retarded growth of the exposed fish was observed and thus consistent with the findings linked to F_0. Notably, the reduction of growth in terms of total length was evident during development from larvae to adult male fish. More precisely, at day 35 pf, the total length was significantly reduced compared to control only at the highest treatment level, whereas measurement at day 63 pf as well as male total length at test end revealed a significant reduction starting even at 34.7 µg Dexamethasone/L. However, female total length at test end were not affected by Dexamethasone exposure, but male and female wet weights at test end were significantly reduced at ≥34.7 µg Dexamethasone/L.

There were no effects on the reproduction capability of the F_1. Although the growth performance was impacted, there was no delay observed with regard to the date of spawning start, i.e. day of first spawning. The reproduction parameters fecundity and fertility were not negatively impacted due to test substance exposure. Other apical endpoints like survival or sex ratio as well as Vitellogenin concentration in blood plasma and the histopathological pattern of the examined gonads were not affected by the test item.

Hatching success of the second filial generation (F_2) was found to be significantly reduced at 10.5 and 34.7 µg Dexamethasone/L. However, as the hatching rates achieved 90% and a concentration-response is missing, a biological relevance of this observation can be excluded.

Based on the endpoint growth in terms of wet weight and total length the NOEC was determined to be 10.5 µg Dexamethasone/L. The LOEC was set at 34.7 µg Dexamethasone/L.

Conclusion and Discussion

Studies with progestins and glucocorticoids

For synthetic progestins, the scientific literature describes several examples of effect patterns for fish similar to other potent endocrine acting substances, e.g. estrogens and androgens. From the substances assessed in this project, Dienogest is reported as anti-androgenic. As the database for effects of Dienogest on fish (and other aquatic organisms) is still very poor, the presented results can help to close existing data gaps. Published data was mostly linked to well described natural and synthetic molecules e.g. Progesterone or Levonorgestrel, respectively. Most articles described effects on egg numbers or observations related to masculinisation or changes in sex ratios towards males.

The relevant effects derived from the ZEOGRT linked to Dienogest exposure were represented by decreased survival of early life stages (F_1-generation) and a reduced hatching success of F_2 generation, while F_2 hatch was clearly more sensitive. Also, the fertilisation success of the adult spawning groups (F_1) was significantly reduced while the fecundity was not affected. It can be assumed that the observed effect was the result of an affected male performance, resulting in inefficient fertilisation capacity. However, based on the histopathological evaluation, no effects on male gonadal tissue were found. Also, the Vitellogenin measurements did not reveal any effect, thus, no information on the underlying mechanism could be derived.

The Chironomid Life Cycle conducted showed no effects in the test concentration range applied. In a reproduction test with the prosobranch snail *Potamopyrgus antipodarum*, no effect on reproduction in terms of the number of embryos in the applied concentration range was detected. Also, no effect on growth and survival was detected. Finally, only the exposed zebrafish expressed sufficient sensitivity to derive an information of a potential endocrine impact of the substance.

There are various studies describing possible impact of glucocorticoid exposure to aquatic organisms including fish. In this context, many studies report direct or indirect impacts on the immune system.

Reported effects on the apical endpoints include reduced survival, reduced growth performance and spawning success. Physiological effects were characterized – beside others - by decreased vitellogenin and estradiol concentrations.

In the ZEOGRT study with Dexamethasone conducted in the course of this project, effects on growth were observed for all life stages applied, i.e. the parental F_0 generation, but also for the early, juvenile and adult stages of F_1 generation. The hatch of F_2 was impacted, but did not show a clear concentration effect response. Interestingly, a decrease of both length and weight was

already observed for the parental fish (F_0). Further impacts on F_0 and F_1 generation, e.g. reduction of reproductive performance, sex ratio or the plasma Vitellogenin concentration were not recorded in the ZEOGRT.

A reproduction test with *Potamopyrgus antipodarum* was conducted at nominal concentrations of 1.0, 3.2, 10, 32, and 100 µg Dexamethasone/L and showed no effect. Further invertebrate studies from the literature showed a higher sensitivity of e.g. crustacea compared to algae and furthermore reported a decline of daphnia populations in a multigeneration setup.

New methodical approaches like the lipopolysaccharides (LPS) – challenge, conducted with zebrafish embryos, were reported as suitable tools to detect the immunosuppressive potential of the glucocorticoids impacting the survival especially of early life stages. However, further research is needed to sufficiently close the gap between immunosuppressive mechanisms and the resulting effects on the apical level.

Testing strategy

From the results obtained from the fish studies conducted it can be postulated that the ZEOGRT provides an appropriate setup to detect the relevant apical endpoints. For the test substances applied the test showed sufficient sensitivity. Moreover, the defined validity criteria were all met for both studies conducted confirming the robustness of the protocol applied.

Sexual development and reproduction capability represent main apical endpoints with regard to the identification of potential endocrine acting substances. A continuous spawning performance of the fish groups was observed for both studies, with low variability in the data sets which allowed a robust statistical evaluation. A decrease of egg numbers was detected in comparison of F_0 and F_1 generation, however, this can be linked to age and thus a different stage of maturation. The parameter sex ratio is a critical one for zebrafish, and might be impeded by high between-replicate variability. For both ZEOGRT studies conducted, the control means were in line with the acceptance criteria for zebrafish sex ratio, i.e. 30-70% of respective sex (males or females). However, the data expressed bias for one fish sex. Genetic variability of zebrafish cultures is discussed to be a reason for variability. To meet this issue, the ZEOGRT protocol was adapted in that way, that fertilised eggs collected to prepare the F_1 generation were sampled and pooled over two successive days.

The egg-yolk precursor protein Vitellogenin represents an established biomarker in endocrine related fish test guidelines. However, for specific modes of action, e.g. thyroid disruption or general impacts on steroidogenesis, Vitellogenin may not always represent the sensitive physiological parameter. Moreover, the discrimination of effects linked to systemic toxicity against potential endocrine mediated effects is not possible by solely standing VTG results. Moreover, the evaluation of VTG measurements can be impeded by high variation of individual measurement results, also in control groups. To prevent significant bias of data set due to single outliers, it was decided for the ZEOGRT studies presented here to calculate geometric mean values of the VTG concentrations per replicate tank.

The identification of underlying mode of actions is a key issue for the development of tailored risk assessment strategies for all kind of chemical regulation including pharmaceuticals. The conducted fish studies were shown to be appropriate to identify sensitive apical endpoints. By comparison with tests on other aquatic organisms, it became obvious that fish represent the most sensitive organism group related to the very direct route of exposure via the respiratory organs. With regard to the EU definition of Endocrine disruptors to include physiological parameters (Vitellogenin) and also histopathological parameters to assist the identification of underlying mechanisms and to create a plausible link between cause and effect. However, as

presented in this report, both were not sufficiently sensitive for the test substances applied. Nevertheless, there are promising methodical approaches which can improve the identification of underlying modes of action.

Fish test protocols like the ZEOGRT allow the collection of appropriate tissue material without extending the number of animals used. As the Adverse Outcome Pathway (AOP) concept became an important tool for the assessment of endocrine acting substances, available -omic methods provide valuable information on the molecular initiating event (MIE) and the following Key Events (KE). The collected tissues allow to generate this data for different life stages of exposed fish.

E.g. for the progestins, gene expression analysis can help to discriminate anti-estrogenic or glucocorticoid-like MoA (related to Progesterone analogues) or anti-estrogenic or androgenic MoA (more related to the Nortestosterone-analogues). Beside the described methods for detection of immunosuppressive mechanisms, i.e. LPS or PAMPs challenge, available -omic methods can be used to discriminate the direct impacts from secondary indirect effects due to immune suppression.

Further research is needed to focus the application of -omic methods with respect to the relevant mechanisms of action and thus improve acceptance in the regulatory context.

Zusammenfassung

Der Eintrag von Arzneistoffen in die aquatische Umwelt ist ein zentrales Thema der Umweltforschung, das angesichts des stetig wachsenden Bedarfs an Human- und Tierarzneimitteln, immer mehr an Bedeutung gewinnt.

Humanarzneistoffe gelangen hierbei vor allem durch menschliche Ausscheidungen und infolge unsachgemäßer Entsorgung in das kommunale Abwassersystem. Kläranlagen können die eingeschwemmten Rückstände nur unzureichend beseitigen, sodass diese schließlich nahezu unverändert in den Wasserkreislauf eintreten. Tierarzneimittel wiederum, werden hauptsächlich durch das Ausbringen von Gülle und Mist in die Umwelt eingetragen.

Arzneimittelrückstände weisen vielfach eine hohe Stabilität auf. Sie zeichnen sich folglich durch eine lange Verweildauer (Persistenz) aus und können daher über einen langen Zeitraum hinweg Nichtzielorganismen schädigen. Daneben gilt es auch anreichernde (kumulative) sowie einander verstärkende (synergistische) Effekte zu berücksichtigen. Ein weiteres Risiko stellt der Trend hin zur Entwicklung hochpotenten Wirkstoffe dar. So üben z.B. hormonaktive Substanzen bereits in kleinsten Mengen eine schädliche Wirkung aus und werden deshalb als besonders umweltgefährlich bezeichnet.

Ziel des Projektes war die Entwicklung einer angepassten Strategie zur Bewertung des Umweltrisikos neuartiger hormoneller Wirkstoffe für aquatische Organismen, insbesondere synthetischer Gestagene und Glukokortikoide. In einer ausführlichen Literaturrecherche wurden die aktuellen Erkenntnisse zu den Wirkungen der beiden Substanzklassen gesammelt und ausgewertet. Anhand der Ergebnisse wurden zwei Kandidatensubstanzen identifiziert, die beide Klassen repräsentieren. In einem nächsten Schritt wurden mit den beiden Testsubstanzen zwei Langzeitwirkungsstudien mit aquatischen Wirbeltieren durchgeführt, für das Gestagen zusätzlich eine Studie mit einem aquatischen Wirbellosen.

Die Ergebnisse der Laborversuche wurden gesammelt und mit den Ergebnissen aus der Literatur verglichen und diskutiert. Daraus wurde anhand der beiden Beispielwirkstoffe eine Bewertungsstrategie entwickelt, mit dem Ziel, eine maßgeschneiderte Risikobewertung zu erstellen.

Auf der Grundlage der Ergebnisse der Literaturrecherche, aber auch unter Berücksichtigung praktischer Aspekte, wurde beschlossen, die ökotoxikologische Studie mit Dienogest als Vertreter der Gestagene und Dexamethason als Vertreter der Glukokortikoide durchzuführen. Beide Substanzen erwiesen sich im wässrigen Medium als stabil und wurden daher als geeignet für eine Langzeitstudie unter Durchflussbedingungen angesehen. Für beide Substanzen wurde ein Zebrafisch Extended One Generation Reproduction Test (ZEOGRT) durchgeführt. Für Dienogest wurde zusätzlich ein Chironomiden-Lebenszyklustest gemäß der OECD-Richtlinie 233, für Dexamethason wurde nur die Fischstudie durchgeführt. Beide Studienprotokolle zielen darauf ab, potenzielle endokrine Wirkungen in Bezug auf populationsrelevante Endpunkte zu ermitteln.

Das ZEOGRT-Protokoll deckt einen Mehrgenerationenansatz ab, der parentale adulte Laicher, eine vollständige Filialgeneration 1 (F_1), beginnend mit Embryonen und einschließlich Jungtierwachstum sowie Reproduktion adulter Tiere, und darüber hinaus eine Filialgeneration 2 (F_2) inklusive Embryonalentwicklung und Schlupf umfasst. Das Verfahren ist in einem Protokollentwurf beschrieben, der derzeit von der OECD validiert wird.

Um mögliche endokrine Wirkungen der Substanz auf wirbellose Tiere zu erfassen, wurde ein Chironomiden-Lebenszyklustest durchgeführt. Dieser Aufbau umfasst zwei Mücken-generationen, beginnend mit Larven im ersten Altersstadium der ersten Generation (F_1),

Emergenz von F_1-Adulten, Transfer von F_1-Eiersträngen zur Vorbereitung einer zweiten Generation (F_2) und Beibehaltung dieser bis zur Emergenz der erwachsenen F_2-Tiere. Diese Studie wird in einem Wasser-Sediment-System durchgeführt.

Im Rahmen des Projekts wurden zusätzlich analytische Messungen von wässrigen Proben aus zwei *Daphnia magna* Reproduktionstests mit denselben Testsubstanzen durchgeführt und berichtet.

Studien mit Dienogest

Zebrafish, Extended one generation reproduction test

Die eingesetzten nominalen Testkonzentrationen lagen bei 3,2, 10, 32, 100 und 320 ng Dienogest/L. Ausgehend von einer regelmäßigen Messung der Prüfsubstanzkonzentrationen einmal pro Woche wurden die mittleren gemessenen Konzentrationen mit 3,51, 10,3, 31,7, 105 und 335 ng Dienogest/L berechnet. Die Messung der physikalischen Wasserparameter, d.h. Wassertemperatur, Sauerstoffgehalt und pH-Wert, bestätigte stabile Testbedingungen während der gesamten Studie. Während eines begrenzten Zeitraums von acht Tagen in der Anfangsphase der Studie war die Wassertemperatur jedoch höher als der empfohlene Grenzwert von 27,5 °C, überstieg aber nicht 28 °C. Die Auswirkungen dieser höheren Temperatur wurden daher als geringfügig eingestuft. Es wurden keine Auswirkungen auf den Schlupf und Überleben der Larven beobachtet.

Für die **Elterngeneration (F_0)** wurde kein statistisch signifikanter Effekt von Dienogest auf einen populationsrelevanten Endpunkt festgestellt. Das Wachstum der Fische in Bezug auf Länge und Gewicht wurde nicht beeinflusst. Die Untersuchung der Fischgonaden auf phänotypische Geschlechtsmerkmale bestätigte die korrekte Zusammenstellung der Laichgruppen, d.h. 5 Männchen und 5 Weibchen, für die meisten der verwendeten Fischgruppen. Darüber hinaus veränderte Dienogest die Vitellogeninkonzentration im Blutplasma sowohl der Männchen als auch der Weibchen nicht. Die histopathologische Analyse der Reifungsstadien und weiterer Läsionen ergab keinen Effekt im Zusammenhang mit der Testsubstanz-Exposition.

Im Gegensatz dazu wurde die Reproduktion in Bezug auf die Fruchtbarkeit und das Überleben der frühen Larven in der **ersten Filialgeneration (F_1)** durch Dienogest beeinträchtigt. Genauer gesagt war die Fruchtbarkeit im Vergleich zur Kontrolle bei der höchsten Behandlungsstufe von 335 ng Dienogest/L signifikant reduziert, die sich daraus ergebende NOEC für diesen Endpunkt betrug somit 105 ng Dienogest/L. Eine signifikante Verringerung der Überlebensrate nach dem Schlüpfen am 21. und 35. Tag wurde bei Konzentrationen von ≥105 ng Dienogest/L beobachtet, der entsprechende NOEC-Wert für diesen Endpunkt betrug somit 31,7 ng Dienogest/L. Hervorzuheben ist, dass die Mortalität der Larven hauptsächlich vor dem Tag 21 pf auftrat, während der Phase des Übergangs von der Dottersackzehrung zur externen Futteraufnahme. Danach, d. h. während der Phase des juvenilen Wachstums und der adulten Phase, wurde bis zum Versuchsende keine weitere Mortalität beobachtet.

Der Schlupferfolg der **zweiten Filialgeneration (F_2)** war im Vergleich zur Kontrolle in allen Behandlungsstufen mit Ausnahme der niedrigsten Konzentration von 3,51 ng Dienogest/L signifikant reduziert. Es kann davon ausgegangen werden, dass eine geringe Qualität der F_1-Eier der Grund für den verminderten Schlupferfolg der F_2-Generation war. Die beobachtete Verringerung der F_1-Fertilität unterstreicht diese Annahme. Eine verringerte VTG-Konzentration im Blutplasma der F_1-Weibchen wurde jedoch nicht gemessen, was darauf schließen lässt, dass die Dottermenge in den Eiern nicht beeinträchtigt wurde.

Basierend auf dem Endpunkt Schlupferfolg der zweiten Filialgeneration (F_2), dem empfindlichsten Endpunkt der gesamten Studie, wurde die Gesamt-NOEC des ZEOGRT auf

3,51 ng Dienogest/L und die LOEC auf 10,3 ng Dienogest/L festgelegt. Die beobachtete Auswirkung auf das Überleben der F_1 im frühen Lebensstadium unterstreicht die allgemeine Beobachtung, dass insbesondere die Embryonal- und Larvenstadien direkt durch diese Substanz beeinträchtigt wurden.

Lebenszyklustest mit Chironomiden

Es wurde ein Chironomiden-Lebenszyklustest durchgeführt, um die Auswirkungen einer Dienogest-Exposition auf zwei Generationen von *Chironomus riparius* unter statischen Bedingungen zu ermitteln. Die eingesetzten nominalen Testkonzentrationen lagen bei 6,25, 12,5, 25, 50 und 100 mg/kg Sedimentgewicht Dienogest. Die Prüfsubstanz wurde durch Aufdotieren des Sediments appliziert.

Die aufgezeichneten Wasserparameter bestätigten stabile Testbedingungen während der Studie. Die gemessenen Anfangskonzentrationen der Prüfsubstanz im Sediment lagen bei 6,33, 12,0, 20,1, 49,6 und 93,3 mg/kg Sediment (trocken) Dienogest für die F_1-Generation und 5,41, 11,9, 23,6, 46,7 und 94,7 mg/kg Sediment (trocken) Dienogest für die F_2-Generation. Die Wasserkonzentrationen von Dienogest waren zu Beginn des Tests und auch nach 4 Wochen niedrig, was auf eine stabile Bindung der Substanz an die Sedimentmatrix schließen lässt. Die relevante Substanzexposition fand also während der Larvenentwicklung der Mücken im Sediment statt.

Es wurden keine Auswirkungen auf die Entwicklungsstadien der exponierten Mücken beobachtet. Der Zeitpunkt des Auftretens der adulten F_1-Stadien war im Vergleich zur Kontrolle nicht signifikant verschoben. Die Auswertung des Geschlechterverhältnisses der geschlüpften Mücken ergab keinen substanzbezogenen Effekt. Die Anzahl der F_1-Eigelege und die berechnete Anzahl der Gelege pro Weibchen ergaben keinen substanzbedingten Effekt. Es wurde kein Einfluss auf den F_2-Schlupf und das Geschlechterverhältnis festgestellt.

Da in dieser Studie keine biologischen Wirkungen festgestellt wurden, wurde die Gesamt-NOEC auf ≥ 100 mg Dienogest/kg sed. dw (Nominalkonzentration) festgelegt.

Studien mit Dexamethason

Zebrafish, Extended one generation reproduction test

Die verwendeten nominalen Testkonzentrationen lagen bei 0,32, 1,0, 3,2, 10, 32 und 100 µg Dexamethason/L. Ausgehend von einer regelmäßigen Messung der Substanzkonzentrationen einmal pro Woche wurden die mittleren gemessenen Konzentrationen mit 0,33, 0,91, 3,2, 10,5, 34,7 und 100 µg Dexamethason/L berechnet. Die Messung der physikalischen Wasserparameter, d.h. Wassertemperatur, Sauerstoffkonzentration und pH-Wert, bestätigte stabile Testbedingungen während der gesamten Studie.

In der **Elterngeneration (F_0)** wurden keine Auswirkungen auf die Reproduktion in Bezug auf Eizahl und Befruchtungsrate festgestellt. Die Bestimmung des phänotypischen Geschlechts bestätigte die korrekte Zusammenstellung von Laichgruppen für die meisten Testgefäße, d. h. 5 Männchen und 5 Weibchen. Auswirkungen im Zusammenhang mit der Prüfsubstanz-Exposition wurden hauptsächlich bei den Wachstumsparametern festgestellt. Das Nassgewicht der Männchen war im Vergleich zur Kontrolle ab einer Konzentration von 34,7 µg Dexamethason/L signifikant reduziert. Daher wurde die NOEC auf 10,5 µg Dexamethason/L festgelegt. Das Nassgewicht der Weibchen sowie die Gesamtlänge wurden in Gegenwart von Dexamethason ebenfalls reduziert, allerdings nur bei der höchsten Behandlungsstufe von 100 µg Dexamethason/L. Dementsprechend wurde die NOEC auf 34,7 µg Dexamethason/L festgelegt. Die Vitellogeninkonzentration im männlichen und weiblichen Blutplasma wurden durch die

Prüfsubstanz nicht beeinflusst. Die histopathologische Untersuchung der Gonaden der exponierten Tiere ergab keinen Befund eines Substanzeffekts.

Auch in der **ersten Filialgeneration (F_1)** wurde ein verzögertes Wachstum der exponierten Fische beobachtet. Insbesondere die Verringerung der Gesamtlänge war während der Entwicklung von der Larve zum erwachsenen männlichen Fisch offensichtlich. Genauer gesagt war die Länge am Tag 35 pf im Vergleich zur Kontrolle nur bei der höchsten Behandlungsstufe signifikant reduziert, während die Messung am Tag 63 pf sowie die Gesamtlänge der Männchen am Ende des Tests eine signifikante Reduktion ab 34,7 µg Dexamethason/L ergab. Das Nassgewicht der Männchen und Weibchen am Testende war bei ≥34,7 µg Dexamethason/L signifikant reduziert.

Es gab keine Auswirkungen auf die Reproduktionsfähigkeit der F_1. Obwohl die Wachstumsleistung beeinträchtigt war, wurde keine Verzögerung in Bezug auf den Zeitpunkt des ersten Ablaichens beobachtet. Die Reproduktionsparameter Fruchtbarkeit und Fertilität wurden durch die Substanzexposition nicht negativ beeinflusst. Andere apikale Endpunkte wie Überleben oder Geschlechterverhältnis sowie die Vitellogenin-Konzentration im Blutplasma und das histopathologische Muster der untersuchten Gonaden wurden durch den Prüfgegenstand nicht beeinflusst.

Es wurde festgestellt, dass der Schlupferfolg der **zweiten Filialgeneration (F_2)** bei 10,5 und 34,7 µg Dexamethason/L signifikant reduziert war. Die Schlupfrate bei der höchsten Konzentration unterschied sich jedoch nicht signifikant von der Kontrolle. Daher sollte die angegebene NOEC als Worst-Case-Wert betrachtet werden.

Auf der Grundlage der beobachteten empfindlichen Endpunkte, d.h. reduziertes Gewicht und Längenwachstum, wurde die NOEC auf 10,5 µg Dexamethason/L festgelegt. Die LOEC wurde auf 34,7 µg Dexamethason/L festgelegt.

Zusammenfassung und Schlussfolgerung

Studien mit Gestagenen und Glukokortikoiden

Für synthetische Gestagene werden in der wissenschaftlichen Literatur mehrere Beispiele für Wirkungsmuster bei Fischen beschrieben, die denen anderer potenter endokrin wirkender Substanzen, z. B. Östrogene und Androgene, ähneln. Von denen in diesem Projekt untersuchten Substanzen wird Dienogest in der Literatur als anti-androgen wirksam beschrieben. Da die Datenbasis für die Auswirkungen von Dienogest auf Fische (und andere aquatische Organismen) noch sehr begrenzt ist, können die hier berichteten Ergebnisse dazu beitragen, bestehende Datenlücken zu schließen. Die vorhandenen Literaturdaten bezogen sich zumeist auf gut beschriebene natürliche bzw. synthetische Moleküle, z. B. Progesteron oder Levonorgestrel. Die meisten Artikel beschrieben Auswirkungen auf die Eizahl oder Beobachtungen im Zusammenhang mit der Maskulinisierung oder der Veränderung des Geschlechterverhältnisses zugunsten der Männchen.

Die aus dem ZEOGRT abgeleiteten relevanten Wirkungen im Zusammenhang mit einer Dienogest-Exposition wurden durch eine verringerte Überlebensrate der frühen Lebensstadien (F_1-Generation) und einen verringerten Schlupferfolg der F_2-Generation charakterisiert, wobei der F_2-Schlupf deutlich empfindlicher war. Auch der Befruchtungserfolg der erwachsenen Laichgruppen (F_1) war signifikant reduziert, während die Eizahlen nicht beeinflusst wurde. Es kann davon ausgegangen werden, dass der beobachtete Effekt das Ergebnis einer beeinträchtigten Leistung der Männchen war, was zu einer ineffizienten Befruchtungsfähigkeit führte. Die histopathologische Auswertung ergab jedoch keine Hinweise auf eine Schädigung des

männlichen Keimdrüsengewebes. Da auch die Vitellogenin-Messungen keinen Effekt ergaben, konnten keine Informationen über den zugrunde liegenden Mechanismus abgeleitet werden.

Der durchgeführte Chironomiden-Lebenszyklus zeigte in dem angewandten Konzentrationsbereich keine Effekte. In einem Reproduktionstest mit der Prosobranchier-Schnecke *Potamopyrgus antipodarum* wurde keine Auswirkung auf die Reproduktion in Bezug auf die Anzahl der Embryonen im angewandten Konzentrationsbereich festgestellt. Auch wurde keine Auswirkung auf Wachstum und Überleben detektiert. Letztendlich zeigten nur die exponierten Zebrabärblinge eine ausreichende Empfindlichkeit, um Informationen über eine mögliche endokrine Wirkung der Substanz abzuleiten.

Es gibt verschiedene Studien, die mögliche Auswirkungen einer Glucocorticoid-Exposition auf Wasserorganismen, einschließlich Fischen, beschreiben. In diesem Zusammenhang berichten viele Studien über Auswirkungen auf das Immunsystem.

Zu den beschriebenen Effekten auf die apikalen Endpunkte gehörten eine verringerte Überlebensrate, eine geringere Wachstumsleistung sowie ein geringerer Laicherfolg. Physiologische Auswirkungen wurden unter anderem durch verringerte Vitellogenin- und reduzierte Östradiol-Konzentrationen charakterisiert.

In der ZEOGRT-Studie mit Dexamethason, die im Rahmen dieses Projekts durchgeführt wurde, wurden Auswirkungen auf das Wachstum für alle angewandten Lebensstadien beobachtet, d. h. für die elterliche F_0-Generation, aber auch für die frühen, juvenilen und adulten Stadien der F_1-Generation. Der Schlupferfolg der F_2-Generation wurde beeinträchtigt, zeigte aber keine eindeutige Konzentrationsabhängigkeit. Interessanterweise wurde bereits bei den Elternfischen (F_0) eine Abnahme von Länge und Gewicht beobachtet.

Ein Reproduktionstest mit *Potamopyrgus antipodarum* wurde im Konzentrationsbereich von 1,0, 3,2, 10, 32 und 100 µg Dexamethason/L durchgeführt und zeigte keine Wirkung. Weitere Invertebraten-Studien aus der Literatur zeigten eine höhere Empfindlichkeit von z.B. Crustaceen im Vergleich zu Algen und berichteten zudem über einen Rückgang von Daphnien-Populationen in einem Mehrgenerationentest.

Neue methodische Ansätze wie die sogenannte Lipopolysaccharid (LPS)-*Challenge*, die mit Zebrafischembryonen durchgeführt wurde, wurden als geeignete Instrumente zum Nachweis des immunsuppressiven Potenzials von Glukokortikoiden mit Auswirkungen auf das Überleben insbesondere der frühen Lebensstadien von Fischen berichtet. Es sind jedoch weitere Forschungsarbeiten erforderlich, um die Erklärungslücken zwischen den immunsuppressiven Mechanismen und den daraus resultierenden Auswirkungen auf der apikalen Ebene ausreichend zu schließen.

Prüfstrategie

Aus den Ergebnissen der durchgeführten Fischstudien kann abgeleitet werden, dass der ZEOGRT ein geeignetes Testdesign bietet, um die relevanten apikalen Endpunkte nachzuweisen. Für die verwendeten Testsubstanzen zeigte der Test eine ausreichende Sensitivität. Darüber hinaus wurden die definierten Validitätskriterien für die beiden durchgeführten Studien erfüllt, was die Robustheit des angewandten Protokolls bestätigt.

Die sexuelle Entwicklung und die Reproduktionsfähigkeit stellen die wichtigsten apikalen Endpunkte im Hinblick auf die Identifizierung potenziell endokrin wirksamer Substanzen dar. In beiden Studien wurde eine kontinuierliche Laichleistung der Fischgruppen beobachtet, mit geringer Variabilität in den Datensätzen, was eine aussagekräftige statistische Auswertung ermöglichte. Im Vergleich zwischen der F_0- und der F_1-Generation wurde ein Rückgang der

Eizahlen festgestellt, der jedoch mit dem Alter und damit einem unterschiedlichen Reifungsstadium der exponierten Tiere in Verbindung gebracht werden kann.

Der Parameter Geschlechterverhältnis ist ein kritischer Parameter für Zebrabärblinge, der durch eine hohe Variabilität zwischen den Beckenreplikaten beeinträchtigt werden kann. Bei beiden durchgeführten ZEOGRT-Studien entsprachen die Kontrollmittelwerte den Akzeptanzkriterien für das Geschlechterverhältnis bei Zebrabärblingen, d. h. 30-70 % des jeweiligen Geschlechts (Männchen oder Weibchen). Die Daten zeigten jedoch jeweils eine Tendenz zu einem der beiden Fischgeschlechter. Die genetische Variabilität der Zebrabärblingskulturen wird als ein Grund für die Variabilität diskutiert. Um diesem Problem zu begegnen, wurde der ZEOGRT so angepasst, dass befruchtete Eier, die zur Vorbereitung der F_1-Generation gesammelt wurden, an zwei aufeinanderfolgenden Tagen entnommen und gepoolt werden.

Das Eidotter-Vorläuferprotein Vitellogenin (VTG) ist ein etablierter Biomarker in den Testrichtlinien zur Detektion endokriner Wirkungen auf Fische. Für bestimmte Wirkweisen, z. B. Störungen der Schilddrüsenfunktion oder allgemeine Auswirkungen auf die Steroidogenese, ist Vitellogenin jedoch nicht immer der empfindliche physiologische Parameter. Darüber hinaus ist die Unterscheidung zwischen Wirkungen, die mit systemischer Toxizität zusammenhängen, und potenziellen endokrin vermittelten Wirkungen nicht allein anhand der VTG-Ergebnisse möglich. Darüber hinaus kann die Auswertung von VTG-Messungen durch eine hohe Streuung der einzelnen Messergebnisse, auch in Kontrollgruppen, erschwert werden. Um eine Verzerrung der Datensätze durch einzelne Ausreißer zu vermeiden, wurde für die hier vorgestellten ZEOGRT-Studien beschlossen, geometrische Mittelwerte der VTG-Konzentrationen pro Replikatbecken zu berechnen.

Die Identifizierung der zugrundeliegenden Wirkmechanismen (*Mode of action*, MoA) ist ein zentraler Faktor für die Entwicklung von maßgeschneiderten Risikobewertungsstrategien für alle Arten von Chemikalien, einschließlich Pharmazeutika. Die im Rahmen des Projekts durchgeführten Fischstudien haben sich als geeignet erwiesen, empfindliche apikale Endpunkte zu identifizieren. Durch den Vergleich mit Tests an anderen Wasserorganismen wurde deutlich, dass Fische die empfindlichste Organismengruppe darstellen, was auf den sehr direkten Expositionsweg über die Atmungsorgane zurückzuführen ist. Im Hinblick auf die EU-Definition von endokrin aktiven Substanzen sollten physiologische Parameter (z.B. Vitellogenin) und auch histopathologische Parameter einbezogen werden, um die Identifizierung der zugrunde liegenden Mechanismen zu unterstützen und eine plausible Verbindung zwischen Ursache und Wirkung herzustellen. Wie in diesem Projektbericht dargestellt, waren beide Untersuchungsparameter jedoch nicht ausreichend empfindlich für die verwendeten Testsubstanzen. Dennoch gibt es vielversprechende methodische Ansätze, die die Identifizierung der zugrundeliegenden Wirkmechanismen verbessern können.

Fischtestprotokolle wie der ZEOGRT erlauben die Gewinnung von geeignetem Gewebematerial, ohne die Zahl der verwendeten Tiere zu erhöhen. Da das Konzept des Adverse Outcome Pathway (AOP) zu einem wichtigen Instrument für die Bewertung endokrin wirkender Substanzen geworden ist, liefern die verfügbaren *-omic* Methoden wertvolle Informationen über das molekulare auslösende Ereignis (*Molecular Initiating Event*, MIE) und die nachgelagerten Schlüsselereignisse (*Key Event*, KE). Die gesammelten Gewebe erlauben es, diese Daten für verschiedene Lebensstadien der exponierten Fische zu generieren.

Bei den Gestagenen z.B. kann eine Genexpressionsanalyse helfen, antiöstrogene oder Glukokortikoid-ähnliche MoA (verwandt mit Progesteron-Analoga) oder antiöstrogene oder androgene MoA (mehr verwandt mit den Nortestosteron-Analoga) zu unterscheiden. Neben den beschriebenen Methoden zum Nachweis immunsuppressiver Mechanismen, d.h. LPS- Challenge

o.a., können verfügbare -*omic* Methoden verwendet werden, um die direkten Auswirkungen der Substanzexposition von sekundären indirekten Effekten aufgrund von Immunsuppression zu unterscheiden.

Weitere Forschungsarbeit ist notwendig um die Anwendung der -*omic* Methoden bezüglich der relevanten Wirkmechanismen zu fokussieren und damit die Akzeptanz im regulatorischen Kontext zu verbessern.

1 Introduction

The entry of pharmaceuticals into the aquatic environment became a central topic in environmental research. The environmental relevance arises from the fact that the amount of pharmaceutical residues via municipal waste water and hospital waste water in the aquatic environment is steadily increasing due to increased demand. On the other hand, due to advances in medical research, it is it possible to design and produce active ingredients with very specific targets. These drugs are often based on natural molecules in their structure. Modifications of the molecular structure often make these active substances more stable, specific and potent in their mode of action. There are numerous examples, in particular of hormone-active substances like Ethinylestradiol or Levonorgestrel, which can trigger adverse effects in aquatic environmental organisms even in a very low water concentration (Länge, Hutchinson et al. 2001, Teigeler, Schaudien et al. 2021).

Following the Regulation of the European Medical Agency, EMA (EMEA and CHMP 2006), the environmental risk assessment of human pharmaceuticals considers a two-tiered approach. Within the first step, relevant and potentially problematic substances are first filtered out.

On the basis of model calculations, a predicted environmental concentration is determined. If this exceeds a certain trigger value, further studies (level 2) are necessary, in which, among other aspects, the chronic toxicity to aquatic organisms is to be investigated. For fish, this is usually a fish early life stage toxicity test, which examines the early life phase, i.e. embryo and larval stage, of the animals.

Level 2 of testing also includes active ingredients with a high bioaccumulation potential. In addition, substances known to be effective in very low concentrations are also treated separately. The latter group includes the hormone-active substances, for which further environmental tests are necessary in addition to the standard tests. For hormone-active substances, these are e.g. life cycle tests with fish, which, in addition to sexual development, also investigate the reproductive capacity of fish.

Although a life cycle test is considered to represent an appropriate study to assess the effects of hormonal substance on fish, the apical endpoints recorded usually do not provide a single clear picture of the cause of an effect on the animals. The identification of underlying mechanisms of action often is not the main intention of the life cycle tests. The examination of the biomarker Vitellogenin and the histopathological examination of fish gonads, which are provided by default, can only deliver very basal interpretation approaches.

The lack of in-depth studies on the mechanistic cause of effects makes it difficult for the authority to assess the risk of the substances.

Hormone-like and hormonally active substances are used in a wide variety of areas. The focus of this project is on newly developed, mostly synthetically produced hormone-active substances, such as glucocorticoids and progestins. Glucocorticoids are highly anti-inflammatory and immunosuppressive agents, thus widely used in the treatment of inflammatory, allergic or immunological disorders. This includes a variety of indications like asthma, chronic obstructive pulmonary disease (COPD), allergic rhinitis, eczema and autoimmune diseases like rheumatoid arthritis or multiple sclerosis. Furthermore, they play an important role in preventing rejection after organ transplantation. However, glucocorticoid are also known to cause several dose- and time-dependant side effects like a delayed wound healing, Cushing`s syndrome as well as an increased risk for diabetes, osteoporosis, ulcerations and infections in general.

Progestins are mainly used in hormonal contraception, either alone or in combination with estrogens. Another area of application are hormone replacement therapies during menopause, but also the treatment of e.g. endometriosis or in vitro fertilization.

The aim of the project was to develop an adapted strategy for assessing the environmental risk of novel hormonal active substances to aquatic organisms. During a detailed literature research, the current findings on the effects of synthetic progestins and glucocorticoids were collected and evaluated. Based on the results, two candidate substances were identified, representing both substance classes. In a next step, two long-term effect studies with aquatic vertebrates with the two identified test substances were carried out, for the progestin a study with an aquatic invertebrate was also conducted.

The results of the laboratory tests were collected and compared with the results from the literature and discussed. From this, an evaluation strategy was developed on the basis of the two example active ingredients, with the aim to develop a tailored risk assessment.

2 Materials and methods

2.1 Literature research

The literature research aimed to gather the existing findings regarding the effects of glucocorticoids and synthetic progestins. This included new endpoints that have not yet been considered in the test guidelines, such as specific biomarkers, but also optimized test methods. A critical evaluation of the literature was carried out and finally an evaluation was carried out to identify the parameters that can be used for an adapted risk assessment. In addition, suitable test substances were identified, which are then tested in the second work package in aquatic laboratory tests. In addition to the effects on fish, other relevant aquatic environmental organisms such as amphibians and invertebrates were included in the literature research. Articles from specialist journals that were subject to a peer-review process were primarily used to build up a collection of literature data. The literature research was carried out using different scientific databases.

The selection of the search engines used includes the following databases, but was not limited to these:

PubMed

http://www.ncbi.nlm.nih.gov/pubmed

Scopus

https://www.elsevier.com/solutions/scopus

Science Direct

http://www.sciencedirect.com/

Web of Science:
http://apps.webofknowledge.com/UA_GeneralSearch_input.do?product=UA&search_mode=GeneralSearch&SID=S2lRkUdwKdY8zSZuMxd&preferencesSaved=

BioMedCentral

http://www.biomedcentral.com/

Google Scholar

https://scholar.google.de/

In order to ensure the quality of the selected literature, it should meet the following main criteria:

- Comprehensible results with a comprehensive presentation of the methodology used and critical discussion on one or more of the following points:
  - Modes of action of glucocorticoids and their effects on the aquatic environment
  - Modes of action of synthetic progestins and their effects on the aquatic environment
  - new assessment strategies and biomarkers to characterise the underlying modes of action
  - information from preclinical studies, where available

- Attempt to classify progestins according to their parent compound (basic chemical structure) and the resulting effect
- Environmental relevance of the identified substances with regard to measured concentrations in environmental media

- Review-literature should display the current articles should reflect the current state of science on the respective topic.

The search strategy included regulatory, ecotoxicological and molecular biological aspects. Restrictions in the search arose initially due to the topicality. Review articles were intended to reflect the current state of the science of the respective topic. The search period was suggested to be the last 10 years, with a focus on the last 5 years. In individual cases, older literature was used, e.g. for the definition of established terms.

The potential efficacy of these substance groups is worked out, among other things, on the basis of example substances for amphibians and fish and furthermore invertebrates.

As initial search criteria the following terms were used:

- progestins/progesterone/gestagen
- glucocorticoid
- sexual endocrine
- anti-/estrogenic (estrogen*)
- anti-/androgenic (androgen*)
- aromatase
- endocrine dysfunction
- immuno suppression

and combinations of these terms. The listed terms were combined with the different taxa, e.g. progestins AND fish, or progestins AND human. The final list of search terms and the searching strategy was agreed in a kick-off meeting with the contract partner UBA.

The relevance of a publication was first checked on the basis of its title, the possibly published highlights and then on the basis of the abstract. When a publication was added to the bibliography, a second step was to search for publications by the same first author or author. Usually, the latter author represents the head of the working group or the question, so that even if the first author changed and the topic was continued, further work could be found in the working group. Other references cited in a publication were also included in the literature collection if they contain additional relevant information.

If a specific article was examined more closely online, the search engines often displayed other relevant articles parallel to the hits. These ads were also checked. For some journals, the keywords assigned to the articles by the authors were indicated. These were checked and integrated into the search as further relevant keywords. The literature research was performed during October 2017 to March 2018.

In the second part of this work package 1, the references were evaluated. For evaluation, the articles were processed in a tabular form. In addition to the scientific statement and relevance, the usability of the results with regard to the identified representatives of the mentioned

substance classes was also considered. The advantage of the tabular evaluation was its clarity. All articles used were managed via the literature management program EndNote. The data collection was handed over to the project partner UBA. A list of relevant literature identified during the literature research are presented in Appendix C of this report.

2.2 Laboratory tests

This work package included the performance of studies with aquatic vertebrates and invertebrates. For both candidate substances, a zebrafish extended one generation reproduction test (ZEOGRT) was conducted. For the progestin identified, a Chironomid life cycle test with *Chironomus riparius*, following the OECD guideline 233 (OECD 2010, TG 233) was conducted. Both tests are introduced in the following chapter.

2.2.1 Zebrafish extended one generation reproduction test (ZEOGRT)

The basic test design of the ZEOGRT considers a broad range of different life stages which are covered by existing fish test guidelines, i.e. the early life stages (OECD 2013, TG 210), the sexual development (OECD 2011, TG 234) and reproduction (OECD 2012, TG 229). However, the protocol also considers existing procedures to cover a fish full life cycle, e.g. the Detailed Review Paper on Fish Life-Cycle Tests (OECD 2008) or the zebrafish life cycle test procedure as published by Nagel et al. (Nagel 1998). There is currently an initiative of Germany at OECD underway to validate the ZEOGRT protocol as a new test guideline as a part of the OECD framework to identify endocrine acting compounds.

The ZEOGRT protocol was developed following the adopted Medaka procedure. The objective of this method is assessing the effects of continuous exposure to the test item on different life stages and life performances of zebrafish (*Danio rerio*) during a life cycle. The primary focus is the assessment of the impacts on population relevant endpoints.

This list of relevant life stages includes the spawning period of the parental generation (F_0 generation), early life stages, juvenile growth, sexual maturation, and reproduction of the first first filial generation (F_1 generation), and the early embryonal stage of the second filial generation (F_2 generation). Endpoints determined are hatching success, mortalities during different life stages, and juvenile growth (F_1 generation, only). The sex ratio of the exposed fish groups is determined either by macroscopic inspection of gonads and confirmed by a histological analysis (F_1 generation) of the fish.

Spawning performance (fecundity) and fertilization rate are recorded for adult fish of F_0- and F_1-generation and the time to first spawning for the F_1-generation.

To obtain mechanistic data for further interpretation of the observed effects the study includes the measurement of biomarkers and a histopathological examination.

Blood plasma samples of the adult fish of F_0- and F_1 generation are taken and measured for Vitellogenin (VTG) concentrations. Furthermore, a histopathological examination of the fish gonads of the adult fish of F_0- and F_1- generation was performed.

Table 1 gives an overview on the basic test design of the ZEOGRT.

Table 1: Zebrafish extended one generation reproduction test (ZEOGRT), basic test design

Time of exposure	Fish age	Phase	Course	Endpoint*
0 d	approx. 15 weeks	Reproduction F_0 generation	Start with spawning groups 5 male/5 female fish	Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility) (Cumulative egg no.)
21 d	0 d	Early life stage	Start with 36 fertilised eggs per vessel (2x18 eggs in stainless steel fry cages)	Time to hatch Hatching success
24 d	3 d		Begin of hatch (hatch completion between 4 to 6 dpf)	
27 d	6 d	F_1 generation	Feeding with breeding food ad libitum	
5 w	14 d		Feeding with *Artemia salina* (Lifefood)	
6 w	21 d	F_0 generation	Termination	Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) Vitellogenin content in males and females
6 w	21 d	Early life stage	Photographic determination of survival; Transfer to main aquaria	Post-hatch survival
8 w	35 d	F_1 generation	Photographic determination of length and survival Random reduction to 20 individuals	Post-hatch survival Length
12 w	63 d	Juvenile growth F_1 generation	Photographic determination of length and survival	Survival Length
13 - 18 w	70 - 105 d	Reproduction	Introduction of spawning trays	Time to first spawning
15 - 20 w	84 d - 119 d	F_1 generation	Daily evaluation of egg numbers and fertilization rates	Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility)
20 w	0d	F_2 generation	Start with 20 fertilised eggs per vessel (2x10 eggs in fry cages)	Time to hatch Hatching success
	96 h		hatch	

Time of exposure	Fish age	Phase	Course	Endpoint*
20 – 22 w	119 – 133 d	Test termination F_1 generation	End of F_1 generation	Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) Vitellogenin content in males and females

2.2.1.1 Fish strain

The presented studies were conducted with the zebrafish strain established at Fraunhofer IME, commonly identified as the "West aquarium strain".

A barcoding analysis of cultured fish was performed to analyse the genetic characteristics of the strain used and the genetic similarity to other zebrafish strains. The analysis revealed a 100% similarity with other common zebrafish lines like AB or HL strain. The analysed DNA barcode regions included parts of the mitochondrial genome (published sequences under the gene assessing numbers KT_624623 and KT_624627).

The test fish which serve as parental generation (F_0) in this study (Dienogest: 8 to 9 months of age; Dexamethasone: max. age of 12 month) have been reared from fertilised eggs at the test facility and were maintained in glass aquaria (150-L max) under conditions which ensure a good reproductive status.

2.2.1.2 Number of animals used

The parental generation (F_0) was started with spawning groups of 10 fish (5 males, 5 females). To obtain the F_1 generation, 36 embryos from the parental group were kept for hatching. To prepare spawning groups of equal numbers, the F_1 groups were reduced to 20 juvenile fish per test vessel at day 35 pf. The surplus fish were anaesthetized and measured for the growth parameters total length and wet weight. The F_2 generation was prepared of 20 fertilised eggs from the F_1 spawning groups.

The presented fish numbers referred to one test vessel.

Figure 1 illustrates the outline of the protocol referred to the animal numbers per treatment concentration.

If a setup with 5 test concentrations and a dilution water control is considered, the total fish numbers used were finally:

- 240 fish for parental generation (F_0)
- 864 embryos for first filial generation (F_1) generation.

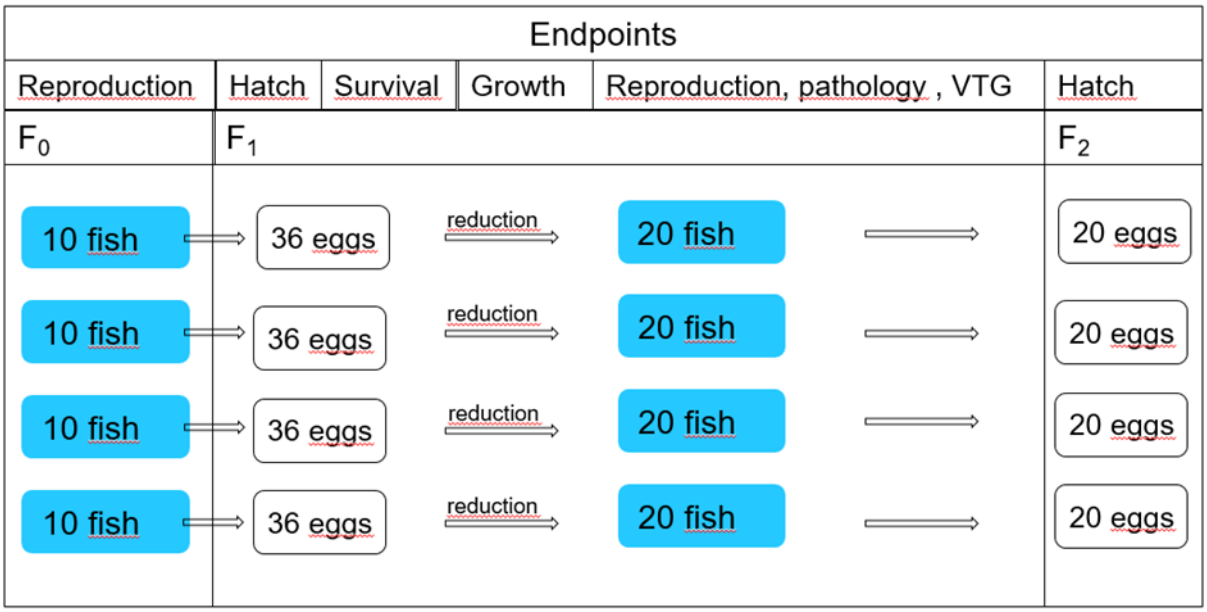
The total fish number is thus 1104 animals. The study with the glucocorticoid was conducted with 6 test concentrations. Thus, the total fish number was finally 1288.

As the embryos for second filial generation (F_2) generation were terminated before they enter a stage of free-feeding, they were not considered as protective life stage according to the European Animal welfare legislation (EEC 2010) and thus not part of the calculation.

2.2.1.3 Animal welfare application

Both studies have been registered and approved with the Federal Animal Welfare Authority of North Rhine Westphalia, under the reference number 81-02-04.2018.A031.

Figure 1: ZEOGRT, fish numbers applied per treatment and in control



Source: Own illustration, Fraunhofer IME

The basic setup of ZEOGRT considered full and true replication throughout the study. There was no pooling of eggs, larvae, juveniles and spawning adults between the test vessels at any time during the study.

2.2.1.4 Test substances

As result of the literature search (see section 3.1 for details), the following substances were used in the studies and applied in the concentrations as shown in Table 2.

Table 2: Test substance and test concentrations

Test substance	CAS number	Purity	Supplier	Nominal test concentrations	
Dienogest	65928-58-7	99.9%	Selleckchem, USA	[ng/L]	3.2, 10, 32, 100, 320
Dexamethasone	50-02-2	98%	ABCR GmbH, Germany	[µg/L]	0.32, 1.0, 3.2, 10, 32, 100

2.2.1.5 Preparation of test solutions

All experiments were conducted under flow through conditions. Pre-dissolved test substance was continuously pumped into the test vessels to ensure stable test concentrations throughout the test period. For application of the substances, appropriate stock solutions were prepared and diluted and stored in glass tanks. The final dilution was achieved by a dosing pump system, mixing dilution water and application solution to the target concentration. The amounts of stock and application solutions were calculated to ensure an exchange of 5 test vessel volumes per day. All substances were stable under the aqueous conditions, thus, the intended basic

application regime, e.g. including a 5-fold exchange per day and test vessel, was suitable for all studies conducted.

2.2.1.6 Chemical analysis of test substance concentrations

The concentrations of the applied substances were measured in regular intervals throughout every study. Before starting the in-life phases, the methods were validated following the basic requirements of the guideline SANCO/3029/99 rev. 4 (EC 2000). This included the measurement of fortification samples at the LOQ determined for each substance and the 10-fold LOQ. A further fortification step was set to encompass the calibration range of each method. Table 3 presents the used analytical methods for each substance and the corresponding LOQ values.

All analyses were conducted at the analytical laboratories of Fraunhofer IME, Schmallenberg, Germany.

Table 3: Chemical analysis of the test substances: Analytical method and LOQ applied.

Test substance	Analytical method	Limit of quantification (LOQ)		Mean RSD [%] of validation (fortification experiment)	Lowest test concentration applied	
Dienogest	LC-MS/MS	[ng/L]	1.5	5.76	[ng/L]	3.2
Dexamethasone	LC-MS/MS	[µg/L]	0.10	4.88	[µg/L]	0.32

2.2.1.7 Test acceptability criteria

The following criteria were defined to assess the test performance and data quality.

Test conditions

- Dissolved oxygen: ≥ 60%
- Mean water temperature: 26 ± 1.5 °C

Biological parameters

- Parental generation (F_0)
  - Successful reproduction: at least 10 eggs per female and day, 80% fertilisation
  - Survival of adults: ≥ 90%
- First filial generation (F_1)
  - Hatching success: ≥ 80%
  - Post hatch survival: ≥ 75%
  - Survival of juvenils and adults: ≥ 90%
  - Sex ratio: between 30% to 70% (males or females)

Chemical analysis: An analytical measurement of the test concentrations is compulsory.

2.2.1.8 Test performance

2.2.1.8.1 Test vessel

The test vessels were glass aquaria with a total volume of 28 L and approximately 25 L of test solution. Each replicate group was kept in an individual test vessel. Each test chamber was labelled with the study number, the test item and test concentrations. Each test vessel was equipped with fry cages for initiation of the filial generations, being glass cylinders with a diameter of 8 cm and a brim height of 10 cm. The bottom of each cage was a Teflon gaze with a mesh width of approximately 350 µm.

2.2.1.8.2 Test conditions

The light regime was 12 hours light / 12 hours dark. The water temperature was maintained by placing the vessels into a temperature-controlled water bath set to 26.0 °C ± 1.5 °C. The pH and oxygen concentration of the water were measured in each aquarium directly before adding the fish and thereafter at least twice a week. The water temperature was measured on workdays. The oxygen concentration of the test solution should not be lower than 60%. There was no additional aeration of the test vessels. Uneaten food and faeces were removed from the fry cages and the test aquaria at regular intervals.

2.2.1.8.3 Flow through conditions

For two replicates each, an individual dosage system was used, i.e., two dosage systems for each treatment concentration. The dilution water was delivered into a mixing chamber placed on a magnetic stirrer via a membrane pump (Prominent, Heidelberg, Germany). A corresponding amount of the application solution was applied to the mixing chamber using a membrane pump with stainless steel head (Prominent, Heidelberg, Germany).

At test start, the flow through system was served by test solutions at least 24 hours before introducing the fish. An appropriate amount of stock solution was prepared, which was renewed on a daily basis. The prepared test solution flowed into the test vessels via flexible Teflon tubes, distributed to the two vessels by an electronically regulated distributor driven by compressed air. Controls were served by dilution water only.

Control and all test concentrations were run in 4 replicate aquaria, each. For every test vessel, a water flow rate of approx. 5 L/h was adjusted (max. variability of flow rate of 10% throughout the test), resulting in a daily turnover of approx. 5 volumes/day. The pumping device as well as the consumption of the stock solution were checked daily.

2.2.1.8.4 Initiation of the study

At test start, 5 male and 5 female fish were allocated to each replicate. Prior to test start, the fish groups were held under test conditions for at least 14 days to record spawning success during the pre-treatment phase.

Females and males were taken from batches of the same age. If single fish from the main spawning groups had to be replaced, they were taken from the same batch. The spawning groups were composed by randomized distribution of males and females.

When fish in all test vessels achieved daily spawning of at least 10 eggs per female and fertilization rates equal to or above 80%, the exposure phase was started. One spawning group each was placed in each test vessel. The fish groups were randomly distributed to the test vessels. MS Excel was used for the randomised allocation of the spawning groups to the individual control and treatment vessels.

2.2.1.8.5 Duration of exposure

From the test start onwards, parental fish from F_0 generation were exposed during a spawning period of three weeks. In week 4, fertilised eggs were kept to prepare a F_1 generation. The F_1 fish were monitored over 5 weeks during their early life stage phase, followed by a juvenile growth phase for further 4 weeks. After reaching sexual maturity, start of reproduction and spawning success was observed and recorded over a period of approx. 5-8 weeks. Finally, an F_2 generation was prepared by keeping eggs of F_1 parental animals, and were exposed until hatch.

For details on the basic test design and time schedule, refer to Table 1.

2.2.1.8.6 Feeding regime

Adult animals of both F_0 and F_1 generation were fed once daily ad libitum with commercially available flake food and brine shrimp nauplii (*Artemia salina*).

The early life stages of the F_1 generation received a specific feeding regime. When hatch was finished, e.g. from day 5 dpf onwards, larvae were fed twice daily with ground larval diet (TetraMin Baby, Tetra Werke, Melle, Germany) and liquid rearing feed (Nobil fluid, JBL, Neuhofen, Germany). Approximately from day 14 pf onwards, brine shrimp nauplii (*Artemia salina*) was added ad libitum to the daily food. From approximately day 21 pf onwards, breeding food was exchanged by ground flake food (ad libitum).

2.2.1.9 Observations and biological endpoints

Observations on fish of all life stages were made daily. Dead eggs, larvae, fry; dead juvenile and adult fish were recorded and removed immediately. Any abnormal behaviour was recorded.

2.2.1.9.1 Parental generation (F_0)

After start of exposure, spawned eggs were collected daily from each test vessel for at least 20 days. Total egg numbers per replicate as well as the number of fertilised eggs were determined. Eggs were collected daily without interruption as this may cause variability in the data set. At the end of this phase, fertilised eggs were collected from each fish tank to prepare the F_1 generation. Eggs from two successive days were pooled per replicate and then systematically redistributed to the fry chamber placed in the test tank.

After successful start of the F_1 generation, based on fry survival recorded after three weeks post fertilization, the fish of the F_0 generation were terminated. All fish were anaesthetised using chloro-butanol. A blood sample was taken from each fish via heart puncture. After successful blood sampling the fish were killed humanly with a dorsal cut.

The phenotypic sex of each fish was determined by macroscopic inspection of fish gonads, i.e. ovary structure for females and testicular tubes for males. Afterwards, individual lengths and weights were measured. Finally, the fish were transferred to appropriate fixative to allow a histopathological analysis of fish tissue according to the OECD guidance document 123 (OECD 2010) The phenotypic sex of each fish was confirmed by histological evaluation of fish gonads.

2.2.1.9.2 First filial generation (F_1)

At start of the F_1 generation, 36 (2 x 18) fertilised eggs, collected on two consecutive days (i.e. 18 eggs per day), were placed randomised in the suitable fry cages fixed under the water surface of each test vessel. Each aquarium was equipped with two fry cages. 144 eggs were used for each test concentration and the control.

Hatch of zebrafish fry started around day 3 post fertilization (pf) and was finished after 6 to 7 days pf. Hatching rates was estimated by daily counting of non-hatched eggs between day 2 and 90% hatch.

After 21 and 35 days pf, survival was determined by photographic counting. After 21 days pf, the fish from the two fry chambers were pooled and released into the test vessels. After 35 days pf, length of each individual fish was measured (photographic length determination) and the fish was reduced randomly to 20 individuals. After 63 days pf, the length of individual fish was again measured (photographic length determination). From day 56 pf onwards, spawning trays were introduced into the aquaria. Spawned eggs were collected daily, counted and the number of fertilised eggs were determined.

The reproductive endpoints like egg number and fertilization rate were observed for 20 daily counts.

The criteria for starting the quantitative evaluation of the parameters was a daily egg production of minimum 15 eggs and a fertilization rate of ≥80% for controls. Both parameters should be found on three consecutive days.

2.2.1.9.3 Second filial generation (F_2)

For the start of the second filial generation (F_2), 20 (2 x 10) fertilised eggs spawned by the adult fish of each replicate were placed randomized in fry cages (two per vessel) and were fixed under the water surface of each test vessel. Hatching success and time to hatch were observed daily and was finished after 96 h. Hatching rates were estimated by daily counting of non-hatched eggs between day 2 and 90% hatch. After 96 h, all fry were terminated.

2.2.1.9.4 Test termination

Fish not further used in the study and all fish not being subject to blood collection and tissue sampling at the end of the study were euthanized after over-dosage with chloro-butanol.

At test termination, the fish of the F_1-generation were sacrificed and prepared as described below. All fish will be anaesthetised using chloro-butanol. A blood sample was taken from each fish via heart puncture. After successful blood sampling the fish were killed humanly with a dorsal cut.

The phenotypic sex of each fish was determined by macroscopic inspection of fish gonads. Individual lengths and weights were measured.

After determination of length and weight, the fish were transferred to appropriate fixative to allow a histopathological analysis of fish tissue according to the OECD guidance document 123 (OECD 2010). The phenotypic sex of each fish was confirmed by histological evaluation of fish gonads.

2.2.1.10 Specific methods

2.2.1.10.1 Egg collection

During the reproduction phase, spawned eggs were collected daily in a glass spawning tray which was placed at the bottom of the test vessels. The tray was covered with a stainless-steel lattice to prevent adult fish from eating the eggs. An artificial plant substrate (modified method according to (Nagel 1986) made of glass pearls was attached to the lattice to stimulate spawning into the tray. The artificial substrate was leached for at least one week in purified tap water in order to remove residual contaminants.

Spawning was finished soon after switch on of light. Within two hours after spawning, the eggs were transferred into a sieve, rinsed with clean water in order to remove debris, put into glass dishes, and counted as total number and number of fertilised eggs on a replicate basis. Fecundity were expressed as the total number of eggs per female and day. The number of fertilised eggs was related to the total number and the respective percentage values of the fertilization rate will

be calculated. Fertilization was determined by observing cleavage stages (> four cell stage) using a binocular.

2.2.1.10.2 Photography and image analysis

For fry counts and total length measurements, photographs were made using the digital camera: Canon Cybershot (Canon, Tokio, Japan). Digital image processing was performed by using UTHSCSA ImageTool Version 3.0 (University of Texas Health Science Center at San Antonio, USA). Fish were netted and placed in rectangular vessels with a low water level. This was placed in the photo device (light plate with additional illumination from above). A visible ruler was placed in every vessel. The level of water was adjusted to be the same in each vessel.

From the fish fry, the total length was measured to the nearest 0.1 cm. After photographing, the fry were carefully re-introduced into the test vessel.

2.2.1.10.3 Blood collection and preparation

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 (at least 2TIU/mL). As ingredients for the buffer, heparin as ammonium-salt (Sigma-Aldrich, Darmstadt, Germany) and lyophilised aprotinin (Roth, Karlsruhe, Germany) was used. For blood sampling a syringe (1 mL) with a fixed needle was used. The syringe was prefilled with buffer (approximately 300µL) to completely elute the small blood volumes from each fish. Blood samples ranged from 15-30 µL and were taken by cardiac puncture. At first the fish were anaesthetised with chloro-butanol (20 g/L). Plasma was separated from the blood via centrifugation (30 min; approx. 5000 g; 4°C) and immediately stored at -80°C until further analysis.

2.2.1.11 Measurement of biomarker Vitellogenin

The measurements of zebrafish Vitellogenin were performed according to the method published by Holbech et al. (Holbech, Andersen et al. 2001). All measurements were conducted at the laboratory of Henrik Holbech at the University of Southern Denmark (SDU) at Odense, Denmark.

2.2.1.12 Histopathology

The evaluation of fish gonads of F_0 and F_1-generation was conducted at the Fraunhofer Institute for Toxicology and Experimental medicine (ITEM). The tissue slides were finally evaluated and results were reported by Dr. Dirk Schaudien as the responsible pathologist. The examination was performed following the OECD guidance document 123 (OECD 2010).

2.2.1.12.1 Tissue fixation procedure for histology

The fish carcasses were fixated following the method described in the OECD guidance document 123 (OECD 2010). After blood collection the fish were opened ventrally by an incision along the midline of abdomen with dissection scissors taking care not to damage the gonads or other organs. Sexing of fish was carried out by macroscopical observation of gonads. Afterwards the fish were fixated to allow a histopathological evaluation.

The whole fish was 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 fixation was done overnight (approx. for 24 h) followed by the transfer on the next day into 10% neutral buffered formalin according to OECD guidance document 123 (OECD 2010).

Of each group, all fish were prepared for histological investigation. The samples were consecutively labelled according to their respective treatment level, replicate and sample number. The samples were sent to the test site at ambient conditions, together with a sample description, by courier as soon as possible after termination of the respective generation.

2.2.1.12.2 Evaluation of gonad tissue

The occurrence of female, male, undifferentiated, hermaphrodite and ovotestis was recorded based on histological observation for F_0 and F_1 fish. In order to assign the fish to the different categories, the following description was considered. During normal sexual development, all zebrafish develop an indifferent gonad with primordial germ cells ('juveniles'). From this stage, all individuals develop so called 'protogynic gonads', with only undeveloped ovaries with oogonia to perinucleolar oocytes (Takahashi 1977, Maack and Segner 2003). Male zebrafish afterwards enter a transition phase, in which the oocytes degenerate. At this stage, no morphologically distinguishable male germ cells are present. Until this stage, the fish are defined as 'undifferentiated', as the sex could not be determined unequivocally. During progression in the transition phase, degenerative oocytes are still present, however also spermatogonia are present. These fish are defined as in 'transition phase', and could be ascribed to the sex category 'male'. In contrast, 'ovotestis' is defined as presence of more than one oocyte in mature testis or spermatogenic cells in mature ovaries. This is a pathological observation in mature gonads. Fish with more mature ovaries are ascribed to the category 'female'. The results were statistically evaluated.

Furthermore, a detailed histopathological examination of gonads was conducted for the males and females of control and all treatment concentrations. During the histologic examination the sex of the animals as well as the stage of the development of the gonads were determined according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010).

The results and a report of the histological sex determination for fish treated with Dienogest are presented in 3.2.1.3 (F_0), 3.2.1.4 (F_1) and in detail in the Appendices B.1.5. (F_0) and B.1.9. (F_1). For fish treated with Dexamethasone the data are presented in 3.3.1.3 (F_0), 3.3.1.4 (F_1) and in detail in the Appendices B.2.5 (F_0) and B.2.9 (F_1).

The results and a report of the detailed histopathological gonad analysis for fish treated with Dienogest are presented in 3.2.1.7.1 (F_0), 3.2.1.7.2 (F_1), and in the Appendices B.1.13 (F_0) and B.1.14 (F_1). For fish treated with Dexamethasone the corresponding data are presented in 3.3.1.7.1 (F_0), 3.3.1.7.2 (F_1), and in the Appendices B.2.13 (F_0) and B.2.14 (F_1).

The morphologic criteria for staging ovaries and testes are given in the following table:

Table 4: Criteria for staging gonads according to the OECD guidance document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010)

	Criteria for staging ovaries	Criteria for staging testes
Stage 0	**Undeveloped**: entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli	**Undeveloped**: entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
Stage 1	**Early spermatogenic:** vast majority (e.g., > 90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar	**Early spermatogenic**: immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
Stage 2	**Mid-development**: at least half of observed follicles are early and mid-vitellogenic.	**Mid-spermatogenic**: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1, but thicker than Stage 3.
Stage 3	**Late development**: majority of developing follicles are late vitellogenic.	**Late spermatogenic**: all stages may be observed; however, mature sperm predominate; the germinal epithelium is thinner than it is during Stage 2.
Stage 4	**Late development/hydrated**: majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.	**Spent**: loose connective tissue with some remnant sperm.
Stage 5	**Post-ovulatory**: predominately spent follicles, remnants of theca externa and granulosa.	-

Furthermore, the occurrence of the following lesions was investigated as well.

Ovaries:

- Increased oocyte atresia
- Egg debris
- Granulomatous inflammation
- Increased post-ovulatory follicles

Testes:

- Testis-ova
- Increased testicular degeneration
- Interstitial cell hypertrophy/hyperplasia
- Increased proportion of spermatogonia
- Increased interstitial proteinaceous fluid
- Asynchronous germ cell development
- Granulomatous inflammation

These lesions were graded according to the following schema:

Grade 0: Not observable. This grade is used if there are no findings associated with a particular diagnostic criterion.

Grade 1: Minimal. Ranging from inconspicuous to barely noticeable, but so minor, small, or infrequent as to warrant no more than the least assignable grade. For discrete changes, grade 1 is used when there are fewer than 2 occurrences per section. For multifocal or diffusely-distributed alterations, this grade is used for processes where ≤ 20 % of the tissue in the section is involved.

Grade 2: Mild. A noticeable feature of the tissue. For discrete changes, grade 2 is used when there are 3 - 5 occurrences per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 20 - 50 % of the tissue in the section are involved.

Grade 3: Moderate. A dominant feature of the tissue. For discrete changes, grade 3 is used when there are 6 - 8 occurrences per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 50 - 80 % of the tissue in the section are involved.

Grade 4: Severe. An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per tissue section. For multifocalor diffusely-distributed alterations, this grade is used for processes where > 80 % of the tissue in the section are involved.

2.2.1.13 Chemical analysis

2.2.1.13.1 Equilibration of the flow through system and sampling

Prior to the proposed test start, the flow through system was equilibrated and samples from all test vessels were taken for chemical analysis. This was performed concomitantly to ensure the right test concentrations and the correct adjustment of the dosing pump system. Samples will immediately be transferred to the chemical analysis laboratory and measured.

If it turned that samples could not be measured within 24 h after sampling, they were immediately be kept frozen at ≤ -18 °C.

If the test concentrations could not be confirmed, the dosing system was re-adjusted and after a further equilibration phase with at least one total exchange of test media, samples of the vessels served by the adjusted dosing system were taken, and the procedure was repeated.

After confirmation of the right test concentrations, the experimental phase was started. Samples from all test vessels including controls were taken at test start, one vessel per replicate pair was sampled weekly thereafter. The two vessels of one vessel pair were sampled alternately.

2.2.1.14 Statistical calculations

For each endpoint, the NOEC and LOEC values were determined. Alternatively, EC_{10} and EC_{50} concentrations were calculated, if possible and reasonable.

To allow a decision for the appropriate post-hoc test, the data were initially checked for normality and variance homogeneity. No Observed Effect Concentrations (NOEC) were calculated, using ANOVA, followed by appropriate post hoc tests, e.g. Dunnett's (Dunnett 1955, Dunnett 1964), or Williams test (Williams 1971, Williams 1972) or respective non-parametric approaches (e.g. Jonckheere-Terpsta test (Jonckheere 1954), (Terpstra 1952)).

The computer software ToxRat® Professional 3.3.0 (ToxRat® Solutions GmbH) was used for statistical evaluations.

For NOEC / LOEC-determination, quantal data were arcsine-transformed prior to analysis.

2.2.2 Chironomid life cycle test, according to OECD guideline 233

The test guideline was developed to assess the effects of long-term exposure to the life cycle of the freshwater dipteran *Chironomus sp.*, this includes two generations. The test was performed in test beakers containing a layer of formulated sediment and a water body. The test substance could be applied by spiking either the sediment or the water column. The first generation was initiated by placing first instar chironomid larvae into the test beakers. The emergence, time to emergence and sex ratio of the fully emerged midges were recorded and assessed.

The adult midges were transferred to a breeding cage which facilitates mating behaviour and finally egg production. The number of egg clutches as well as the fertilisation success was recorded. From the clutches new first instar larvae of the 2nd generation were kept. These larvae were transferred to a new freshly prepared test beaker containing sediment. Also, for this generation, emergence, time to emergence and sex ratio of the emerged animals were evaluated.

2.2.2.1 Test acceptability criteria

The following criteria were defined to assess the validity of the test

- the mean emergence in the control treatment should be at least 70% at the end of the exposure period for both generations;

- for *Chironomus riparius*, 85% of the total emerged adult midges from the control treatment in both generations should occur between 12 and 23 days after the insertion of the first instar larvae into the vessels
- the mean sex ratio of fully emerged and alive adults (as female or male fraction) in the control treatment of both generations should be at least 0.4, but not exceed 0.6;
- for each breeding cage the number of egg ropes in the controls of the 1st generation should be at least 0.6 per female added to the breeding cage;
- the fraction of fertile egg ropes in each breeding cage of the controls of the 1st generation should be at least 0.6;
- at the end of the exposure period for both generations, pH and the dissolved oxygen concentration should be measured in each vessel. The oxygen concentration should be at least 60% of the air saturation value (ASV), and the pH of overlying water should be between 6 and 9 in all test vessels.
- the water temperature should not differ by more than ± 1.0°C.

2.2.2.2 Test performance

The test was conducted between October and December 2018. Only one Chironomid life cycle test was part of the project. It was agreed to perform the study with the synthetic progestin Dienogest.

The test was conducted following the spiked-sediment approach.

2.2.2.2.1 Test concentrations

The following test concentrations were applied:

6.25, 12.5, 25, 50 and 100 mg/kg sediment (related to dry mass).

2.2.2.2.2 Test medium

In all test beakers a water sediment system was applied. The beakers were filled with approximately 1.5 cm Sediment and 6 cm of overlaying water to achieve a final height of 7.5 cm.

Artificial sediment was prepared consisting of 75% quartz sand, 20% kaolin and 5% white peat. The pH of the sediment (in deionised water) was finally 7.0.

2.2.2.2.3 Spiking of sediment

The test substance was applied on the quartz sand using an acetonic application solution. After mixing with the sand, the acetone was completely evaporated. Afterwards, the spiked quartz sand was mixed with the artificial sediment. The amount of spiked sand was considered in the total sand amount. For the overlaying water, test water of the test facility was used. The final pH in the water was 8.1.

During the study, the water body was slightly aerated.

2.2.2.2.4 Test setup and procedure

At test start, 20 larvae with two days of age (first larvae stage) were allocated to each replicate. Eight replicate beakers were applied for control and each concentration level.

The emerged animals of the F_1 generation were transferred to breeding cages. For each treatment level, two breeding cages were applied, i.e. four replicate beakers per cage. From the egg clutches obtained in each cage, four replicates were applied to prepare the F_2 generation. To

obtain the egg clutches, each breeding cages was equipped with a flat crystalline dish, containing water and spiked sediment in a ratio of 4:1.

All egg clutches collected were transferred to microtiter plates, i.e. 12 well plates. Each well of the plate was filled with water and received only one egg rope. The well plates were covered with a lid to prevent evaporation of the water. The egg ropes were kept for observation for at least six days to observe the fertility status of the eggs.

For the start of the F_2 generation, the egg ropes were allowed to hatch. Again 20 larvae with an age of 2 days were transferred to each replicate beaker. For both F_1 and F_2, the transfer of larvae was conducted two days after spiking of the sediment. The total number of larvae for each generation was 160 per concentration and control.

2.2.2.2.5 Test conditions

Water temperature, pH and oxygen concentrations were measured continuously using a data logger system. At test start and end of each of the two generations, total hardness and ammonia concentration was measured in the test water of control and highest test concentration.

2.2.2.2.6 Chemical analysis

The concentration of the test chemical was analytically measured in both sediment and water. Sediment samples were measured at start of the F_1 generation in the test beakers of all test concentrations and of the two highest test concentrations at the end of F_1. The same sampling regime was chosen for the F_2 generation.

Samples of the overlaying water were measured of all test concentrations at test start and of the two highest concentrations at the end of the F_1 generation. Following the guideline requirement, Water samples were analysed from the beakers presented for oviposition and placed in the breeding cages. Water samples were taken at the date of application and at date the egg clutches will be distributed to the microtiter plates. At start of the F_2 generation again water samples were analysed at start of all test concentrations and finally at the end of the test at the two highest concentration levels.

The limits of quantification were set at 0.02 mg/kg sediment and 0.0015 mg/L, for sediment and water, respectively. Details on the analytical methods can be found in the Appendix to this report.

2.2.3 *Daphnia magna* reproduction test, according to OECD guideline 211

In addition to this project, two *Daphnia magna* reproduction tests were conducted at the Umweltbundesamt test facilities in Berlin-Marienfelde.

Fraunhofer IME was contracted to conduct the analytical measurements of samples from that studies. The results of the measurements were summarized in the result section of this report. Details on the analytical method are presented in the Appendix A of this report.

3 Results

3.1 Literature search

The list of pharmaceuticals finally assessed during the literature research are listed.

For the groups of progestins and gestagens, data for the following substances were available:

Megestrol, Progesterone, Hydroxyprogesterone, Medroxyprogesterone, Norethindrone, Etonogestrel. Furthermore: Dydrogesterone, Medrogestone, Chlormadinone acetate, Cyproterone acetate, Medroxyprogesterone acetate, Megestrol acetate, Nomegestrol, Promegestone, Trimegestone, Nesterone, Norethisterone, Lynestrenol, Levonorgestrel, Desogestrel, Gestodene, Norgestimate and Dienogest.

For the groups of glucocorticoides, data for the following substances were available:

Dexamethasone, Prednisolone, Triamcinolone, Deflazacort, Prednisone, Budesonide, Betamethasone, Hydrocortisone, Cortisone, Methylprednisolone, Clobetasol, Clobetasone, Difluprednate, Triamcinolone acetonide, Fluticasone propionate.

Also data for natural glucocorticoids were available, represented by 11-Desoxycortisol, Cortisol and Cortisone.

The literature data should allow a reliable estimate of the appropriate concentration ranges to be applied in the fish test. The data obtained from the literature research was extended by the internal data collection of the Umweltbundesamt for different pharmaceuticals. Also, information on the consumption amount per year in Germany triggered the final decision.

Based on the available research findings, it was finally agreed to perform the studies with Dienogest, representing the progestins, and Dexamethasone as representative for the glucocorticoids. The existing gap of public available effect data was crucial for the choice of Dienogest as test substance.

For the glucocorticoids, the amount of fish test data, especially on population relevant parameters was limited. Effects identified for Dexamethasone were effects on reproduction and growth effects for larval stages. Also, survival effects were found in a fish early life stage toxicity test. Reduced fecundity was observed after exposure of fathead minnows to 500 µg/L Dexamethasone. Fry growth was impacted at the same concentration level (LaLone, Villeneuve et al. 2012). Due to a remaining insecurity with regard to the expected effects on fish, it was decided to extend the ZEOGRT by a sixth concentration step.

Beside the research results, also technical considerations triggered the decision for the chosen substances. With regard to the scheduled flow through application regime for the long-term fish tests, it was considered reasonable to choose substances with appropriate stability in the aqueous medium which also allows a long-term storage. Both Dienogest and Dexamethasone fulfilled these requirements, confirmed by the analytical results which proved stable application conditions with low variability (see result section for details).

All results of the literature research are summarized in Appendix C.

3.2 Ecotoxicological Studies with Dienogest

3.2.1 Zebrafish extended one generation reproduction test (ZEOGRT)

3.2.1.1 Test conditions

During the study there was a timely limited deviation from the recommended range of water temperature. Over a period between day 2 and 10 after test start, single temperatures exceeded the recommended maximum level of 27.5 °C, but were not higher than 28 °C. High water temperatures were measured at all treatment levels including controls. After technical adjustment, the temperature was in the recommended range until the end of the study, leading to mean values between 26.6 and 27.0°C in controls and treatments. The mean dissolved oxygen concentrations were determined to be between 95 and 98%. The corresponding single values were between 83 and 107% and thus in line with the acceptance criteria of >60%. The mean pH levels were calculated to be in the range of 8.01 and 8.27 in controls and treatments. The single pH measurements were between 7.70 and 8.48. There was no impact of the substance application on the pH level.

All mean values and standard deviations are summarized in Table 5 to Table 7. For more details please refer to the Appendices B.1.1 to B.1.3.

Table 5: Zebrafish EOGRT with Dienogest: Test conditions, water temperature [°C]

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	RSD	Min value	Max value
control	**A**	26.8	0.2	0.9	26.4	27.5
	B	26.8	0.2	0.8	26.4	27.6*
	C	26.7	0.4	1.3	26.1	28.0*
	D	26.7	0.4	1.4	26.1	28.0*
3.20	**A**	26.7	0.2	0.9	26.4	27.6*
	B	26.7	0.2	0.9	26.4	27.5
	C	26.7	0.3	1.2	26.3	27.9*
	D	26.7	0.3	1.2	26.3	27.9*
10.0	**A**	26.7	0.2	0.9	26.2	27.4
	B	26.7	0.2	0.9	26.3	27.6*
	C	26.7	0.3	1.2	26.3	28.0*
	D	26.8	0.3	1.2	26.3	28.0*
32.0	**A**	26.8	0.2	0.8	26.3	27.6*
	B	27.0	0.2	0.8	26.5	27.7*
	C	26.8	0.3	1.1	26.3	28.0*
	D	26.9	0.3	1.1	26.3	28.0*
100	**A**	27.0	0.3	1.2	26.5	27.7*
	B	26.9	0.3	1.0	26.3	27.8*
	C	26.6	0.3	1.2	26.1	27.8*
	D	26.6	0.3	1.2	26.0	27.8*
320	**A**	26.9	0.3	1.1	26.2	27.8*
	B	26.9	0.3	1.1	26.2	27.9*
	C	26.8	0.3	1.1	26.2	27.8*
	D	26.7	0.3	1.2	26.2	27.9*

SD = Standard deviation, RSD = Relative standard deviation

*During the initial phase of the study with Dienogest, the water temperature was exceeded on single days. After technical adjustment, the temperature was in the recommended range until the end of the study.

Table 6: Zebrafish EOGRT with Dienogest: Test conditions, oxygen concentration [%]

Nominal concentration Dienogest [ng/L]	replicate	Mean	SD	RSD	Min value	Max value
control	**A**	96	5	5.4	83	103
	B	98	5	5.6	84	107
	C	97	6	5.7	83	107
	D	96	6	5.9	83	106
3.20	**A**	97	5	5.5	83	107
	B	97	5	5.3	85	107
	C	97	5	5.3	83	107
	D	97	5	5.1	83	106
10.0	**A**	96	5	5.4	83	105
	B	96	5	5.4	83	105
	C	96	5	5.5	83	106
	D	96	5	5.2	85	105
32.0	**A**	96	5	5.2	86	105
	B	96	5	5.4	84	105
	C	95	5	5.1	83	105
	D	95	4	4.6	83	105
100	**A**	95	5	4.8	90	105
	B	96	4	4.7	86	103
	C	96	4	4.2	88	103
	D	96	4	4.1	87	104
320	**A**	95	4	4.6	84	104
	B	95	4	4.7	85	103
	C	95	4	4.6	85	102
	D	95	3	3.6	87	102

SD = Standard deviation; RSD = Relative standard deviation

Table 7: Zebrafish EOGRT with Dienogest: Test conditions, pH value

Nominal concentration Dienogest [ng/L]	replicate	Mean	SD	RSD	Min value	Max value
control	**A**	8.01	0.17	2.07	7.70	8.32
	B	8.06	0.17	2.06	7.80	8.35
	C	8.04	0.15	1.90	7.78	8.33
	D	8.03	0.17	2.13	7.72	8.31
3.20	**A**	8.09	0.15	1.91	7.80	8.33
	B	8.10	0.16	1.95	7.82	8.32
	C	8.08	0.16	2.03	7.78	8.33
	D	8.10	0.15	1.88	7.80	8.35
10.0	**A**	8.07	0.17	2.06	7.80	8.36
	B	8.08	0.17	2.16	7.80	8.35
	C	8.09	0.17	2.14	7.80	8.34
	D	8.09	0.16	2.03	7.80	8.35
32.0	**A**	8.11	0.19	2.31	7.79	8.35
	B	8.11	0.19	2.34	7.78	8.40
	C	8.09	0.19	2.38	7.75	8.40
	D	8.10	0.18	2.27	7.78	8.40
100	**A**	8.27	0.11	1.33	8.07	8.48
	B	8.12	0.19	2.35	7.75	8.40
	C	8.15	0.17	2.08	7.75	8.42
	D	8.15	0.17	2.04	7.75	8.40
320	**A**	8.13	0.18	2.28	7.77	8.42
	B	8.12	0.18	2.23	7.75	8.41
	C	8.12	0.18	2.27	7.77	8.39
	D	8.14	0.18	2.21	7.75	8.42

SD = Standard deviation, RSD = Relative standard deviation

3.2.1.2 Chemical analysis

It was possible to apply the test chemical Dienogest without the use of a solvent vehicle. Decrease of test substance concentration, e.g. due to degradation or adsorption of the substance could not be observed.

The mean measured concentration were calculated to be 3.51, 10.3, 31.7, 105, and 335 ng Dienogest/L. These correspond to 110, 103, 99.1, 105 and 105% of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations of Dienogest.

All results of the chemical analysis are shown in Table 8. For more details please refer to the Appendix A.

Table 8: Zebrafish EOGRT with Dienogest: Chemical analysis

Nominal concentration Dienogest [ng/L]	replicate	Measured concentration Dienogest							
		[ng/L]/vessel		[%]/vessel		[ng/L]/ treatment		[%]/ treatment	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	A	<LOQ	-	-	-	-	-	-	-
	B	<LOQ	-	-	-				
	C	<LOQ	-	-	-				
	D	<LOQ	-	-	-				
3.20	A	3.66	2.10	114	65.7	3.51	0.30	110	8.64
	B	3.22	0.69	101	21.5				
	C	3.87	2.81	121	87.9				
	D	3.30	0.77	103	24.0				
10.0	A	11.0	3.91	110	39.1	10.3	0.51	103	4.99
	B	10.0	1.38	100	13.8				
	C	10.3	3.45	103	34.5				
	D	9.85	1.28	98.5	12.8				
32.0	A	31.1	11.2	97.2	35.0	31.7	2.53	99.1	7.96
	B	28.7	3.68	89.7	11.5				
	C	34.8	12.4	109	38.7				
	D	32.3	4.40	101	13.8				
100	A	95.5	23.5	95.5	23.5	105	7.01	105	6.71
	B	102	12.8	102	12.8				
	C	110	19.3	110	19.3				
	D	110	8.60	110	8.60				
320	A	318	65.3	99.3	20.4	335	13.3	105	3.95
	B	343	30.7	107	9.59				
	C	333	67.7	104	21.2				
	D	347	56.2	109	17.6				

SD = Standard deviation
LOQ = 1.5 ng/L

3.2.1.3 Biological results, parental generation (F_0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. Reproduction was recorded in terms of egg numbers and fertilisation rates. For the controls, a mean of 64 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be between 64 (at 3.51 ng/L) and 84 (at 31.7 ng/L). The mean egg numbers and standard deviation are shown in Table 9. The mean fertilisation rate for controls was calculated to be 90.7%. For the exposed groups, the fertilisation rates were determined to be between 90.1% (at 10.3 ng/L) and 93.7% (at 105 ng/L). A concentration related impact on the reproductive capability of the fish could not be observed.

After successful initiation of the first filial generation, the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection and histopathological examination of the fish gonads. The inspection revealed that most of the groups were set correctly, i.e. 5 males and 5 females were present. However, 4 male and 6 female fish were found in replicates B and D at 3.51 ng Dienogest/L and in replicate C at 10.3 ng Dienogest/L, while 6 male and 4 female fish were found in replicate A at 3.51 ng Dienogest/L, in replicate D at 105 ng Dienogest/L and in replicate B at 335 ng Dienogest/L. Due to mortality or technical failure, the fish numbers were reduced down to nine fish only in two tanks, namely in replicate C of the control (four male and five female fish were counted) and in replicate D at 31.7 ng Dienogest/L (five male and four female fish were counted). At 10.3 ng Dienogest/L, a single fish was identified as hermaphrodite. This individual fish was excluded from further calculation of the mean values.

The mean fish total length in controls was determined to be 3.9 cm for both, males and females. The mean fish total lengths of males in the treatments were determined to be between 3.8 cm (at 3.51, 10.3 and 31.7 ng Dienogest/L) and 3.9 cm (at 105 and 335 ng Dienogest/L). The mean fish total lengths of females in the treatments were determined to be between 3.9 cm (at 3.51, 10.3, 105 and 335 ng Dienogest/L) and 4.0 cm (at 31.7 ng Dienogest/L). The mean wet weight in controls was calculated to be 0.503 g for males, and 0.711 g for females. The mean wet weights of males in the treatments were determined to be between 0.479 g (at 3.51 ng Dienogest/L) and 0.518 g (at 105 ng Dienogest/L). The mean wet weights of females in the treatments were determined to be between 0.657 g (at 105 ng Dienogest/L) and 0.713 g (at 31.7 ng Dienogest/L).

Statistical evaluation of endpoint data could not reveal any statistically significant difference between control and treatment with Dienogest.

All mean values and standard deviation of all biological parameters are summarized in Table 9. For more details please refer to the Appendices B.1.4 to B.1.5.

Table 9: Zebrafish EOGRT with Dienogest: Biological results for parental generation (F_0)

	Mean measured concentration Dienogest [ng/L]					
Parameters	**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean egg number per day and female [n] (± SD)	64 ± 11	64 ± 24	73 ± 12	84 ± 13	64 ± 11	77 ± 16
Mean fertilisation rate [%] (± SD)	90.7 ± 3.4	91.5 ±.2.2	90.1 ±.0.9	91.5 ±.3.3	93.7 ±.2.0	90.2 ±.3.8
Mean total length, males [cm] (± SD)	3.9 ± 0.1	3.8 ± 0.0	3.8 ± 0.1	3.8 ± 0.0	3.9 ± 0.1	3.9 ± 0.0
Mean total length, females [cm] (± SD)	3.9 ± 0.2	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.1	3.9 ± 0.0	3.9 ± 0.1
Mean wet weight, males [g] (± SD)	0.503 ± 0.019	0.479 ± 0.018	0.491 ± 0.036	0.497 ± 0.025	0.518 ± 0.063	0.498 ± 0.012
Mean wet weight, females [g] (± SD)	0.711 ± 0.055	0.680 ± 0.118	0.683 ± 0.046	0.713 ± 0.064	0.657 ± 0.040	0.665 ± 0.054
Mean sex ratio, males [%] (± SD)	48.6 ± 2.8	47.5 ± 9.6	47.5 ± 5.0	51.4 ± 2.8	52.5 ± 5.0	52.5 ± 5.0
Mean sex ratio, females [%] (± SD)	51.4 ± 2.8	52.5 ± 9.6	50.0 ± 8.2	48.6 ± 2.8	47.5 ± 5.0	47.5 ± 5.0
Mean hermaphrodite ratio [%] (± SD)	0.0	0.0	2.50 ± 5.0	0.0	0.0	0.0

SD = Standard deviation

3.2.1.4 Biological results, first filial generation (F_1)

The filial 1 (F_1) generation was initiated by keeping fertilised eggs from the parental group and placing them in separate brood chambers placed in each test vessel. The allocation of eggs, in total 36, was splitted over two subsequent days, which means that 18 eggs were kept on day 1 and a second set of eggs was kept on day 2. This approach was chosen to prevent that the whole clutch of eggs originated from the same female fish.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80% in all test vessels (related to the number of eggs introduced). The mean hatching rate in controls and treatments was calculated to be 100%.

The number of surviving fish larvae were determined on day 21 and 35 post fertilisation (pf). For this, all remaining larvae were catched and photographed. The photos from day 35 pf were also evaluated for total fish length. Fish counting and length measurement were performed computer-aided. After each evaluation the fish were carefully re-introduced to the test vessel. The post hatch survival rate in controls was calculated to be 93.1% and 92.4% for day 21 and 35 pf, respectively. The acceptance criteria, taken from OECD 210, Fish early life stage toxicity test (OECD 2013), i.e. 75%, was thus fulfilled. In the exposed groups, the mean post hatch survival rates were determined to be 80.6, 70.8, 72.9, 38.2 and 47.9% at day 21 pf in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. At day 35 pf the mean post hatch survival rates were determined to be 79.2, 70.8, 71.5, 37.5 and 47.2% in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Statistical analyses evaluating both time points revealed a significant reduction in larval survival between controls and treatment with 105 and 335 ng Dienogest/L (Williams test, $p \leq 0.050$, one-sided smaller).

On day 35 pf, the total length of all remaining fish larvae was measured computer-aided. The mean total length in controls was calculated to be 1.85 cm. The fish lengths in the exposed groups were between 1.73 cm (at 10.3 ng Dienogest/L) and 1.95 cm (at 335 ng Dienogest/L). The statistical evaluation revealed a significant difference at 10.3 ng Dienogest/L compared to the control, however, due to a missing concentration related response, this finding was considered to have no biological relevance.

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish size. On day 63 pf, the fish groups were again photographed, revealing a surviving rate of 98.8% in the controls and 100% in all Dienogest concentrations tested. The mean total length at day 63 pf was measured to be 3.1 cm in the controls and 3.1, 3.1, 3.1, 3.3 and 3.2 cm in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Neither survival nor total length at day 63 pf showed any statistical differences between control and treatment with Dienogest.

From day 64 pf onwards, reproduction was recorded in terms of egg numbers and fertilisation rates. For the controls, a mean of 31 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration) were determined to be 30, 26, 37, 50 and 43, respectively. The mean fertilisation rate was calculated to be 86.4% in the controls and 88.8, 88.5, 84.1, 80.5 and 72.1% in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Compared to control, the treatment with Dienogest did not show any statistically significant effect on the mean egg number per day and female. However, a trend towards an increased egg number could be observed at the top concentrations. The fertilisation

rate was significantly reduced in treatment with 335 ng Dienogest/L (Williams test, p≤0.050, one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The obtained results indicated no test item induced shift of the sex ratio. In controls, the ratio was at 60.9% males/39.1 % females. Under treatment conditions, the percentage of males ranged between 42.9% (at 105 ng Dienogest/L) and 56.8% (at 3.51 ng Dienogest /L). Correspondingly, the number of females remained at similar levels in controls and treatments.

The mean fish total length in males was determined to be 3.7 cm in controls and 3.8, 3.8, 3.8, 3.9 and 3.8 in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean fish total length in females was determined to be 3.6 cm in controls and 3.7, 3.7, 3.7, 4.0 and 3.8 cm in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

The mean fish wet weight of males was determined to be 0.436 g in controls and 0.461, 0.449, 0.445, 0.516 and 0.495 g in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean wet weight in females was determined to be 0.512 g for the controls and 0.534, 0.565, 0.549, 0.700 and 0.648 g in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Neither the total fish length nor the wet weight showed any statistical difference between control and treatment with Dienogest. Notably, a trend for an increase of wet weights was observed for the female fish at the two top concentrations.

All results, i.e. mean values and standard deviations, are shown in Table 10. For more details please refer to the Appendices B.1.6 to B.1.9.

Table 10: Zebrafish EOGRT with Dienogest: Biological results for first filial generation (F_1)

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean hatching rate [%] (± SD)	100	100	100	100	100	100
Mean post hatch survival at day 21 pf [%] (± SD)	93.1 ± 3.6	80.6 ± 2.3	70.8 ± 12.9	72.9 ± 13.5	38.2 ± 37.4 [a])	47.9 ± 25.8 [a])
Mean post hatch survival at day 35 pf [%] (± SD)	92.4 ±4.7	79.2 ± 2.8	70.8 ± 12.9	71.5 ± 14.9	37.5 ± 36.2 [a])	47.2 ± 24.5 [a])
Mean total length, day 35 pf [cm] (± SD)	1.85 ± 0.05	1.78 ± 0.02	1.73 ± 0.05 [b])	1.78 ± 0.01	1.88 ± 0.20	1.95 ± 0.17
Mean survival day 63 pf [%] (± SD)	98.8 ± 2.5	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

Parameter	Mean measured concentration Dienogest [ng/L]					
Mean total length, day 63 pf [cm] (± SD)	3.1 ± 0.1	3.1 ± 0.0	3.1 ± 0.0	3.1 ± 0.0	3.3 ± 0.3	3.2 ± 0.2
Time to first spawning [fish age days] (± SD)	A 65	≤64	≤64	≤64	-	≤64
	B ≤64	≤64	≤64	≤64	≤64	≤64
	C ≤64	≤64	≤64	≤64	≤64	≤64
	D ≤64	65	≤64	≤64	≤64	≤64
	As most fish groups were already spawning from the first day of tray introduction, no mean value can be presented.					
Mean egg number per day and female [n] (± SD)	31 ± 9	30 ± 8	26 ± 7	37 ± 11	50 ± 18	43 ± 15
Mean fertilisation rate [%] (± SD)	86.4 ± 3.6	88.8 ± 3.3	88.5 ± 2.5	84.1 ± 2.2	80.5 ± 13.3	72.1 ± 11.8 [a])
Mean survival, adult stage [%] (± SD)	100 ± 0.0	98.8 ± 2.5	100 ± 0.0	95.0 ± 10.0	98.3 ± 2.9	97.9 ± 4.2
Mean total length, males, adult stage [cm] (± SD)	3.7 ±0.1	3.8 ± 0.1	3.8 ± 0.0	3.8 ± 0.1	3.9 ± 0.4	3.8 ± 0.1
Mean total length, females, adult stage [cm] (± SD)	3.6 ± 0.1	3.7 ± 0.0	3.7 ± 0.1	3.7 ± 0.0	4.0 ± 0.4	3.8 ± 0.2
Mean wet weight, males, adult stage [g] (± SD)	0.436 ± 0.027	0.461 ± 0.044	0.449 ± 0.010	0.445 ± 0.044	0.516 ± 0.161	0.495 ± 0.056
Mean wet weight, females, adult stage [g] (± SD)	0.512 ± 0.063	0.534 ± 0.015	0.565 ± 0.027	0.549 ± 0.060	0.700 ± 0.284	0.648 ± 0.087
Mean sex ratio, males [%] (± SD)	60.9 ± 10.7	56.8 ± 9.5	43.8 ± 12.5	51.4 ± 15.5	42.9 ± 13.9	53.3 ± 13.8
Mean sex ratio, females [%] (± SD)	39.1 ± 10.7	43.2 ± 9.5	56.3 ± 12.5	48.6 ± 15.5	57.1 ± 13.9	46.7 ± 13.8

[a]) Statistically significant reduction compared to control, $p<0.05$, Williams test, one-sided smaller.

[b]) Statistically significant reduction compared to control, $p<0.05$, Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, one-sided-smaller, heterogenous variances. Due to a missing concentration related response, this finding was considered to have no biological relevance.

3.2.1.5 Biological results, second filial generation (F_2)

The mean hatching success in the controls was 83.8% and 75.0, 53.8, 46.3, 56.7 and 40.0% in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. For more details please refer to the Appendix B.1.10.

A statistically significant difference versus control was determined for all treatment levels ≥10.3 ng Dienogest/L (Williams test, one-sided smaller, $p<0.05$).

Table 11: Zebrafish EOGRT with Dienogest: Biological results for F_2 generation

	Mean measured concentration Dienogest [ng/L]					
Parameter	**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean hatching rate [%] (± SD)	83.8 ± 11.1	75.0 ± 12.2	53.8 ± 26.9 [a])	46.3 ± 14.4 [a])	56.7 ± 10.4 [a])	40.0 ± 29.7 [a])

SD = Standard deviation

[a]) Statistically significant reduction compared to control, $p<0.05$, Williams test, one-sided smaller.

3.2.1.6 Biomarker results

The mean Vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 12 and Table 13. All single values are given in the Appendices B.1.11 (F_0) and B.1.12 (F_1).

In parental generation (F_0) the mean Vitellogenin values in controls were determined to be 62.01 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 451.93, 207.48, 346.49, 138.67 and 339.60 ng Dienogest/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean VTG values in controls were determined to be 1.64E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 1.43E+07, 1.94E+07, 2.70E+07, 2.19E+07 and 1.34E+07 ng Dienogest/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments, neither for males nor for females of parental generation.

Table 12: Zebrafish EOGRT with Dienogest: Biomarker results for parental generation (F_0); Vitellogenin concentration in blood plasma [ng/ml]

	Mean measured concentration Dienogest [ng/L]					
Parameter	**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
Parental generation (F_0)						
Mean VTG concentration, males [ng/ml] (± SD)	62.01 ± 21.52	451.93 ± 439.43	207.48 ± 131.87	346.49 ± 423.59	138.67 ± 56.20	339.60 ± 330.27
Mean VTG concentration, females [ng/ml] (± SD)	1.64E+07 ± 2.58E+06	1.94E+07 ± 7.01E+06	2.70E+07 ± 9.40E+06	2.19E+07 ± 6.11E+06	1.43E+07 ± 3.28E+06	1.34E+07 ± 4.76E+06

SD = Standard deviation

In first filial generation (F_1) the mean Vitellogenin (VTG) values in controls were determined to be 177.70 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 55.16, 84.10, 228.48, 13638.42 and 295.18 ng VTG/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean VTG values in controls were determined to be 1.81E+07 ng VTG/L for females. The mean VTG values of females in treatments 1.36E+07, 1.73E+07, 1.62E+07, 3.62E+07 and 2.93E+07 ng Dienogest/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments, neither for males nor for females of first filial generation.

Table 13: Zebrafish EOGRT with Dienogest: Biomarker results for first filial generation (F_1); Vitellogenin concentration in blood plasma [ng/ml]

	Mean measured concentration Dienogest [ng/L]					
Parameter	**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
First filial generation (F_1)						
Mean VTG concentration, males [ng/mL] (± SD)	177.70 ±75.77	55.16 ± 19.26	84.10 ± 45.42	228.48 ± 316.75	13638.42 ± 22986.12	295.18 ± 301.40
Mean VTG concentration, females [ng/mL] (± SD)	1.81E+07± 1.11E+07	1.36E+07 ±4.19E+06	1.73E+07 ± 5.70E+06	1.62E+07 ± 3.72E+06	3.62E+07 ±2.31E+07	2.93E+07 ± 8.57E+06

SD = Standard deviation

3.2.1.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in 2.2.1.12.2.

Median values were calculated for each test vessel. The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the Appendices B.1.13 for parental generation (F_0) and in B.1.14 for first filial generation (F_1). In 3.2.1.7.1 and in 3.2.1.7.2 the results are sorted according to the corresponding control or treatment level for parental generation (F_0) and first filial generation (F_1), respectively.

Summarizing the results, neither parental generation (F_0) nor first filial generation (F_1) showed any treatment-related differences in male or female gonad staging. The respective median values are presented in Table 14 and Table 15.

Table 14: Zebrafish EOGRT with Dienogest: Median maturation stages for F_0 generation

		Mean measured concentration Dienogest [ng/L]					
Parameter		**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
Parental generation (F_0)							
Maturation stages, male [1)]		Stage 1 (2/19) Stage 2 (16/19) Stage 3 (1/19)	Stage 2 (16/19) Stage 3 (3/19)	Stage 1 (2/19) Stage 2 (15/19) Stage 3 (2/19)	Stage 2 (14/20) Stage 3 (6/20)	Stage 2 (18/21) Stage 3 (3/21)	Stage 1 (1/21) Stage 2 (19/21) Stage 3 (1/21)
Maturation stage, male, median value/ replicate	A	2	2	2	2	2	2
	B	2	2	2	2	2	2
	C	2	2	2	2	2	2
	D	2	2	2	2	2	2
Maturation stages, female [2)]		Stage 2 (5/20) Stage 3 (13/20) Stage 4 (2/20)	Stage 1 (1/21) Stage 2 (9/21) Stage 3 (11/21)	Stage 2 (6/20) Stage 3 (14/20)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 1 (1/19) Stage 2 (5/19) Stage 3 (13/19)
Maturation stage, female, median value/ replicate	A	3	3	2	3	3	3
	B	3	2	2	3	3	3
	C	3	3	3	3	3	3
	D	3	3	3	3	3	2

[1)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory;

[2)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

Table 15. Zebrafish EOGRT with Dienogest: Median maturation stages for F_1 generation

		Mean measured concentration Dienogest [ng/L]					
Parameter		**Control**	**3.51**	**10.3**	**31.7**	**105**	**335**
First filial generation (F_1)							
Maturation stages, male 1)		Stage 1 (2/48) Stage 2 (39/48) Stage 3 (7/48)	Stage 2 (35/45) Stage 3 (10/45)	Stage 1 (2/35) Stage 2 (28/35) Stage 3 (5/35)	Stage 1 (1/38) Stage 2 (31/38) Stage 3 (6/38)	Stage 2 (13/17) Stage 3 (4/17)	Stage 1 (3/32) Stage 2 (24/32) Stage 3 (5/32)
Maturation stage, male, median value/ replicate	A	2	2	2	2	-	2
	B	2	2	2	2	2	2
	C	2	2	2	2	3	2
	D	2	2	2	2	2	2
Maturation stages, female 2)		Stage 2 (17/31) Stage 3 (17/31) Stage 4 (1/31)	Stage 2 (9/34) Stage 3 (24/34) Stage 4 (1/34)	Stage 1 (1/45) Stage 2 (15/45) Stage 3 (28/45) Stage 4 (1/45)	Stage 2 (6/37) Stage 3 (31/37)	Stage 2 (6/26) Stage 3 (20/26)	Stage 2 (7/26) Stage 3 (18/26) Stage 4 (1/26)
Maturation stage, female, median value/ replicate	A	2	3	3	3	-	3
	B	3	3	3	3	3	3
	C	3	3	3	3	3	3
	D	3	3	3	3	3	3

[1)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory;

[2)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

Furthermore, neither parental generation (F_0) nor first filial generation (F_1) showed any treatment-related differences in the occurrence of the investigated lesions. In fact, there were only a few findings at all, subsequently resulting in median values of zero.

As male fish of parental generation (F_0) did not show any gonadal lesions at all, only the evaluations of female fish lesions are presented in this report.

Very similar results were obtained for the examined first filial generation. Increased testicular degeneration, interstitial cell hyperthrophy/ hyperplasia, increased proportion of spermatogonia and interstitial proteinaceous fluid were not found in any male fish. Asynchronous germ cell development was only observed in one single fish (4/3-5) and granulomatous inflammation was found to be present in three animals (2/1-6, 4/3-4, 5/3-17). Only testis-ova was a little more often observed in male fish and thus presented in this report along with the female fish findings.

Table 16: Zebrafish EOGRT with Dienogest: Grading of lesions severity for F_0 generation

Parameter		Mean measured concentration Dienogest [ng/L]					
		Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Parental generation (F_0)							
Increased oocyte atresia, female, findings [1)]		Grade 1 (4/20) Grade 2 (1/20)	Grade 1 (3/21) Grade 2 (1/21)	Grade 1 (2/20)	Grade 1 (2/19) Grade 2 (1/19)	Grade 1 (1/19) Grade 2 (1/19)	-
Increased oocyte atresia, female, median value/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
Egg debris, female, findings [1)]		Grade 1 (2/20)	Grade 1 (2/21) Grade 2 (6/21)	Grade 1 (4/20) Grade 2 (1/20)	Grade 1 (3/19) Grade 2 (1/19)	Grade 1 (1/19) Grade 2 (1/19)	Grade 2 (1/19)
Egg debris, female, median value/ replicate	A	0.0	1.0	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.5	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
Granulomatous inflammation, female, findings [1)]		Grade 1 (2/20) Grade 2 (1/20)	Grade 1 (1/21)	Grade 1 (2/20) Grade 2 (3/20)	Grade 1 (2/19) Grade 2 (1/19) Grade 3 (1/19)	Grade 1 (1/19)	Grade 1 (3/19) Grade 4 (1/19)
Granulomatous inflammation, female, median value/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.5
	C	0.0	0.0	0.5	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0

Parameter		Mean measured concentration Dienogest [ng/L]					
Increased post-ovulatory follicles, female, findings [1)]		Grade 1 (3/20) Grade 2 (2/20)	Grade 1 (4/21) Grade 2 (1/21)	Grade 1 (4/20) Grade 2 (1/20)	Grade 1 (2/19) Grade 2 (1/19)	Grade 1 (1/19) Grade 2 (1/19)	Grade 1 (3/19) Grade 2 (3/19) Grade 3 (1/19)
Increased post-ovulatory follicles, female, median value/ replicate	A	0.0	0.5	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	1.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0

Criteria for grading lesions: grade 0 = not observable; grade 1 = minimal; grade 2 = mild; grade 3 = moderate; grade 4 = severe

[1)] Only findings with a severity grades of 1 and higher are presented.

Table 17. Zebrafish EOGRT with Dienogest: Grading of lesions severity for F_1 generation

Parameter		Mean measured concentration Dienogest [ng/L]					
		Control	**3.51**	**10.3**	**31.7**	**105**	**335**
First filial generation (F_1)							
Testis-ova, male, findings [1)]		Grade 1 (5/48) Grade 2 (1/48)	Grade 1 (3/45)	Grade 1 (4/35)	Grade 1 (1/38)	Grade 1 (1/17) Grade 2 (1/17)	Grade 4 (1/32)
Testis-ova, male, median value/ replicate	A	0.0	0.0	0.0	0.0	-	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
Increased oocyte atresia, female, findings [1)]		Grade 1 (1/31)	-	-	-	-	Grade 1 (3/26)
Increased oocyte atresia, female, median value/ replicate	A	0.0	0.0	0.0	0.0	-	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
Egg debris, female, findings [1)]		Grade 1 (4/31) Grade 2 (1/31)	Grade 1 (3/34) Grade 2 (2/34)	Grade 1 (2/45)	Grade 1 (3/37)	Grade 2 (3/26)	Grade 1 (4/26) Grade 2 (2/26) Grade 4 (1/26)
Egg debris, female, median value/ replicate	A	0.0	0.0	0.0	0.0	-	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0

Parameter		Mean measured concentration Dienogest [ng/L]					
Granulomatous inflammation, female, findings [1)]		Grade 1 (2/31) Grade 2 (1/31)	Grade 1 (3/34)	Grade 1 (3/45)	Grade 1 (3/37)	Grade 1 (2/26)	Grade 1 (1/26) Grade 2 (1/26) Grade 3 (1/26)
Granulomatous inflammation, female, median value/ replicate	A	0.0	0.0	0.0	0.0	-	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0
Increased post-ovulatory follicles, female, findings [1)]		Grade 1 (3/31)	Grade 1 (4/34)	Grade 1 (1/45) Grade 2 (3/45)	Grade 1 (2/37) Grade 2 (2/37)	Grade 1 (6/26) Grade 2 (1/26)	Grade 1 (4/26) Grade 2 (3/26)
Increased post-ovulatory follicles, female, median value/ replicate	A	0.0	0.0	0.0	0.0	-	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0

Criteria for grading lesions: grade 0 = not observable; grade 1 = minimal; grade 2 = mild; grade 3 = moderate; grade 4 = severe

[1)] Only findings with a severity grades of 1 and higher are presented.

3.2.1.7.1 Histopathology results, parental generation (F_0)

Control – group 0

The sex of one animal of the group 0 was determined macroscopically as male but was changed to female based on histology.

Five female animals (5/20) had the ovary stage 2, thirteen animals (13/20) the ovary stage 3 and two animals (2/20) the ovary stage 4.

Five female fish showed increased oocyte atresia (four females grade 1 and one female grade2), two females egg debris (grade 1), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and five females increased post-ovulatory follicles (three females grade 1 and two females grade 2).

Two male fish (2/19) were diagnosed with the testis stage 1, sixteen males (16/19) with the stage 2 and one male (1/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

3.51 ng Dienogest/L (mean measured) – group 1

The sex of one fish of the group 1 was determined macroscopically as male but was changed to female based on histology.

One female animal (1/21) had the ovary stage 1, nine females (9/21) the ovary stage 2 and eleven females (11/21) the ovary stage 3.

Four females showed increased oocyte atresia (three females grade 1 and one female grade 2), eight females egg debris (two females grade 1 and six females grade 2), one female a grade 1 granulomatous inflammation and five females increased post-ovulatory follicles (four females grade 1 and one female grade 2).

Sixteen male fish (16/19) were diagnosed with the testis stage 2 and three males (3/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

10.3 ng Dienogest/L (mean measured) – group 2

One fish was diagnosed as a hermaphrodite with two ovaries of stage 1 and one testis of stage 1.

The sex of two animals of the group 1 was determined macroscopically as male but was changed to female based on histology.

Of these 20 female animals, six (6/20) had the ovary stage 2 and fourteen (14/20) females the ovary stage 3.

Two female fish showed increased oocyte atresia (both with grade 1), five animals egg debris (four females grade 1 and one female grade 2), five females a granulomatous inflammation (two females grade 1 and three females grade 2) and five females increased post-ovulatory follicles (four females grade 1 and one female grade 2).

Two male fish (2/19) were diagnosed with the testis stage 1, fifteen males (15/19) with the stage 2 and two males (2/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

31.7 ng Dienogest/L (mean measured) – group 3

Three female animals (3/19) had the ovary stage 2, fifteen females (15/19) the ovary stage 3 and one female (1/19) the ovary stage 4.

Three female fish showed increased oocyte atresia (two females grade 1 and one female grade 2), four females egg debris (three females grade 1 and one female grade 2), four females a granulomatous inflammation (two females with grade 1, one female grade 2 and one female grade 3) and three females increased post-ovulatory follicles (two females grade 1 and one female grade 2).

Fourteen male fish (14/20) were diagnosed with the testis stage 2 and six males (6/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

105 ng Dienogest/L (mean measured) – group 4

Three female animals (3/19) had the ovary stage 2, fifteen animals (15/19) the ovary stage 3 and one female (1/19) the ovary stage 4.

Two female fish showed increased oocyte atresia (one female grade 1 and one female grade 2), two females egg debris (one female grade 1 and one female grade 2), one female a granulomatous inflammation of grade 1 and two females increased post-ovulatory follicles (one female grade 1 and one female grade 2).

Eighteen male fish (18/21) were diagnosed with the testis stage 2 and three males (3/21) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

335 ng Dienogest/L (mean measured) – group 5

One female fish (1/19) had the ovary stage 1, five females (5/19) the ovary stage 2 and thirteen females (13/19) the ovary stage 3.

One female fish showed egg debris (grade 2), four females a granulomatous inflammation (three females grade 1 and one female grade 4) and seven females increased post-ovulatory follicles (three females grade 1, three females grade 2 and one female grade 3).

One male fish (1/21) was diagnosed with the testis stage 1, nineteen male fish (19/21) were diagnosed with the testis stage 2 and one male (1/21) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

3.2.1.7.2 Histopathology results, first filial generation (F_1)

Control - group 0

The sex of one fish of the group 0 was determined macroscopically as male but was changed to female based on histology. Another animal was determined macroscopically as female but was changed to male based on histology.

Thirteen female fish (13/31) had the ovary stage 2, seventeen females (17/31) the ovary stage 3 and one female (1/31) the ovary stage 4.

One female fish showed increased oocyte atresia (grade 1), five females egg debris (four females grade 1 and one female grade 2), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (all grade 1).

Two male fish (2/48) were diagnosed with the testis stage 1, thirty-nine males (39/48) with the stage 2 and seven males (7/48) with the stage 3.

Six male fish showed a testis-ova (five males grade 1 and one male grade 2). Additional diagnoses were not observed in the male fish of this group.

3.51 ng Dienogest/L (mean measured) – group 1

Nine female fish (9/34) had the ovary stage 2, twenty-four females (24/34) the ovary stage 3 and one female (1/34) the ovary stage 4.

Five female fish showed egg debris (three females grade 1 and two females grade 2), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (all grade 1).

Thirty-five male fish (35/45) were diagnosed with the testis stage 2 and ten males (10/45) with the stage 3.

Three male fish showed a testis-ova (all grade 1). Additional diagnoses were not observed in the male fish of this group.

10.3 ng Dienogest/L (mean measured) – group 2

The sex of one fish of the group 2 was determined macroscopically as male but was changed to female based on histology.

One female fish (1/45) had the ovary stage 1, fifteen females (15/45) the ovary stage 2, twenty-eight females (28/45) the ovary stage 3 and one female (1/45) the ovary stage 4.

Two female fish showed egg debris (both with grade 1), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (one female grade 1 and three females grade 2).

Two male fish (2/35) were diagnosed with the testis stage 1, twenty-eight males (28/35) with the stage 2 and five males (5/35) with the stage 3.

Four male fish showed a testis-ova (all grade 1). One male had a grade 1 granulomatous inflammation.

31.7 ng Dienogest/L (mean measured) – group 3

Six female animals (6/37) had the ovary stage 2 and thirty-one females (31/37) the ovary stage 3.

Three female fish showed egg debris (all grade 1), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (two females grade 1 and two females grade 2).

One male fish (1/38) were diagnosed with the testis stage 1, thirty-one males (31/38) with the testis stage 2 and 6 males (6/38) with the stage 3.

One male fish showed a testis-ova (grade 1). Additional diagnoses were not observed in the male fish of this group.

105 ng Dienogest/L (mean measured) – group 4

The sex of one animal of the group 4 was determined macroscopically as female but was changed to male based on histology. The sex of another animal of the group 4 was not determinable macroscopically. This fish was diagnosed as female based on histology

Six female fish (6/26) had the ovary stage 2 and twenty females (20/26) the ovary stage 3.

Three female fish showed egg debris (all grade 2), two females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (six females grade 1 and one female grade 2).

Thirteen male fish (13/17) were diagnosed with the testis stage 2 and four males (4/17) with the stage 3.

Two male fish showed a testis-ova (one male grade 1 and the other grade 2). One male fish had a grade 2 asynchronous germ cell development and another male a grade 2 granulomatous inflammation.

335 ng Dienogest/L (mean measured) – group 5

Seven female fish (7/26) had the ovary stage 2, eighteen females (18/26) the ovary stage 3 and one female (1/26) the ovary stage 4.

Three female fish showed increased oocyte atresia (all grade 1), seven females egg debris (four females grade 1, two females grade 2 and one female grade 4), three females a granulomatous inflammation (one female grade 1, one female grade 2 and one female grade 3) and seven females increased post-ovulatory follicles (four females grade 1 and three females grade 2).

Three male fish (3/32) were diagnosed with the testis stage 1, twenty-four males (24/32) with the testis stage 2 and five males (5/32) with the stage 3.

One male fish showed a testis-ova (grade 4) as well as a granulomatous inflammation (grade 1). The contralateral gonad of this animal showed a unilateral atrophy/hypoplasia (grade 4). Additional diagnoses were not observed in the male fish of this group.

3.2.1.1 Summary/Conclusion

The ZEOGRT study aimed to assess the effects of continuous exposure to Dienogest on different life stages and life performances of zebrafish (*Danio rerio*) during a life cycle, including adult fishes representing a parental generation (P/F_0), a complete filial generation (F_1) and embryo stages of a second filial generation (F_2). The measured water parameters as well as the results of the chemical analysis of Dienogest concentration in the applied test media suggested stable test conditions throughout the study.

For the **parental generation (F_0)**, the evaluation and statistical analysis revealed no significant effect of Dienogest on any population-relevant endpoint determined. The measurement of growth in terms of lengths and weights did not show an effect. The inspection of the fish gonads confirmed the correct setting of the spawning groups for most of the fish groups applied. Furthermore, Dienogest did not alter the Vitellogenin concentration in blood plasma of both males and females. The histopathological analysis of maturation stages and further lesions did not reveal an effect linked to test substance exposure.

In contrast to that, reproduction in terms of fertility and early larval survival was affected by Dienogest in **first filial generation (F_1)**. More precisely, fertility was significantly reduced compared to control at the highest treatment level of 335 ng Dienogest/L (mean measured concentration; Williams test, $p<0.05$, one-sided smaller). The resulting NOEC for this endpoint was thus 105 ng Dienogest/L (mean measured concentration). A significant reduction of post hatch survival at day 21 pf and 35 pf was observed starting at a concentration of 105 ng Dienogest /L (mean measured concentration; Williams test, $p<0.05$, one-sided smaller). The corresponding NOEC was thus 31.7 ng Dienogest/L (mean measured concentration). Notably, mortality of larvae occurred mainly before day 21 pf, during the phase of feed transition from yolk sac feeding to external feeding. Thereafter, i.e. during the phase of juvenile growth, no further mortality was observed until test end.

Hatching success of the **second filial generation (F_2)** was significantly reduced compared to control in all treatment levels except the lowest concentration at 3.51 ng Dienogest/L (mean measured concentration; Williams test, $p<0.05$, one-sided smaller). It can be assumed that a low quality of F_1 eggs was the reason for a reduced hatching success of the F_2 generation larvae. The observed reduction of F_1 fertility underlines this assumption. However, a reduced VTG concentration in the blood plasma of F_1 females was not measured, suggesting that the yolk amount in the eggs was not impacted.

Based on the endpoint hatching success of the second filial generation (F_2), which was the most sensitive endpoint throughout the study, the NOEC was determined to be 3.51 ng Dienogest/L and the LOEC was 10.5 ng Dienogest/L. The observed effect on the early life stage survival of F_1 underlines the overall observation that especially embryonic and larval stages were directly impacted by this substance.

All results of the ZEOGRT are summarized in Table 18.

Table 18: ZEOGRT with Dienogest: Summary of NOEC / LOEC determination during the course of the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dienogest [ng/L]	NOEC / LOEC Mean measured concentration Dienogest [ng/L]
F_0-Reproduction	Fecundity (Egg number per day and female)	≥ 320 / >320	≥ 335 / >335
	Fertilisation rate	≥ 320 / >320	≥ 335 / >335
F_0-Growth	Wet weight males	≥ 320 / >320	≥ 335 / >335
	Wet weight females	≥ 320 / >320	≥ 335 / >335
	Total length males	≥ 320 / >320	≥ 335 / >335
	Total length females	≥ 320 / >320	≥ 335 / >335
F_0-Sex ratio	Sex ratio (% males)	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% females)	≥ 320 / >320	≥ 335 / >335
F_0-Histopathology	Maturity stage ovary	≥ 320 / >320	≥ 335 / >335
	Maturity stage testis	≥ 320 / >320	≥ 335 / >335
	Histopathology (females)	≥ 320 / >320	≥ 335 / >335
	Histopathology (males)	≥ 320 / >320	≥ 335 / >335
F_0-Biomarker	Vitellogenin females	≥ 320 / >320	≥ 335 / >335
	Vitellogenin males	≥ 320 / >320	≥ 335 / >335
F_1-ELS	Hatch day 5	≥ 320 / >320	≥ 335 / >335
	Survival day 21*	**32.0 / 100**	**31.7 / 105**
	Survival day 35*	**32.0 / 100**	**31.7 / 105**
	Total length day 35 [1])	≥ 320 / >320	≥ 335 / >335
F_1-Juveniles	Survival day 35 – day 63	≥ 320 / >320	≥ 335 / >335
	Total length day 63	≥ 320 / >320	≥ 335 / >335
F_1-Reproduction	Time to first spawning	≥ 320 / >320	≥ 335 / >335
	Fecundity (Egg number per day and female)	≥ 320 / >320	≥ 335 / >335
	Fertilisation rate*	**100 / 320**	**105 / 335**

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dienogest [ng/L]	NOEC / LOEC Mean measured concentration Dienogest [ng/L]
F_1-Termination	Survival (test end)	≥ 320 / >320	≥ 335 / >335
	Total length males	≥ 320 / >320	≥ 335 / >335
	Total length females	≥ 320 / >320	≥ 335 / >335
	Wet weight males	≥ 320 / >320	≥ 335 / >335
	Wet weight females	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% males)	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% females)	≥ 320 / >320	≥ 335 / >335
F_1-Biomarker	Vitellogenin females	≥ 320 / >320	≥ 335 / >335
	Vitellogenin males	≥ 320 / >320	≥ 335 / >335
F_1-Histopathology	Maturity stage ovary	≥ 320 / >320	≥ 335 / >335
	Maturity stage testis	≥ 320 / >320	≥ 335 / >335
	Histopathology (females)	≥ 320 / >320	≥ 335 / >335
	Histopathology (males)	≥ 320 / >320	≥ 335 / >335
F_2-Embryo	**Hatch day 4***	**3.20 / 10.0**	**3.51 / 10.3**

* Williams test, $p<0.05$, one-sided smaller

[1]) The statistical evaluation revealed a significant difference of fish lengths at 10.3 ng Dienogest/L compared to the control, however, due to a missing concentration related response, this finding was considered to have no biological relevance.

3.2.2 Chironomid Life Cycle Test, according to OECD Guideline 233

3.2.2.1 Test conditions

The water temperature was measured continuously in the water bath serving the test beakers. Throughout the test period, the temperature was measured between 19.5 and 21.5 °C. The oxygen saturation was measured to be between 90 and 101% in the test beakers and in the crystalline dishes presented for oviposition in the breeding cages. The pH values in the same beakers and dishes were measured to be between 7.7 and 8.6 throughout the test period. The water parameters confirm stable test conditions throughout the study.

Total hardness was determined to be at 1.1 mmol/L at test start and 1.7 mmol/L at test end. Ammonia in water of the test beakers and dishes was between 0.3 mg/L at test start and 30 mg/L at test end. Ammonia is a product of microbial degradation of feed and peat in the sediment layer appearing throughout the study. The Corg content of the artificial sediment used was measured to be 2.0%.

3.2.2.2 Chemical analysis of sediment and water

The measurement of Dienogest concentrations in both sediment and water were in good compliance with the nominal concentrations. All results are summarised in the following table.

All effect concentrations of the biological parameters were related to the mean measured initial concentrations of Dienogest in the sediment.

Table 19: Chironomid Life Cycle test with Dienogest: Results of the chemical analysis of test chemical concentrations in sediment and water

	Nominal concentration [mg/kg sed. dw]					
	control	**6.25**	**12.5**	**25**	**50**	**100**
F_1 generation, test start	**sediment**					
Measured concentration [mg/kg sed. dw]	<LOQ	6.33	12.0	20.1	49.6	93.3
Measured concentration [%]	-	101.3	95.9	80.4	99.2	93.3
	water					
Measured concentration [mg/L]	<LOQ	0.03	0.06	0.12	0.33	0.67
Measured concentration [%]	-	1.5	1.6	1.5	2.0	2.1
F_1 generation, day 28 of age	**sediment**					
Measured concentration [mg/kg sed. dw]					41.1	82.8
Measured concentration [%]					82.2	82.8
	water					
Measured concentration [mg/L]					0.28	0.80
Measured concentration [%]					1.7	2.5

	Nominal concentration [mg/kg sed. dw]					
Dishes for oviposition, day of application	**water**					
Measured concentration [mg/L]	<LOQ	0.02	0.05	0.13	0.28	0.49
Measured concentration [%]	-	1.2	1.2	1.6	1.7	1.5
Dishes for oviposition, finish	**water**					
Measured concentration [mg/L]					0.41	1.15
Measured concentration [%]					2.6	3.6
F_2 generation, test start	**sediment**					
Measured concentration [mg/kg sed. dw]	<LOQ	5.41	11.9	23.6	46.7	94.5
Measured concentration [%]		86.6	95.5	94.4	93.4	94.5
	water					
Measured concentration [mg/L]	<LOQ	0.02	0.06	0.16	0.35	0.82
Measured concentration [%]		1.2	1.6	2.0	2.2	2.5
F_2 generation, test end (day 27 of age)	**sediment**					
Measured concentration [mg/kg sed. dw]					45.9	87.0
Measured concentration [%]					91.8	87.0
	water					
					0.31	0.77
					1.9	2.4
	sediment					
Mean measured initial concentration [mg/kg sed. dw]	**<LOQ**	**5.87**	**12.0**	**21.9**	**48.2**	**93.9**
[%] of nominal	-	94.0	95.7	87.4	96.3	93.9

Limit of quantification (LOQ) Sediment: 0.02 mg/kg sed dw; LOQ Water: 0.0015 mg/L.

3.2.2.3 Biological results

3.2.2.3.1 Effects on F_1 generation

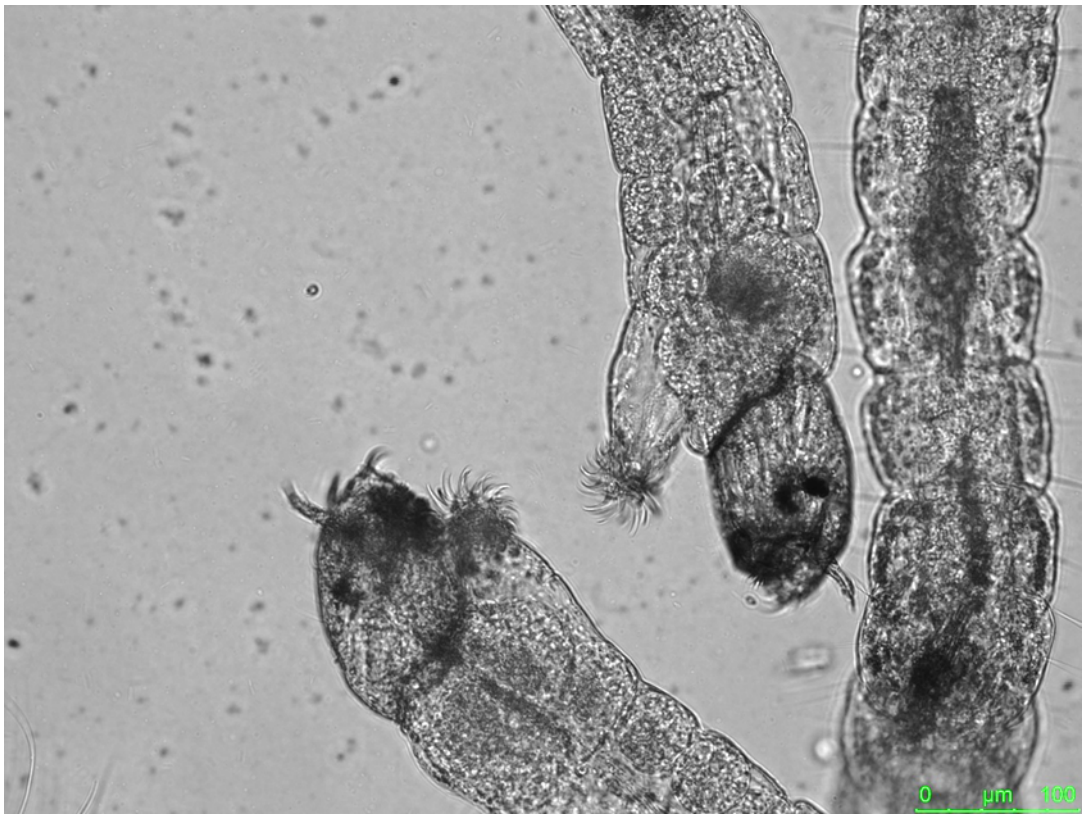
After introduction of the instar larvae (see Figure 2) the course of emergence was recorded daily over 28 days. Emerged adult midges were classified for either male or female. Based on the number of emerged animals recorded, an emergence rate [%] and a development rate was calculated following the guideline recommendations.

For the calculation of the sex-specific emergence rate, the following approach was applied. The number of introduced males or females at test start is not known and was estimated with 50% of the total number, i.e. 80 animals. In at least one test concentration, more than 50% of males or females emerged. Therefore, for the calculation of the emergence rate of each sex, the difference between assumed number of males (or females) and maximum number of males (or females) actually emerged was summed up to the number of assumed introduced males (or females) and subtracted from the number of assumed introduced females (or males).

The statistical evaluation of emergence, developmental rate and sex ratio revealed no statistically significant difference between control and treatment groups. The course of F_1 emergence is shown in Figure 3.

All results for the F_1 generation are shown in Table 20.

Figure 2: Chironomid life cycle test: Picture of newly hatched first instar larvae



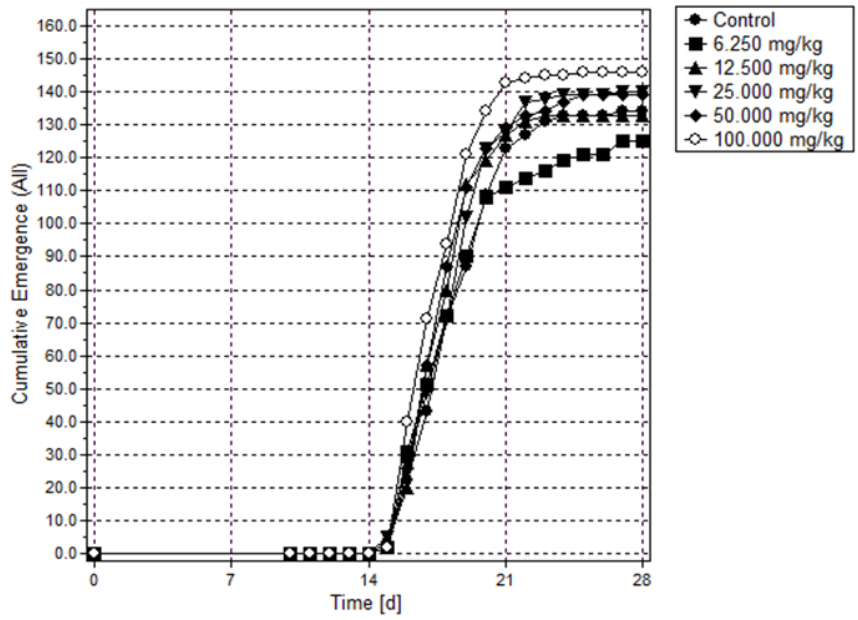
Source: Katja Mock, Fraunhofer IME

Table 20: Chironomid Life Cycle Test with Dienogest: Biological results for F_1 generation

Parameter	Mean measured initial concentration [mg/kg sed. dw]					
	control	**5.87**	**12.0**	**21.9**	**48.2**	**93.9**
Number of males [n]	55	70	71	70	70	81
Recalculated number of males introduced [n] *	73	84	80	79	80	87
Emergence rate males [%] *	75.3	83.3	88.7	88.6	87.5	93.1
Number of females [n]	79	55	62	70	69	65
Recalculated number of females introduced [n] *	87	76	80	81	80	73
Emergence rate females [%] *	90.8	72.4	77.5	86.4	86.3	89.0
Sex ratio [% females]	59.0	44.0	46.6	50.0	49.6	44.5
Total number of midges [n]	134	125	133	140	139	146
Total number of larvae introduced [n]	160	160	160	160	160	160
Total emergence rate [%]	83.8	78.1	83.1	87.5	86.9	91.3
Total Developmental rate [1/d] (+/- SD)	0.053 ± 0.002	0.052 ± 0.006	0.054 ± 0.001	0.053 ± 0.001	0.054 ± 0.001	0.055 ± 0.001

* Rate based on the recalculated theoretical number of individuals from both sexes introduced. Generally expected are 50% for each sex. However, actually rates differ and, in some replicates, more than 50 % from the expected absolute numbers occur. In this case, ToxRat® recalculates the number of individuals for both sexes introduced based on the sex surpassing the expected 50 % value.

Figure 3: Chironomid Life Cycle Test with Dienogest: Course of emergence of midges from F_1



Source: ToxRatPro Version 3.3.0

On the day of emergence, the adult midges were transferred to breeding cages. For each treatment two cages were applied serving the animals from in total 4 replicate beakers set for F_1 generation. From the date of transfer, the record of egg clutches was started. The number of egg ropes was finally related to the number of female midges transferred to each cage.

The number of females transferred may differ from the number of females emerged, e.g. due to loss of animals during transfer to mating cages. Since the total number of egg-clutches depends on the number of females actually transferred, possible differences are irrelevant, since cannot be traced back to the test substance.

To assess the effects on F_1 reproduction, the number of egg clutches recorded was related to the number of females transferred. The number of clutches per female was calculated to be between 1.53 to 1.79 in controls and treatments. The statistical evaluation did not show a statistically significant difference between control and treatments. All results are shown in Table 21.

Table 21: Chironomid Life Cycle Test with Dienogest: Total number of eggs and number of female midges transferred for F_1 generation

Parameter	Mean measured initial concentration [mg/kg sed. dw]					
	control	**5.87**	**12.0**	**21.9**	**48.2**	**93.9**
Total number of egg clutches [n]	51 / 65 116	50 / 35 85	48 / 44 92	70 / 51 121	69 / 53 122	55 / 50 105
Number of females transferred [n]	33 / 43 76	29 / 23 52	31 / 28 59	37 / 31 68	36 / 32 68	32 / 31 63
Egg clutches per female [n]	1.53 ± 0.02	1.62 ± 0.14	1.56 ± 0.02	1.77 ± 0.17	1.79 ± 0.18	1.67 ± 0.08

3.2.2.3.2 Effects on F_2 generation

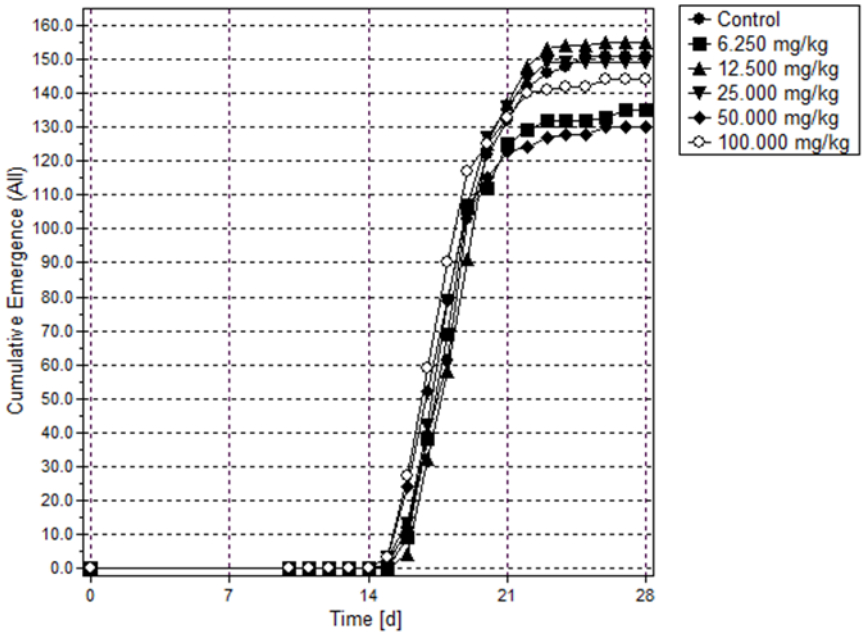
First instar larvae, collected from the timepoint of maximum egg production observed for F_1, were applied to prepare the Filial 2 Generation (F_2). All egg clutches applied were collected on the same day. For all test beakers, there was a daily recording of emerged male and female midges for a period of 28 days. Based on the statistical analysis, no significant effect was found for emergence rate and sex ratio. The total emergence rate was calculated to be between 81 and 97%. The sex ratio was calculated to between 45 and 54% for females and the developmental rate was determined to be between 0.051 – 0.054.

Table 22: Chironomid Life Cycle Test with Dienogest: Biological results for F_2 generation

Parameter	Mean measured initial concentration [mg/kg sed. dw]					
	control	**5.87**	**12.0**	**21.9**	**48.2**	**93.9**
Number of males [n]	70	66	74	79	72	71
Recalculated number of males introduced [n] *	76	80	76	84	85	80
Emergence rate males [%]*	92.1	82.5	97.4	94.0	84.7	88.7
Number of females [n]	81	69	81	70	58	73
Recalculated number of females introduced [n] *	84	80	84	76	75	80
Emergence rate females [%]*	96.4	86.3	96.4	92.1	77.3	91.3
Sex ratio [% females]	53.6	51.1	52.3	47.0	44.6	50.7
Total number of midges [n]	151	135	155	149	130	144
Total number of larvae introduced [n]	160	160	160	160	160	160
Total emergence rate [%]	94.4	84.4	96.9	93.1	81.2	90.0
Total Developmental rate [1/d] (+/- SD)	0.052 ± 0.001	0.053 ± 0.002	0.051 ± 0.002	0.053 ± 0.001	0.054 ± 0.002	0.054 ± 0.001

* Rate based on the recalculated theoretical number of individuals from both sexes introduced. Generally expected are 50% for each sex. However, actually rates differ and, in some replicates, more than 50% from the expected absolute numbers occur. In this case, ToxRat® recalculates the number of individuals for both sexes introduced based on the sex surpassing the expected 50% value.

Figure 4: Chironomid Life Cycle Test with Dienogest: Course of emergence of midges from F_2



Source: ToxRatPro Version 3.3.0

3.2.2.4 Validity criteria

All acceptance criteria given by the OECD Guideline 233 were fulfilled:

- The mean emergence for controls was 75% and 94% for F_1 and F_2 generation, respectively (TG 233: at least 70%).
- The emergence in control was at 85% of total emergence at day 21 for both generations. According to OECD 233 this should be achieved between day 12 to 23
- The sex ratio was at 59 and 54% for females of F_1 and F_2 generation, respectively (between 40 to 60% according to OECD 233)
- The number of control egg clutches per female was at 1.51 and 1.55 and thus higher than 0.6 as specified by the guideline.
- The ratio of fertilised egg clutches was higher than 60%.
- The oxygen concentrations in the test beakers was between 97 to 100% (F_1) and 95 to 99% and thus higher than 60% as specified by OECD 233.
- The mean water temperature did not differ by more than ± 1.0°C.

3.2.2.5 Conclusion and Summary, Chironomid Life Cycle Test

A chironomid life cycle test according to OECD 233 was conducted the effects of a Dienogest exposure to two generations of *C. riparius* under static conditions. The water parameters recorded confirm stable test conditions during the study. The test substance was applied by spiking the sediment.

The mean measured initial concentrations of the test substance in sediment were at 5.87, 12.0, 21.9,48.2 and 93.9 mg/kg sed. dw, corresponding to 94.0, 95.7, 87.4, 96.3 and 93.9 % of the

nominal concentrations. The water concentrations of Dienogest were low at test start and also after 4 weeks, suggesting a stable binding of the substance at the sediment matrix. Thus, the major exposure was during the larval development of the midges.

No effect on the developmental stages of the exposed midges was observed. The time of emergence of adult F_1 stages was without statistically significance compared to the control. The evaluation of the sex ratio of the emerged midges did not identify a substance related effect.

The number of F_1 egg clutches and the calculated number of egg clutches per female did not reveal a substance related effect.

No effect on F_2 emergence and sex ratio was detected.

Thus, due to the absence of biological effects in this study, the overall NOEC was determined to be ≥ 100 mg Dienogest/kg sed. dw. All results are summarized in Table 23.

Table 23: Chironomid Life Cycle Test with Dienogest: Summary of effect concentrations

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration [mg Dienogest/kg sed. dw]	NOEC / LOEC Mean measured initial concentration [mg Dienogest/kg sed. dw]
F_1-emergence	Emergence males	≥100 / >100	≥93.9 / >93.9
	Emergence females	≥100 / >100	≥93.9 / >93.9
	Total emergence	≥100 / >100	≥93.9 / >93.9
	Developmental rate, total	≥100 / >100	≥93.9 / >93.9
F_1-Sex ratio	Sex ratio (%males/%females)	≥100 / >100	≥93.9 / >93.9
F_1-Reproduction	Total number of egg clutches	≥100 / >100	≥93.9 / >93.9
	Egg clutches per female	≥100 / >100	≥93.9 / >93.9
F_2-emergence	Emergence males	≥100 / >100	≥93.9 / >93.9
	Emergence females	≥100 / >100	≥93.9 / >93.9
	Total emergence	≥100 / >100	≥93.9 / >93.9
	Developmental rate, total	≥100 / >100	≥93.9 / >93.9
F_2-Sex ratio	Sex ratio (%males/%females)	≥100 / >100	≥93.9 / >93.9

3.3 Ecotoxicological study with Dexamethasone

3.3.1 Zebrafish extended one generation reproduction test (ZEOGRT)

3.3.1.1 Test conditions

The mean water temperatures in controls and treatments were calculated to be between 26.1 and 26.7°C and thus in line with the defined acceptance criteria. The single measurements in all test vessels were between 25.6 and 27.2°C. The mean oxygen concentrations throughout the test period were determined to be between 100 and 104% and based on single measurement between 88 and 115%. The mean pH levels were between 7.93 and 8.08 in controls and treatments. The single pH measurements were between 7.77 and 8.28. There was no impact of the substance application on the pH level.

All in all, stable conditions of exposure could be confirmed.

All mean values and standard deviations are summarized in Table 24 to Table 26. For more details please refer to the Appendices B.2.1 to B.2.3.

Table 24: Zebrafish EOGRT with Dexamethasone: Test conditions, water temperature [°C]

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
control	**A**	26.4	0.19	0.7	25.9	26.8
	B	26.4	0.20	0.8	25.8	26.8
	C	26.4	0.27	1.0	25.9	27.1
	D	26.5	0.25	0.9	26.0	27.1
0.32	**A**	26.2	0.21	0.8	25.6	26.7
	B	26.2	0.23	0.9	25.7	26.7
	C	26.4	0.28	1.1	25.9	27.1
	D	26.4	0.26	1.0	25.9	27.1
1.0	**A**	26.1	0.18	0.7	25.7	26.6
	B	26.1	0.19	0.7	25.7	26.5
	C	26.3	0.25	1.0	25.9	27.0
	D	26.3	0.28	1.1	25.8	27.1
3.2	**A**	26.4	0.23	0.9	25.8	26.9
	B	26.5	0.23	0.9	25.8	27.0
	C	26.4	0.26	1.0	25.9	27.1
	D	26.4	0.24	0.9	25.9	27.2

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
10	**A**	26.2	0.18	0.7	25.8	26.7
	B	26.2	0.20	0.7	25.8	26.7
	C	26.4	0.20	0.8	25.9	27.0
	D	26.4	0.20	0.8	25.9	27.0
32	**A**	26.3	0.20	0.8	25.9	26.8
	B	26.3	0.23	0.9	25.9	26.8
	C	26.4	0.19	0.7	26.0	27.0
	D	26.5	0.19	0.7	26.1	27.1
100	**A**	26.5	0.19	0.7	26.2	26.9
	B	26.5	0.19	0.7	26.1	26.9
	C	26.7	0.16	0.6	26.4	27.2
	D	26.7	0.16	0.6	26.4	27.2

SD = Standard deviation, RSD = Relative standard deviation

Table 25: Zebrafish EOGRT with Dexamethasone: Test conditions, oxygen concentration [%]

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
control	**A**	103	6	5.6	88	112
	B	104	6	5.6	91	115
	C	104	6	5.3	92	113
	D	104	5	5.1	93	113
0.32	**A**	104	5	4.8	95	114
	B	104	5	4.8	96	114
	C	104	5	5.2	96	114
	D	104	6	5.5	93	115
1.0	**A**	103	5	5.0	95	114
	B	103	5	5.0	94	114
	C	103	5	5.1	94	114
	D	103	5	4.7	95	113

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
3.2	**A**	103	5	5.0	94	113
	B	102	5	5.0	95	112
	C	102	5	5.0	95	112
	D	102	5	5.0	94	111
10	**A**	102	5	4.7	95	111
	B	103	5	4.7	95	112
	C	102	5	4.9	95	112
	D	101	5	5.0	92	110
32	**A**	102	5	4.9	94	111
	B	102	5	5.2	94	111
	C	102	5	5.2	95	111
	D	102	5	5.3	94	111
100	**A**	102	6	5.4	95	112
	B	101	5	5.2	94	112
	C	101	5	5.0	94	111
	D	100	5	5.2	93	111

Sub caption of table – for example source, additional information.

Table 26: Zebrafish EOGRT with Dexamethasone: Test conditions, pH value

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
control	**A**	7.93	0.10	1.2	7.73	8.11
	B	7.97	0.12	1.4	7.77	8.30
	C	7.97	0.10	1.2	7.79	8.20
	D	8.00	0.09	1.1	7.85	8.18
0.32	**A**	8.02	0.10	1.2	7.86	8.22
	B	8.02	0.10	1.3	7.84	8.22
	C	8.04	0.11	1.3	7.84	8.22
	D	8.02	0.10	1.2	7.86	8.22

Nominal concentration Dexamethasone [µg/L]	replicate	Mean	SD	RSD	Min value	Max value
1.0	**A**	8.03	0.09	1.1	7.87	8.21
	B	8.02	0.08	1.0	7.88	8.19
	C	8.03	0.08	1.0	7.89	8.19
	D	8.03	0.08	1.0	7.89	8.20
3.2	**A**	8.04	0.09	1.1	7.87	8.19
	B	8.03	0.09	1.1	7.87	8.20
	C	8.05	0.08	1.0	7.90	8.23
	D	8.06	0.09	1.1	7.90	8.22
10	**A**	8.04	0.09	1.1	7.90	8.21
	B	8.04	0.09	1.2	7.90	8.27
	C	8.05	0.09	1.1	7.90	8.28
	D	8.04	0.09	1.1	7.88	8.22
32	**A**	8.06	0.09	1.1	7.92	8.21
	B	8.08	0.10	1.3	7.93	8.25
	C	8.07	0.09	1.1	7.93	8.24
	D	8.08	0.09	1.1	7.94	8.25
100	**A**	8.08	0.09	1.1	7.93	8.26
	B	8.06	0.09	1.1	7.85	8.22
	C	8.08	0.10	1.2	7.83	8.24
	D	8.06	0.10	1.3	7.72	8.23

3.3.1.2 Chemical analysis

It was possible to apply the test chemical Dexamethasone without the use of a solvent vehicle. Decrease of test substance concentration, e.g. due to degradation or adsorption of the substance could not be observed.

The mean measured concentration were calculated to be 0.33, 0.91, 3.2, 10.5, 34.7 and 100.1 µg Dexamethasone/L. These correspond to 102.6, 91.4, 99.7, 105.4, 108.5 and 100.1% of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations.

All results of the chemical analysis are shown in Table 27. For more details please refer to the Appendix A.

Table 27: Zebrafish EOGRT with Dexamethasone: Chemical analysis

Nominal concentration Dexamethasone	replicate	Measured concentration Dexamethasone							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
[µg/L]		Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	A	<LOQ	-	-	-	-	-	-	-
	B	<LOQ	-	-	-				
	C	<LOQ	-	-	-				
	D	<LOQ	-	-	-				
0.32	A	0.32	0.04	99.5	13.0	0.33	0.01	103	2.1
	B	0.33	0.05	103	14.2				
	C	0.33	0.03	104	9.7				
	D	0.33	0.06	104	17.6				
1.00	A	0.87	0.14	87.4	14.3	0.91	0.04	91.4	4.1
	B	0.89	0.13	89.1	12.8				
	C	0.94	0.07	94.2	7.3				
	D	0.95	0.10	95.0	10.4				
3.20	A	3.2	0.3	99.6	9.0	3.2	0.05	99.7	1.7
	B	3.2	0.31	99.3	9.8				
	C	3.1	0.2	97.9	6.7				
	D	3.3	0.38	102	11.8				

Nominal concentration Dexamethasone	replicate	Measured concentration Dexamethasone							
10.0	**A**	10.1	1.0	101	10.1	10.5	0.51	105	4.8
	B	10.1	0.78	101	7.8				
	C	11.1	1.2	111	11.6				
	D	10.9	1.12	109	11.2				
32.0	**A**	35.6	2.7	111	8.5	34.7	1.56	109	4.5
	B	36.5	2.78	114	8.7				
	C	33.2	2.8	104	8.8				
	D	33.7	3.02	105	9.4				
100	**A**	101.2	9.7	101.2	9.7	100	2.7	100	2.7
	B	103.3	11.6	103.3	11.6				
	C	97.5	7.9	97.5	7.9				
	D	98.3	7.8	98.3	7.8				

LOQ=0.10 µg/L

3.3.1.3 Biological results, parental generation (F_0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. Reproduction was recorded in terms of egg numbers and fertilisation rates. For the controls, a mean of 75 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments was determined to be between 53 (at 0.91 µg/L) and 73 (at 10.5 µg/L). The mean egg numbers and standard deviation are shown in Table 28. The mean fertilisation rate for controls was calculated to be 91.1%. For the exposed groups, the fertilisation rates were determined to be between 82.3% (at 10.5 µg/L) and 90.4% (at 100 µg/L). The statistical evaluation revealed a significant reduction of egg numbers at 0.33 and 0.91 µg Dexamethasone/L, and for fertilisation rate at 10.5 and 34.7 µg Dexamethasone/L. However, a concentration related impact on the reproductive capability of the fish could not be observed. Thus, the biological relevance of these observations was considered as minor. See Table 28 for details.

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads. The inspection revealed that most of the groups were set correctly, i.e. 5 males and females were present. For two test vessels, i.e. vessels 3-2 and 3-4 (at 3.20 µg/L), 6 female and 4 male fish were found. Due to mortality or technical failure, the fish numbers were reduced in two tanks, i.e. in vessel 5-1 (at 32.0 µg/L) five male and four female fish were found, and in vessel 6-2 (at 100 µg/L) only four male and five female fish were detected.

The mean fish total lengths of males were determined to be 3.8 cm in controls and 3.8, 3.7, 3.8, 3.8, 3.6 and 3.6 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean fish total lengths of females were determined to be 3.9 cm in controls

and 3.9, 3.8, 3.8, 3.9, 3.8 and 3.8 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

The mean wet weights of males were determined to be 0.482 g in controls and 0.482, 0.465, 0.482, 0.504, 0.430 and 0.406 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean wet weights in females were determined to be 0.704 g in controls and 0.697, 0.689, 0.671, 0.652, 0.638 and 0.559 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Evaluation of data revealed a statistically significant reduction in female fish total length and wet weight compared to control in treatment with 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller). Male fish in treatments with 34.7 and 100 µg Dexamethasone/L showed a significant reduction in wet weight, too (Jonckheere-Terpstra, $p<0.05$, one-sided smaller).

All mean values and standard deviation of all biological parameters are summarized in Table 28.

For more details please refer to the Appendices B.2.4 to B.2.5.

Table 28: Zebrafish EOGRT with Dexamethasone: Biological results for parental generation (F_0)

	Mean measured concentration Dexamethasone [µg/L]						
Parameters	**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Mean egg number per day and female [n] (± SD)	75 ± 9	57 ± 6 [a])	53 ± 11 [a])	62 ± 7	73 ± 8	68 ± 12	67 ± 10
Mean fertilisation rate [%] (± SD)	91.1 ± 2.5	87.2 ± 2.9	86.3 ± 3.6	87.8 ± 1.7	82.3 ± 5.8 [a])	85.3 ± 2.2 [a])	90.4 ± 0.6
Total length, males [cm] (± SD)	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.8 ± 0.1	3.6 ± 0.0	3.6 ± 0.1
Total length, females [cm] (± SD)	3.9 ± 0.1	3.9 ± 0.2	3.8 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.8 ± 0.1 [b])
Wet weight, males [g] (± SD)	0.482 ± 0.036	0.482 ± 0.029	0.465 ± 0.014	0.486 ± 0.024	0.504 ± 0.104	0.430 ± 0.009 [c])	0.406 ± 0.039 [c])
Wet weight, females [g] (± SD)	0.704 ± 0.054	0.697 ± 0.113	0.689 ± 0.052	0.675 ± 0.046	0.652 ± 0.044	0.638 ± 0.060	0.559 ± 0.037 [b])
Mean sex ratio, males [%] (± SD)	50.0 ± 0.0	50.0 ± 0.0	50.0 ± 0.0	47.5 ± 5.0	50.0 ± 0.0	51.4 ± 2.8	48.6 ± 2.8
Mean sex ratio, females [%] (± SD)	50.0 ± 0.0	50.0 ± 0.0	50.0 ± 0.0	52.5 ± 5.0	50.0 ± 0.0	48.6 ± 2.8	51.4 ± 2.8

a) Statistically significant reduction compared to control, $p<0.05$, Dunnett`s Multiple t-test, one-sided smaller.

b) Statistically significant reduction compared to control, $p<0.05$, Williams test, one-sided smaller.

c) Statistically significant reduction compared to control, $p<0.05$, Jonckheere-Terpstra test, one-sided smaller.

3.3.1.4 Biological results, first filial generation (F_1)

The first filial generation (F_1) was initiated by keeping fertilised eggs from the parental group and placing them in separate brood chambers placed in each test vessel. The allocation of eggs, in total 36, was splitted over two subsequent days, which means that 18 eggs were kept on day 1 and a second set of eggs was kept on day 2. This approach was chosen to prevent that the whole clutch of eggs originated from the same female fish.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80% in all test vessels (related to the number of eggs introduced). The mean hatching success in controls was calculated to be 98.6%. In the treatments, the hatching rates were between 89.6% (at 10.5 µg Dexamethasone/L) and 100% (at 100 µg Dexamethasone/L). A concentration related effect on hatching was not evident. All mean hatching rates and standard deviations are shown in Table 29.

The number of surviving fish larvae were determined on day 21 and 35 post fertilisation (pf). For this, all remaining larvae were catched and photographed. The photos from day 35 were also evaluated for total fish length. Fish counting and length measurement was performed computer aided. After each evaluation the fish were carefully re-introduced to the test vessel. The post hatch survival rate in controls was calculated to be 97.2% and 94.4% for day 21 and 35, respectively. The acceptance criteria, taken from OECD 210, Fish early life stage toxicity test (OECD 2013), i.e. at least 75%, was thus fulfilled. In the exposed groups, the mean post hatch survival rates were determined to be between 90.5% (at 10.5 µg/L) and 100% (at 34.7 µg/L). No significant effect on larval survival was observed. Due to a handling mistake in the second control replicate B, the fish larvae were unintendedly released to the main vessels and finally got lost. Thus, the control was continued with only three tank replicates. A re-set of this replicate was considered as not appropriate, as there would have been a time difference of 4 weeks compared to the other groups.

On day 35 pf, the total length of all remaining fish larvae was measured computer-aided. The mean length in controls was calculated to be 1.76 cm. The fish lengths in the exposed groups were between 1.60 cm (at 100 µg/L) and 1.83 cm (at 10.5 µg/L). A decrease of fish lengths was observed and was statistically significant at 100 µg Dexamethasone/L (Jonckheere-Terpstra test, $p<0.05$, one-sided smaller).

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish size. On day 63 pf, the fish groups were again photographed and the number of surviving fishes was determined and the fish total length was measured. The mean control length was determined to be 3.05 cm. In the treatment groups, the mean fish total lengths were calculated to be between 2.74 cm (at 100 µg/L) and 3.07 cm (at 0.91 µg/L). Again, as observed for the larval stages, a decrease of fish growth was also evident for the juvenile stages. The decrease was statistically significant at 34.7 and 100 µg/L (Williams test, $p<0.05$, one-sided smaller). All fish groups were reduced to an equal number of 20 fish per tank (if possible), an effect induced by fish density can be excluded. Thus, it can be postulated that the growth effect was induced by the test chemical itself. There was no effect on survival of the juvenile stages.

From day 57 pf onwards, reproduction was recorded in terms of egg number and fertilisation rates. Evaluation of data did not show any significant differences between treatments and controls. For the controls, a mean value of 21 eggs per female and day was calculated, while mean values in treatments were determined to be between 17 (at 34.7 µg/L) and 23 (at 0.91, 3.20 and 10.5 µg/L) eggs per female and day. The mean fertilisation rate for the controls was

determined to be 89.9%. In treatments with Dexamethasone the fertilisation rates were between 90.0% (at 100 µg/L) and 93.5% (at 0.33 µg/L).

After successful initiation of the second filial generation, the adult fish groups of F1 were sacrificed. Measurement of the male fish total length revealed a mean value of 3.8 cm for the controls and 3.9, 3.8, 3.9, 3.8, 3.6 and 3.5 cm when treated with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. Compared to control, a statistically significant reduction of total fish length was evident in treatments with 34.7 and 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller). In female fish the mean total length was determined to be 3.7 cm in the controls and 3.8, 3.8, 3.8, 3.8, 3.7 and 3.6 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. Female fish did not show any statistical difference compared to control in terms of fish total length.

The mean fish wet weight in males was determined to be 0.425 g for controls and 0.454, 0.432, 0.453, 0.449, 0.361 and 0.296 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. The mean wet weight in females was determined to be 0.528 g for the controls and 0.573, 0.568, 0.534, 0.531, 0.459 and 0.394 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. Male as well as female fish showed a significant reduction in wet weight when treated with 34.7 and 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller.

The sex ratio was first calculated based on the macroscopic inspection of fish gonads and was afterwards verified by histopathological evaluation. The obtained results indicated no test item induced shift of the sex ratio. In controls, the ratio was at 43.3% males/56.7% females. Under treatment conditions, the percentage of males ranged between 29.1% (at 34.7 µg Dexamethasone/L) and 50.0% (at 10.5 µg Dexamethasone/L). Correspondingly, the number of females remained at similar levels in controls and treatments.

All results, i.e. mean values and standard deviations, are shown in Table 29. For more details please refer to the Appendices B.2.6 to B.2.9.

Table 29: Zebrafish EOGRT with Dexamethasone: Biological results for first filial generation (F_1)

Parameter		Mean measured concentration Dexamethasone [µg/L]						
		Control	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Mean hatching rate [%] (± SD)		98.6 ± 2.8	93.1 ± 8.6	95.1 ± 4.7	99.3 ± 1.4	89.6 ± 12.5	92.4 ± 6.5	100± 0.0
Mean post hatch survival at day 21 pf [%] (± SD)		97.2 ± 4.8*	100 ± 0.0	100 ± 0.0	99.3 ± 1.4	99.3 ± 1.4	100 ± 0.0	98.6 ± 2.8
Mean post hatch survival at day 35 pf [%] (± SD)		94.4 ± 9.6*	95.3 ± 5.7	98.5 ± 1.8	97.2 ± 3.9	90.5 ± 11.3	100 ± 0.0	98.6 ± 2.8
Mean total length, day 35 pf [cm] (± SD)		1.76 ± 0.01*	1.80 ± 0.06	1.76 ± 0.04	1.73 ± 0.04	1.83 ± 0.12	1.74 ± 0.06	1.60 ± 0.07 [a])
Mean survival day 63 pf [%] (± SD)		100 ± 0.0*	100 ± 0.0	100 ± 0.0	98.8 ± 2.5	100 ± 0.0	100 ± 0.0	100 ± 0.0
Mean total length, day 63 pf [cm] (± SD)		3.05 ± 0.02*	3.04 ± 0.06	3.07 ± 0.04	3.01 ± 0.09	3.02 ± 0.09	2.91 ± 0.07 [b])	2.74 ± 0.05 [b])
Time to first spawning [fish age days] (± SD)	A	61	58	66	65	57	61	62
	B	-*	60	68	57	58	58	69
	C	58	59	61	58	57	58	61
	D	59	57	68	71	57	60	57
Mean egg number per day and female [n] (± SD)		21 ± 3*	18 ± 7	23 ± 3	23 ±4	23 ± 1	17 ± 5	20 ±4
Mean fertilisation rate [%] (± SD)		89.9 ± 2.5*	93.5 ± 3.1	90.9 ± 3.1	90.2 ± 5.6	92.1 ± 0.8	92.9 ± 2.0	90.0 ± 4.4
Mean survival, adult stage [%] (± SD)		100 ± 0.0*	100 ± 0.0	98.8 ± 2.5	98. 8± 2.5	97.5 ± 2.9	98.8 ± 2.5	96.3 ± 4.8

Parameter	Mean measured concentration Dexamethasone [µg/L]						
Mean total length, males, adult stage [cm] (± SD)	3.8 ± 0.1*	3.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.6 ± 0.1 [b])	3.5 ± 0.1 [b])
Mean total length, females, adult stage [cm] (± SD)	3.7 ± 0.1*	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.2	3.8 ± 0.0	3.7 ± 0.2	3.6 ± 0.1
Mean wet weight, males, adult stage [g] (± SD)	0.425 ± 0.017*	0.454 ± 0.040	0.432 ± 0.030	0.453 ± 0.031	0.445 ± 0.061	0.359 ± 0.009 [b])	0.296 ± 0.002 [b])
Mean wet weight, females, adult stage [g] (± SD)	0.528 ± 0.027*	0.573 ± 0.050	0.568 ± 0.036	0.534 ± 0.059	0.531 ± 0.005	0.459 ± 0.049 [b])	0.394 ± 0.041 [b])
Mean sex ratio, males [%] (± SD)	43.3 ± 7.6*	35.0 ± 8.2	29.3 ± 14.7	38.6 ± 23.0	50.0 ± 8.4	29.1 ± 15.5	30.3 ± 18.8
Mean sex ratio, females [%] (± SD)	56.7 ± 7.6*	65.0 ± 8.2	70.7 ± 14.7	61.4 ± 23.0	50.0 ± 8.4	70.9 ± 15.5	69.7 ± 18.8
Mean sex ratio, hermaphrodite [%] (± SD)	-	-	-	-	1.3 ± 2.5-	1.3 ± 2.5	-

SD = Standard deviation

* Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value drives from three replicates only.

a) statistically significant reduced compared to control, $p<0.05$, Jonckheere-Terpstra test; one-sided smaller

b) statistically significant reduced compared to control, $p<0.05$, Williams test, one-sided smaller

3.3.1.5 Biological results, second filial generation (F_2)

The mean hatching success in the controls was 100% and 100, 100, 97.5, 90.0, 90.0 and 97.5% in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (mean measured concentration), respectively. For more details please refer to the Appendix B.2.10.

Evaluation of hatching data revealed a significant reduction at 10.5 µg Dexamethasone /L and at 34.7 µg Dexamethasone/L (Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, one-sided-smaller, heterogenous variances). However, as the hatching rates achieved 90% and a concentration response is missing, a biological relevance of this observation can be excluded.

Table 30: Zebrafish EOGRT with Dexamethasone: Biological results for F_2 generation

	Mean measured concentration Dexamethasone [µg/L]						
Parameter	**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Mean hatching rate [%] (± SD)	100 ± 0.0*	100 ± 0.0	100 ± 0.0	97.5 ± 2.9	90.0 ± 7.1 [a])	90.0 ± 5.8 [a])	97.5 ± 2.9

SD = Standard deviation

* Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value drives from three replicates only.

a) Statistically significant reduction compared to control, Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, $p<0.05$, one-sided-smaller, heterogeneous variances.

3.3.1.6 Biomarker results

The mean Vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 31and Table 32.All single values are given in the Appendices B.2.11 (F_0) and B.2.12 (F_1).

In parental generation (F_0) the mean Vitellogenin (VTG) value in controls was determined to be 75.36 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 205.72, 394.89, 110.01, 144.64, 2631.22 and 469.51 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

The mean VTG value in controls was determined to be 4.89E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 3.65E+07, 4.07E+07, 4.52E+07, 5.19E+07, 6.28E+07 and 5.35E+07 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments neither for males nor for females of parental generation (F_0), respectively.

Table 31: Zebrafish EOGRT with Dexamethasone: Biomarker results for parental generation (F_0), Vitellogenin concentration in blood plasma [ng/mL]

	Mean measured concentration Dexamethasone [µg/L]						
Parameter	**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Parental generation (F_0)							
Mean VTG concentration, males [ng/mL] (± SD)	75.36 ± 18.48	205.72 ± 135.73	394.89 ± 315.32	110.01 ± 115.58	144.64 ± 115.83	2631.22 ± 4211.09	469.51 ± 231.23
Mean VTG concentration, females (ng/mL] (± SD)	4.89E+07 ± 2.77E+07	3.65E+07 ± 9.16E+06	4.07E+07 ± 1.35E+07	4.52E+07 ± 1.51E+07	5.19E+07 ± 1.45E+07	6.28E+07 ± 3.01E+07	5.35E+07 ± 1.81E+07

SD = Standard deviation

Remark: The data presented is characterised by high variations (+/- SD). This was mostly due to single measurements which can be interpreted as biological outliers. However, no explicit mathematical outlier test was conducted to exclude those values.

In first filial generation (F_1) the mean Vitellogenin (VTG) value in controls was determined to be 28.03 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 41.43, 455.70, 131.22, 48.97, 48.77 and 39.44 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean VTG value in controls was determined to be 2.76E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 1.11E+07, 7.87E+06, 1.33E+07, 1.88E+07, 7.01E+06 and 2.37E+06 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments neither for males nor for females of first filial generation (F_1), respectively.

Table 32: Zebrafish EOGRT with Dexamethasone: Biomarker results for first filial generation (F_1), Vitellogenin concentration in blood plasma [ng/mL]

	Mean measured concentration Dexamethasone [µg/L]						
Parameter	**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
First filial generation (F_1)							
Mean VTG concentration, males [ng/ml] (± SD)	28.03 ± 7.12*	41.43 ± 18.96	455.70 ±534.11	131.22 ± 107.34	48.97 ± 41.11	48.77 ± 59.27	39.44 ± 17.56
Mean VTG concentration, females (ng/ml] (± SD)	2.76E+07 ± 1.78E+07*	1.11E+07 ± 9.25E+06	7.87E+06 ± 5.60E+06	1.33E+07 ± 4.36E+06	1.88E+07 ± 2.92E+06	7.01E+06 ± 3.15E+06	2.37E+06 ± 1.88E+06

SD = Standard deviation

* Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value drives from three replicates only.

Remark: The data presented is characterised by high variations (+/- SD). This was mostly due to single measurements which can be interpreted as biological outliers. However, no explicit mathematical outlier test was conducted to exclude those values.

3.3.1.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010) Criteria for staging and grading are given in detail in 2.2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in B.2.13 for parental generation (F_0) and in B.2.14 for first filial generation (F_1). In 3.3.1.7.1 and in 3.3.1.7.2 the results are sorted according to the corresponding control or treatment level for parental generation (F_0) and first filial generation (F_1), respectively.

Summarizing the results, neither parental generation (F_0) nor first filial generation (F_1) showed any treatment-related differences in male or female gonad staging. The respective median values are presented in Table 33 and Table 34.

Table 33: Zebrafish EOGRT with Dexamethasone: Median maturation stages for F_0 generation

		Mean measured concentration Dexamethasone [µg/L]						
Parameter		**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Parental generation (F_0)								
Maturation stages, male		Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (16/20) Stage 3 (4/20)	Stage 2 (15/19) Stage 3 (4/19)	Stage 2 (18/20) Stage 3 (2/20)	Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (14/19) Stage 3 (5/19)
Maturation stage, male, median value/ replicate	A	2	2	2	2	2	2	2
	B	2	2	2	3	2	2	2
	C	2	2	2	2	2	2	3
	D	2	2	2	2	2	2	2
Maturation stages, female		Stage 2 (6/20) Stage 3 (13/20) Stage 4 (1/20)	Stage 2 (5/20) Stage 3 (15/20)	Stage 2 (4/20) Stage 3 (16/20)	Stage 2 (3/21) Stage 3 (17/21) Stage 4 (1/21)	Stage 2 (2/20) Stage 3 (17/20) Stage 4 (1/20)	Stage 1 (1/19) Stage 2 (4/19) Stage 3 (14/19)	Stage 2 (8/20) Stage 3 (12/20)
Maturation stage, female, median value/ replicate	A	3	3	3	3	3	3	3
	B	3	3	3	3	3	3	3
	C	3	2	3	3	3	3	2
	D	3	3	3	3	3	2	2

1) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory;

2) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 5 = spent

Table 34: Zebrafish EOGRT with Dexamethasone: Median maturation stages for F_1 generation

		Mean measured concentration Dexamethasone [µg/L]						
Parameter		**Control**	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
First filial generation (F_1)								
Maturation stages, male		Stage 2 (26/26)	Stage 1 (1/28) Stage 2 (25/28) Stage 3 (2/28)	Stage 2 (22/23) Stage 3 (1/23)	Stage 1 (1/30) Stage 2 (28/30) Stage 3 (1/30)	Stage 1 (2/38) Stage 2 (35/38) Stage 3 (1/38)	Stage 1 (2/22) Stage 2 (20/22)	Stage 1 (1/23) Stage 2 (22/23)
Maturation stage, male, median value/ replicate	A	2	2	2	2	2	2	2
	B	-	2	2	2	2	2	2
	C	2	2	2	2	2	2	2
	D	2	2	2	2	2	2	2
Maturation stages, female		Stage 2 (25/34) Stage 3 (9/34)	Stage 2 (38/52) Stage 3 (14/52)	Stage 1 (2/56) Stage 2 (41/56) Stage 3 (13/56)	Stage 2 (34/48) Stage 3 (14/48)	Stage 2 (26/39) Stage 3 (13/39)	Stage 2 (35/46) Stage 3 (21/46)	Stage 2 (31/54) Stage 3 (23/54)
Maturation stage, female, median value/ replicate	A	2	2	2	2	2	2	2
	B	-	3	2	2	2	2	2
	C	2	2	2	2	2	3	2
	D	2	2	2	2	2	2	2

1) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late developmet/hydrated; stage 5 = post ovulatory;
2) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 5 = spent

Furthermore, neither parental generation (F_0) nor first filial generation (F_1) showed any treatment-related differences in the occurrence of the investigated lesions. The median values are presented in Table 35 and Table 36.

Notably, increased testicular degeneration, interstitial cell hypertrophy/ hyperplasia, interstitial proteinaceous fluid and asynchronous germ cell development were not found to be present in any male fish of parental generation (F_0). Testis-ova was diagnosed in one male fish (1/1-8), another single animal (6/4-5) displayed granulomatous inflammation. Only the occurrence of increased proportion of spermatogonia was a little more often and thus presented in this report along with the female fish findings of parental generation (F_0).

A very similar pattern was observed for first filial generation (F_1). Increased testicular degeneration, interstitial cell hypertrophy/ hyperplasia, interstitial proteinaceous fluid and asynchronous germ cell development were not found to be present in any male fish of first filial generation (F_1). Increased proportion of spermatogonia was diagnosed in one male fish of the control (0/4-13), another single animal (2/4-10) displayed granulomatous inflammation. Only

the occurrence of testis-ova was a little more often and thus presented in this report along with the female fish findings of first filial generation (F_1).

Table 35: Zebrafish EOGRT with Dexamethasone: Grading of lesions severity for F_0

Parameter		Mean measured concentration Dexamethasone [µg/L]						
		Control	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
		Parental generation (F_0)						
Increased proportion of spermatogonia, males, findings 1)		-	Grade 2 (1/20)	Grade 1 (1/20)	-	Grade 1 (1/20)	-	Grade 1 (3/20)
Increased proportion of spermato-gonia, males, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Increased oocyte atresia, females, findings 1)		Grade 1 (8/20) Grade 2 (6/20)	Grade 1 (5/20) Grade 2 (7/20) Grade 3 (2/20) Grade 4 (1/20)	Grade 1 (7/20) Grade 2 (2/20) Grade 3 (2/20) Grade 4 (1/20)	Grade 1 (7/21) Grade 2 (3/21) Grade 3 (1/21)	Grade 1 (4/20) Grade 2 (1/20) Grade 3 (1/20)	Grade 1 (4/19)	Grade 1 (4/20) Grade 2 (3/20)
Increased oocyte atresia, females, median/ replicate	A	1.0	0.0	1.0	1.0	0.0	0.0	0.0
	B	1.0	2.0	1.0	1.0	0.0	0.0	0.0
	C	2.0	1.0	0.0	0.0	0.0	0.0	1.0
	D	1.0	1.0	1.0	1.0	0.0	0.0	0.0
Egg debris, females, findings 1)		Grade 1 (7/20) Grade 2 (3/20)	Grade 1 (5/20) Grade 2 (5/20) Grade 3 (3/20)	Grade 1 (4/20) Grade 2 (7/20) Grade 3 (1/20) Grade 4 (1/20)	Grade 1 (8/21) Grade 2 (4/21) Grade 3 (1/21)	Grade 1 (3/20) Grade 2 (2/20) Grade 3 (1/20)	Grade 1 (5/19) Grade 2 (5/19) Grade 3 (1/19)	Grade 1 (2/20) Grade 2 (3/20)
Egg debris, females, median/ replicate	A	0.0	0.0	2.0	1.0	0.0	0.5	0.0
	B	1.0	1.0	1.0	1.5	0.0	1.0	0.0
	C	0.0	1.0	0.0	0.0	0.0	0.0	0.0
	D	1.0	2.0	1.0	1.0	0.0	2.0	0.0

Parameter		Mean measured concentration Dexamethasone [µg/L]						
Granulomatous inflammation, females, Findings 1)		Grade 1 (8/20) Grade 2 (1/20)	Grade 1 (3/20) Grade 2 (7/20) Grade 3 (5/20)	Grade 1 (9/20) Grade 2 (2/20) Grade 3 (1/20)	Grade 1 (4/21) Grade 2 (4/21) Grade 4 (1/21)	Grade 1 (8/20) Grade 2 (3/20) Grade 3 (3/20)	Grade 1 (4/19) Grade 2 (4/19)	Grade 1 (3/20)
Granuloma-tous inflammation, females, median/ replicate	A	0.0	0.0	1.0	0.0	1.0	1.5	0.0
	B	1.0	3.0	1.0	2.0	2.0	0.0	0.0
	C	1.0	2.0	0.0	0.0	1.0	0.0	0.0
	D	1.0	2.0	1.0	1.0	1.0	1.0	0.0
Increased post-ovulatory follicles, female, findings 1)		Grade 1 (5/20) Grade 2 (6/20) Grade 3 (2/20)	Grade 1 (4/20) Grade 2 (2/20) Grade 3 (1/20)	Grade 1 (5/20) Grade 2 (2/20)	Grade 1 (2/21) Grade 2 (6/21)	Grade 1 (3/20) Grade 2 (4/20)	Grade 1 (3/19) Grade 2 (3/19)	Grade 1 (4/20) Grade 2 (4/20)
Increased post-ovulatory follicles, female, median/ replicate	A	0.0	0.0	1.0	1.0	0.0	0.0	0.0
	B	1.0	0.0	1.0	0.5	0.0	0.0	0.0
	C	2.0	1.0	0.0	0.0	1.0	0.0	0.0
	D	2.0	0.0	0.0	0.0	0.0	0.0	0.0

Criteria for grading lesions: grade 0 = not observable; grade 1 = minimal; grade 2 = mild; grade 3 = moderate; grade 4 = severe

1) Only findings with a severity grades of 1 and higher are presented.

Table 36: Zebrafish EOGRT with Dexamethasone: Grading of lesions severity for F_1

Parameter		Mean measured concentration Dexamethasone [µg/L]						
		Control	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
		First filial generation (F_1)						
Testis-ova, males, findings 1)		Grade 1 (1/26)	Grade 1 (3/28)	Grade 1 (1/23)	Grade 4 (1/30)	-	-	-
Testis-ova, males, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	-	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Increased oocyte atresia, females, findings 1)		Grade 1 (3/34)	Grade 1 (7/52) Grade 2 (1/52)	Grade 1 (2/56) Grade 2 (1/56)	Grade 1 (2/48)	Grade 1 (4/39)	Grade 1 (7/56)	Grade 1 (3/54)
Increased oocyte atresia, females, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	-	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Egg debris, females, findings 1)		Grade 1 (6/34) Grade 2 (1/34)	Grade 1 (8/52)	Grade 1 (4/56) Grade 2 (2/56)	Grade 1 (6/48) Grade 2 (1/48)	Grade 1 (2/39) Grade 2 (1/39)	Grade 1 (9/56) Grade 2 (2/56) Grade 3 (1/56)	Grade 1 (6/54) Grade 2 (3/54) Grade 3 (2/54)
Egg debris, females, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	-	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Granumatous inflammation, females, findings 1)		Grade 1 (2/34) Grade 2 (1/34)	Grade 1 (9/52)	Grade 1 (8/56)	Grade 1 (5/48) Grade 2 (1/48) Grade 3 (1/48)	Grade 1 (4/39) Grade 2 (3/39)	Grade 1 (12/56)	Grade 1 (10/54) Grade 2 (1/54)
Granuloma-tous inflammation, females, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	-	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Parameter		Mean measured concentration Dexamethasone [µg/L]						
Increased post-ovulatory follicles, female, findings		Grade 1 (3/34)	Grade 1 (8/52)	Grade 1 (4/56) Grade 2 (3/56)	Grade 1 (4/48) Grade 3 (2/48)	Grade 2 (1/39)	Grade 1 (6/56) Grade 2 (1/56)	Grade 1 (2/54) Grade 2 (1/54)
Increased post-ovulatory follicles, female, median/ replicate	A	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	B	-	0.0	0.0	0.0	0.0	0.0	0.0
	C	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Criteria for grading lesions: grade 0 = not observable; grade 1 = minimal; grade 2 = mild; grade 3 = moderate; grade 4 = severe

1) Only findings with a severity grades of 1 and higher are presented.

3.3.1.7.1 Histopathology results, parental generation (F_0)

Control – group 0

Six female fish (6/20) had the ovary stage 2, thirteen females (13/20) the ovary stage 3 and one female (1/20) the ovary stage 4.

Fourteen female fish showed increased oocyte atresia (eight females with grade 1 and six females with grade 2), ten females egg debris (seven females grade 1 and three females grade 2), nine females a granulomatous inflammation (eight females grade 1 and one female grade 2) and thirteen females increased post-ovulatory follicles (five females with grade 1, six females with grade 2 and two females with grade 3).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

0.33 µg Dexamethasone/L (mean measured) – group 1

Five female fish (5/20) had the ovary stage 2 and fifteen females (15/20) the ovary stage 3.

Fifteen female fish showed increased oocyte atresia (five females grade 1, seven females grade 2, two females grade 3 and one female grade 4), thirteen females egg debris (five females grade 1, five females grade 2 and three females grade 3), fifteen females a granulomatous inflammation (three females grade 1, seven females grade 2 and five females grade 3) and seven females increased post-ovulatory follicles (four females grade 1, two females grade 2 and one female grade 3).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

One male fish showed a testis-ova (grade 1) and one male fish showed increased proportion of spermatogonia (grade 2). Additional diagnoses were not observed in the male fish of this group.

0.91 µg Dexamethasone/L (mean measured) – group 2

Four female fish (4/20) had the ovary stage 2 and sixteen females (16/20) the ovary stage 3.

Twelve female fish showed increased oocyte atresia (seven females grade 1, two females grade 2, two females grade 3 and one female grade 4), thirteen females egg debris (four females grade 1, seven females grade 2, one female grade 3 and one female grade 4), twelve females a granulomatous inflammation (nine females grade 1, two females grade 2 and one female grade 3) and seven females increased postovulatory follicles (five females grade 1 and two females grade 2).

Sixteen male fish (16/20) were diagnosed with the testis stage 2 and four males (4/20) with the stage 3.

One male fish showed increased proportion of spermatogonia (grade 1). Additional diagnoses were not observed in the male fish of this group.

3.19 µg Dexamethasone/L (mean measured) – group 3

Three female fish (3/21) had the ovary stage 2, seventeen females (17/21) the ovary stage 3 and one female (1/21) the ovary stage 4.

Eleven female fish showed increased oocyte atresia (seven females grade 1, three females grade 2 and one female grade 3), thirteen females egg debris (eight females grade 1, four females grade 2 and one female grade 3), nine females a granulomatous inflammation (four females grade 1,

four females grade 2 and one female grade 4) and eight females increased post-ovulatory follicles (two females grade 1 and six females grade 2).

Fifteen male fish (15/19) were diagnosed with the testis stage 2 and four males (4/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

10.5 µg Dexamethasone/L (mean measured) – group 4

Two female fish (2/20) had the ovary stage 2, seventeen females (17/20) the ovary stage 3 and one female (1/20) the ovary stage 4.

Six female fish showed increased oocyte atresia (four females grade 1, one female grade 2 and one female grade 3), six females egg debris (three females grade 1, two females grade 2 and one female grade 3), fourteen females a granulomatous inflammation (eight females grade 1, three females grade 2 and three females grade 3) and seven females increased post-ovulatory follicles (three females grade 1 and four females grade 2).

Eighteen male fish (18/20) were diagnosed with the testis stage 2 and two males (2/20) with the stage 3.

One male fish showed increased proportion of spermatogonia (grade 1). Additional diagnoses were not observed in the male fish of this group.

34.7 µg Dexamethasone/L (mean measured) – group 5

One female fish (1/19) had the ovary stage 1, four female fish (4/19) had the ovary stage 2 and fourteen females (14/19) the ovary stage 3.

Four female fish showed increased oocyte atresia (all grade 1), eleven females egg debris (five females grade 1, five females grade 2 and one female grade 3), eight females a granulomatous inflammation (four females grade 1 and four females grade 2) and six females increased post-ovulatory follicles (three females grade 1 and three females grade 2).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

100 µg Dexamethasone/L (mean measured) – group 6

Eight female fish (8/20) had the ovary stage 2 and twelve females (12/20) the ovary stage 3. Seven female fish showed increased oocyte atresia (four females grade 1 and three females grade 2), five females egg debris (two females grade 1 and three females grade 2), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (two females grade 1 and two females grade 2).

Fourteen male fish (14/19) were diagnosed with the testis stage 2 and five males (5/19) with the stage 3.

Three male fish showed increased proportion of spermatogonia (grade 1) and one male a granulomatous inflammation (grade 1)

3.3.1.7.2 Histopathology results, first filial generation (F_1)

Control – group 0

Twenty-five female fish (25/34) had the ovary stage 2 and nine females (9/34) the ovary stage 3.

Three female fish showed increased oocyte atresia (all grade 1), seven females egg debris (six females grade 1 and one female grade 2), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (all grade 1).

All twenty-six male fish (26/26) were diagnosed with the testis stage 2.

One male fish showed a testis-ova (grade 1) and one male fish an increased proportion of spermatogonia (grade 1).

0.33 µg Dexamethasone/L (mean measured) – group 1

Thirty-eight female fish (38/52) had the ovary stage 2 and fourteen females (14/52) the ovary stage 3.

Eight female fish showed increased oocyte atresia (seven females grade 1 and one female grade 2), eight females egg debris (all grade 1), nine females a grade 1 granulomatous inflammation and eight females increased post-ovulatory follicles (all grade 1).

One male fish (1/28) was diagnosed with the testis stage 1, twenty-five males (25/28) with the stage 2 and two males (2/28) with the stage 3.

Three male fish showed a testis-ova (all grade 1). Additional diagnoses were not observed in the male fish of this group.

0.91 µg Dexamethasone/L (mean measured) – group 2

Two female fish (2/56) had the ovary stage 1, forty-one females (41/56) the ovary stage 2 and thirteen females (13/56) the ovary stage 3.

Three female fish showed increased oocyte atresia (two females grade 1 and one female grade 2), six females egg debris (four females grade 1 and two females grade 2), eight females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (four females grade 1 and three females grade 2).

Twenty-two male fish (22/23) were diagnosed with the testis stage 2 and one male (1/23) with the stage 3.

One male fish showed a testis-ova (grade 1). One male had a granulomatous inflammation (grade 1).

3.19 µg Dexamethasone/L (mean measured) – group 3

Thirty-four female fish (34/48) had the ovary stage 2 and fourteen females (14/48) the ovary stage 3.

Two female fish showed increased oocyte atresia (all grade 1), seven females egg debris (six females grade 1 and one female grade 2), seven females a granulomatous inflammation (five females grade 1, one female grade 2 and one female grade 3) and six females increased post-ovulatory follicles (four females grade 1 and two females grade 3).

One male fish (1/30) was diagnosed with the testis stage 1, twenty-eight males (28/30) with the testis stage 2 and one male (1/30) with the stage 3.

One male fish showed a testis-ova (grade 4). Additional diagnoses were not observed in the male fish of this group.

10.5 µg Dexamethasone/L (mean measured) – group 4

Twenty-six female fish (26/39) had the ovary stage 2 and thirteen females (13/39) the ovary stage 3.

Four female fish showed increased oocyte atresia (all grade 1), three females egg debris (two females grade 1 and one female grade 2), seven females a granulomatous inflammation (four females grade 1 and three female grade 2) and one female increased post-ovulatory follicles (grade 2).

One male fish of this group had also an ovary stage 1 ovary separated from the testes. Therefore, this animal was diagnosed as hermaphrodite. Two male fish (2/38) was diagnosed with the testis stage 1, thirty-five males (35/38) with the testis stage 2 and one male (1/38) with the stage 3.

The fish diagnosed as hermaphrodite showed a testis-ova (grade 1). Additional diagnoses were not observed in the male fish of this group.

34.7 µg Dexamethasone/L (mean measured) – group 5

Thirty-five female fish (35/56) had the ovary stage 2 and twenty-one females (21/56) the ovary stage 3.

Seven female fish showed increased oocyte atresia (all grade 1), Twelve females egg debris (nine females grade 1, two females grade 2 and one female grade 3), twelve females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (six females grade 1 and one females grade 2).

One male fish of this group had also an ovary stage 1 ovary separated from the testes. Therefore, this animal was diagnosed as hermaphrodite. Two male fish (2/22) was diagnosed with the testis stage 1 and twenty-one (21/22) with the testis stage 2.

Additional diagnoses were not observed in the male fish of this group.

100 µg Dexamethasone/L (mean measured) – group 6

Thirty-one female fish (31/54) had the ovary stage 2 and twenty-three females (23/54) the ovary stage 3.

Three female fish showed increased oocyte atresia (all grade 1), eleven females egg debris (six females grade 1, three females grade 2 and two females grade 3), eleven females a granulomatous inflammation (ten females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (two females grade 1 and one females grade 2).

One male fish (1/23) was diagnosed with the testis stage 1 and twenty-two (22/23) with the testis stage 2.

Additional diagnoses were not observed in the male fish of this group.

3.3.1.8 Summary and Conclusion

The presented ZEOGRT study aimed to assess the effects of continuous exposure to Dexamethasone on different life stages and life performances of zebrafish (*Danio rerio*) during a multi generation approach. The measured water parameters and the results of the regular chemical analysis confirmed stable test conditions throughout the study.

In the **parental generation (F_0)**, no effects on reproduction in terms of fecundity and fertility were detected. The determination of the phenotypic sex confirmed the correct setting of spawning groups for most of the test vessels. Effects related to test substance exposure were mainly found for the growth parameters. Male wet weight was significantly reduced compared to control starting at a concentration of 34.7 µg Dexamethasone/L (mean measured concentration). Thus, the NOEC was set at 10.5 µg Dexamethasone/L. Female wet weight as well as total length were also reduced in the presence of Dexamethasone, but only at the highest treatment level of 100 µg Dexamethasone/L (mean measured concentration). Accordingly, the NOEC was set as 34.7 µg Dexamethasone/L (mean measured concentration). Vitellogenin concentration in male and female blood plasma and the histopathological pattern of the examined gonads of both males and females were not affected by the test substance.

Also, in the **first filial generation (F_1)**, retarded growth of the exposed fish was observed and thus consistent with the findings linked to F_0. Notably, the reduction of growth in terms of total length was evident during development from larvae to adult male fish. More precisely, at day 35 pf, the total length was significantly reduced compared to control only at the highest treatment level, whereas measurement at day 63 pf as well as male total length at test end revealed a significant reduction starting even at 34.7 µg Dexamethasone/L (mean measured concentration). However, female total length at test end were not affected by Dexamethasone exposure, but male and female wet weights at test end were significantly reduced starting at 34.7 µg Dexamethasone/L (mean measured concentration).

There were no effects on the reproduction capability of the F_1. Although the growth performance was impacted, there was no delay observed with regard to the date of spawning start, i.e. day of first spawning. The reproduction parameters fecundity and fertility were not negatively impacted due to test substance exposure. Other apical endpoints like survival or sex ratio as well as Vitellogenin concentration in blood plasma and the histopathological pattern of the examined gonads were not affected by the test item.

Hatching success of the **second filial generation (F_2)** was found to be significantly reduced at 10.5 and 34.7 µg Dexamethasone/L (mean measured concentration). However, as the hatching rates achieved 90% and a concentration-response is missing, a biological relevance of this observation can be excluded.

Based on the endpoint growth in terms of wet weight and total length the NOEC was determined to be 10.5 µg Dexamethasone/L. The LOEC was set at 34.7 µg Dexamethasone/L (mean measured concentration).

The identified effects linked to the growth performance were in line with recent literature findings reporting that glucocorticoid receptor mediated cortisol signalling in fish can be associated with growth-suppressing effects on muscle tissue (Sadoul and Vijayan 2016). Furthermore, Dexamethasone-induced muscle atrophy in zebrafish had been suggested recently (Faught and Vijayan 2019, Ryu, Je et al. 2021). As muscle contributes to over 50% of adult fish weight, changes in this organ system have a direct impact on the parameter growth.

All results of the ZEOGRT with Dexamethasone are shown in Table 37.

Table 37: Zebrafish EOGRT with Dexamethasone: Summary of NOEC / LOEC determination during the course of the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dexamethasone [µg/L]	NOEC / LOEC Mean measured concentration Dexamethasone [µg/L]
F_0-Growth	**Wet weight males***	**10.0 / 32.0**	**10.5 / 34.7**
	Wet weight females**	**32.0 / 100**	**34.7 / 100**
	Total length males	≥ 100 / >100	≥ 100 / >100
	Total length females**	**32.0 / 100**	**34.7 / 100**
	Sex ratio (% males)	≥ 100 / >100	≥ 100 / >100
	Sex ratio (% females)	≥ 100 / >100	≥ 100 / >100
F_0-Reproduction [1])	Fecundity (Egg number per day and female)	≥ 100 / >100	≥ 100 / >100
	Fertilisation rate	≥ 100 / >100	≥ 100 / >100
F_0-Histopathology	Maturity stage ovary	≥ 100 / >100	≥ 100 / >100
	Maturity stage testis	≥ 100 / >100	≥ 100 / >100
	Histopathology (females)	≥ 100 / >100	≥ 100 / >100
	Histopathology (males)	≥ 100 / >100	≥ 100 / >100
F_0-Biomarker	Vitellogenin females	≥ 100 / >100	≥ 100 / >100
	Vitellogenin males	≥ 100 / >100	≥ 100 / >100
F_1-ELS	Hatch day 5	≥ 100 / >100	≥ 100 / >100
	Survival day 21	≥ 100 / >100	≥ 100 / >100
	Survival day 35	≥ 100 / >100	≥ 100 / >100
	Total length day 35*	**32.0 / 100**	**34.7 / 100**
F_1-Juveniles	Survival day 35 – day 63	≥ 100 / >100	≥ 100 / >100
	Total length day 63**	**10.0 / 32.0**	**10.5 / 34.7**
F_1-Reproduction	Time to first spawning	≥ 100 / >100	≥ 100 / >100
	Fecundity (Egg number per day and female)	≥ 100 / >100	≥ 100 / >100
	Fertilisation rate	≥ 100 / >100	≥ 100 / >100

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dexamethasone [µg/L]	NOEC / LOEC Mean measured concentration Dexamethasone [µg/L]
F_1-Termination	Survival (test end)	≥ 100 / >100	≥ 100 / >100
	Total length males**	**32.0 / 100**	**34.7 / 100**
	Total length females	≥ 100 / >100	≥ 100 / >100
	Wet weight males**	**32.0 / 100**	**34.7 / 100**
	Wet weight females**	**32.0 / 100**	**34.7 / 100**
	Sex ratio (% females)	≥ 100 / >100	≥ 100 / >100
	Sex ratio (% males)	≥ 100 / >100	≥ 100 / >100
F_1-Biomarker	Vitellogenin females	≥ 100 / >100	≥ 100 / >100
	Vitellogenin males	≥ 100 / >100	≥ 100 / >100
F_1-Histopathology	Maturity stage ovary	≥ 100 / >100	≥ 100 / >100
	Maturity stage testis	≥ 100 / >100	≥ 100 / >100
	Histopathology (females)	≥ 100 / >100	≥ 100 / >100
	Histopathology (males)	≥ 100 / >100	≥ 100 / >100
F_2-Embryo	Hatch day 4***	≥ 100 / >100 [2)]	≥ 100 / >100 [2)]

* Step down Jonckheere-Terpstra test, p<0.05, one-sided smaller

** Williams test, p<0.05, one-sided smaller

*** Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, p<0.05, one-sided-smaller.

[1)] The statistical evaluation revealed a significant reduction of egg numbers at 0.33 and 0.91 µg Dexamethasone/L, and for fertilisation rate at 10.5 and 34.7 µg Dexamethasone/L. However, a concentration related impact on the reproductive capability of the fish could not be observed. Thus, the biological relevance of these observations was considered as minor.

[2)] Hatching success was found to be significantly reduced at 10.5 and 34.7 µg Dexamethasone/L (mean measured concentration). However, the hatching rate at the top concentration was not significantly different from control. Thus, a biological relevance of this observation can be excluded.

4 Conclusion and Discussion

The aim of the project was to develop an adapted strategy for assessing the environmental risk of novel hormonal active substances to aquatic organisms, in particular synthetic progestins and glucocorticoids. The following chapters will discuss the overall findings of the studies conducted. In a second step, procedural details of the ZEOGRT design will be discussed aiming to identify a suitable test strategy.

4.1 Progestin: Studies with Dienogest

The 19-norprogestins represent a group of progestins which are used as contraceptives often in combination e.g. with Ethinylestradiol. Examples for the Norprogestins are Levonorgestrel, Gestodene or Desogestrel. Dienogest is the first non-alkylated 19-norprogestin that was placed on the market for contraceptives (Oettel, Breitbarth et al. 1999). Due to a substitution at the 17alpha position, it was reported to have a good bioavailability. Moreover, it was shown in clinical studies that Dienogest has clearly anti-androgenic properties and thus differs from other progestins of that group.

For synthetic progestins there are several examples showing similar effect patterns on fish like other potent endocrine acting substances like estrogen receptor agonists (e.g. Ethinylestradiol) or androgen receptor agonists like Trenbolone, and progestins were found to be effective also at very low concentration levels. However, the effects observed may differ with regard to their mode of action. According to Sitruk-Ware and Nath (Sitruk-Ware and Nath 2010), there are progestins representing partly estrogenic and androgenic action properties (e.g. Norethisterone), partly androgenic (e.g. Levonorgestrel, Gestodene, Desogestrel). In this classification, Dienogest is listed as anti-androgenic acting substance.

In recent years, the amount of available data on potential endocrine related effects of progestins to fish increased slowly. However, published data was mostly linked to well described molecules like the natural progestin progesterone and very prominently Levonorgestrel. Runnals et al. (Runnalls, Beresford et al. 2013) examined the impact of different synthetic progestins to fish considering different potencies. The authors reported that levonorgestrel and gestodene were able to stop spawning of fathead minnows at 100 ng/L, while no effect on reproduction was detected for Desogestrel or Drospirenone at the same concentration. In a second experiment with two progestins it was shown that Gestodene impacted reproduction already at 1 ng/L, while concentration of 1 µg Desogestrel had to be applied to induce an effect on reproduction. Masculinization of females and results from in vitro assays were linked to a clear androgenic mode of action (Runnalls, Beresford et al. 2013).

Effects on sex ratio were reported by Liang et al. (Liang, Huang et al. 2015) describing a shift towards males in exposed zebrafish groups at low concentrations (ng/L) of Norgestrel while exposure of zebrafish to Progesterone was linked to an increased proportion of female fish. Teigeler et al. (Teigeler, Schaudien et al. 2021) reported a shift towards full male groups of zebrafish exposed to very low concentrations of Levonorgestrel. Androgenic effects of levonorgestrel were also published for other fish species, i.e. three spined stickleback. After exposure females expressed androgenic effects i.e. induction of spiggin transcription and reduction of Vitellogenin transcription (Svensson, Fick et al. 2013).

In contrast, the database for effects of Dienogest on fish (and other aquatic organisms) is still very poor. Thus, the results presented in this study can close data gaps.

The relevant effects derived from the ZEOGRT linked to Dienogest exposure were represented by decreased survival of early life stages (F_1-generation) and a reduced hatching success of F_2

generation, while F_2 hatch was clearly more sensitive. Also, the fertilisation success of the adult spawning groups (F_1) was significantly reduced while the fecundity was not affected. It can be assumed that the observed effect was the result of an affected male performance, resulting in inefficient fertilisation capacity. However, based on the histopathological evaluation, no effects on male gonadal tissue were found.

The Vitellogenin measurements did not reveal an effect linked to Dienogest exposure, also, no effect on gonadal tissue was observed, e.g. on the maturation stages of both males and females. Thus, from the physiological data recorded, no information on the underlying mechanism can be derived. Further sensitive apical endpoints like e.g. sex ratio were not impacted in the ZEOGRT. The observed impact on fertility can be presumably linked to the reported anti-androgenic effect.

Unfortunately, studies with other animal species could not improve the data base to be used for the investigation of endocrine mediated effects.

The Chironomid Life Cycle conducted showed no effects in the test concentration range applied. The concentrations were applied via spiked sediment; however, the water concentrations were measured to be higher than the water concentrations in the fish study. No effects were observed for the measurement water concentrations ranging between 0.02 and 1.15 mg/L (min-max range of the available results from measurement of overlaying water).

Additional data is available for molluscs. A reproduction test with the prosobranch snail *Potamopyrgus antipodarum* was conducted at nominal concentrations of 3.2, 10, 32, 100 and 320 ng Dienogest/L. Thus, it was possible to directly compare the observed effects between the aquatic snails and the data derived from the ZEOGRT. The snails were exposed over 28 days under semi-static conditions. The study was conducted in accordance with the OECD guideline 242 (OECD 2016). Reproduction was assessed based on the number of snail embryos prepared from brood pouches of the adult female snails at the end of the study. No effect on reproduction in terms of the number of embryos in the applied concentration range was detected. Also, no effect on growth and survival was detected (Brüggemann 2023).

Finally, only the exposed zebrafish expressed sufficient sensitivity to derive an information of a potential endocrine impact of the substance.

4.2 Glucocorticoides: Studies with Dexamethasone

There are various studies describing possible impact of glucocorticoid exposure to aquatic organisms including fish. However, as in most cases physiological effects are reported, the information on mainly apical endpoints is limited. Notably, many studies are linked to impact on the immune system.

Kugathas et al. (Kugathas, Runnalls et al. 2013) published results from a short-term exposure of fathead minnows to different concentrations of beclomethasone dipropionate. The authors detected an increase of plasma glucose concentration and a decrease of blood lymphocytes. For female fish, an induction of male secondary sex characteristics was observed and moreover a decrease of plasma Vitellogenin (Kugathas, Runnalls et al. 2013).

LaLone et al. (LaLone, Villeneuve et al. 2012) exposed fathead minnow during reproduction and a subsequent early life stage phase to three different concentrations of Dexamethasone, i.e. at 0.1, 50 and 500 µg/L. The authors found a statistically significant reduction of egg numbers at 500 µg/L and reduced estradiol concentrations in female plasma. Abnormally developed fry were observed at the same concentration level. Moreover, plasma Vitellogenin concentrations were reduced. Fry exposed to 500 µg/L expressed a reduced growth performance.

Overturf et al. (Overturf, Overturf et al. 2012) reported a significant decrease of survival after 28 days exposure of fathead minnows to Dexamethasone, i.e. at 577 µg/L.

In the ZEOGRT study with Dexamethasone conducted in the course of this project, effects on growth were observed for all life stages applied, i.e. the parental F_0 generation, but also for the early, juvenile and adult stages of F_1 generation. The hatch of F_2 was impacted, but did not show a clear concentration effect response. Interestingly, a decrease of both length and weight was already observed for the parental fish. Based on experience from other ZEOGRT studies from the validation project and multigeneration studies conducted at the test facility, a growth effect on fully mature fish is usually not expected. Effects on growth were also observed in the study of LaLone et al. (LaLone, Villeneuve et al. 2012), but for fish fry during the early life stage (≤ 29 days of age). Notably, for zebrafish the growth effect was detected already at 34.7 µg Dexamethasone/L for juveniles and adult fishes in the ZEOGRT. Further impacts on F_0 and F_1 generation, e.g. reduction of reproductive performance, sex ratio or the plasma Vitellogenin concentration were not recorded in the ZEOGRT.

Also, for Dexamethasone, additional data is available for molluscs. A reproduction test with *Potamopyrgus antipodarum* was conducted at nominal concentrations of 1.0, 3.2, 10, 32, and 100 µg Dexamethasone/L, i.e. in the same concentration range as the fish study. Following OECD TG 242, the snails were exposed over 28 days under semi-static conditions. Reproduction was assessed based on the number of snail embryos prepared from brood pouches of the adult female snails at the end of the study. No effect on reproduction in terms of the number of embryos in the applied concentration range was detected. However, the comparison of embryo numbers with and without eggshell revealed a significant decrease of embryo numbers in terms of shelled embryos in all treatment levels (NOEC of < 1.06 µg/L). For unshelled embryos, no significant difference between embryo numbers compared to the control was detected; no effect on growth and survival was detected (Brüggemann 2023). Bal et al. (Bal, Kumar et al. 2016) published a 14-days study with the freshwater snail *Physa acuta*. Egg clutches derived from cultured snails were exposed to different concentrations of prednisolone ranging from 15.6 to 1000 µg/L. At concentrations from 125 to 1000 µg/L, significant decline of growth, survival and furthermore heart rate was found. Beside these effects, a significant impact on egg shell development was described and related to lower calcium concentrations in the shells.

Further information on the toxicity of glucocorticoids and especially Dexamethasone to aquatic organisms is limited in the scientific literature. DellaGreca et al. (DellaGreca, Fiorentino et al. 2004) reported on acute and chronic toxicity of the pharmaceutical to algae and invertebrates. The results showed a higher sensitive of a crustacea (*C. dubia*) compared to an algae species with regard to the chronic toxicity. However, the examination of acute toxicity to two other crustaceans and a rotifer revealed effects concentration >10 mg/L and thus considered as low toxic to these non-vertebrate organisms (DellaGreca, Fiorentino et al. 2004). Also, Bal et al. (Bal, Kumar et al. 2017) reported on effects of Dexamethasone on *C. dubia* in a multigeneration setup. An acute effect concentration (48h) was detected at 0.75 mg Dexamethasone/L, while effects showing a population decline were recorded at 48 µg/L (F_0 generation) down to 2.2 µg Dexamethasone/L for F_3 generation.

There are several studies describing the immunosuppressive properties and related effects (reviewed e.g. by Hidasi et al. (Hidasi, Groh et al. 2017)) on fish populations. The exposure to glucocorticoids is assumed to cause secondary effects like reduced growth performance or increased mortality of sensitive life stages. New methodical approaches include a so-called immune challenge aiming to detect a potential immunosuppression as a result of glucocorticoid exposure. Herbomel et al. (Herbomel, Thisse et al. 1999) described a method using zebrafish embryos. By applying bacteria-based lipopolysaccharides (LPS) to these embryos, an

inflammatory reaction was induced. As a reaction, different mediators like interleukins, prostaglandins are released. As for zebrafish the immune system is already developed at this early stage, this procedure can be included in the protocol of a fish embryo test based on OECD guideline 236 (OECD 2013). If LPS is now applied in high concentrations, there is an overproduction of the mediators in the organisms and thus an increased mortality of the fish embryos. The simultaneous administration of glucocorticoids can suppress this immune reaction and thus increase the survival rate of the embryos. Initial studies indicate that the increase in the survival rate after the administration of a glucocorticoid is directly concentration-dependent (Hidasi, Groh et al. 2017).

In a more recent publication, Essfeld et al. (Essfeld, Reinwald et al. 2022) described the change of gene-expression profiles after challenging zebrafish embryos followed by exposure to the glucocorticoid Clobetasol propionate. The challenge was not induced by solely lipopeptides, but also additional potential stressors like bacterial flagellin and viral RNA (summarised as pathogen-associated molecular patterns (PAMPs)). These patterns were directly applied to the zebrafish embryos by microinjection. Finally, RNA was extracted from the exposed animals. The gene expression analysis revealed that the PAMP challenge alone triggered processes linked to immune activation, while the application of Clobetasol propionate induced differing gene expression suggesting an impaired immune induction (Essfeld, Reinwald et al. 2022).

The described approaches illustrate methodical options to detect immunosuppressive mechanisms in fish. Even if the effect in these assays can be considered as inclusive, they should not be regarded as environmentally relevant, but purely from the point of view of effect identification.

Further research is needed to sufficiently close the gap between immunosuppressive mechanisms and the resulting effects on the apical level.

4.3 Testing strategy

From the results obtained from the Fish studies conducted it can be postulated that the ZEOGRT provides an appropriate setup to detect the relevant apical endpoints. For the test substances applied the test showed sufficient sensitivity. Moreover, the defined validity criteria were all met for both studies conducted confirming the robustness of the protocol applied. The comparison with the validity criteria is shown in Table 38.

Table 38: ZEOGRT studies: Compliance with acceptance criteria

Validity criterion	ZEOGRT with Dienogest		ZEOGRT with Dexamethasone	
	value	compliant	value	compliant
Test conditions				
Dissolved oxygen content: >60%	Mean values between 83 and 107%.	yes	Mean values between 100 and 104%.	yes
Water temperature: 26.0 +/- 1.5 °C	Mean values between 26.6 and 27.0 °C.	yes	Mean values between 26.1 and 26.7 °C.	yes
F_0 generation				
Reproduction: at least 10 eggs per female and day	64 +/- 11 [eggs per female and day]	yes	75 +/- 9 [eggs per female and day]	yes
≥ 80% fertility	90.7 +/- 3.4 [%]	yes	91.1 +/-2.5 [%]	yes
F_1 generation				
Early life stage: ≥ 80% hatching success	100 +/- 0.0 [%]	yes	98.6 +/- 2.8 [%]	yes
≥ 75% post hatch survival	92.4 +/- 4.7 [%]	yes	94.4 +/- 9.6 [%]	yes
Survival ≥ 90% (juveniles and adults)	100 +/- 0.0 [%]	yes	100 +/- 0.0 [%]	yes
Sex ratio 30 to 70% (males or females)	60.9 +/- 10.7 [% males]	yes	43.3 +/- 7.6 [% males]	yes
The analytical measurement of the test concentrations is compulsory.	A weekly measurement of at least two tank replicates was conducted.	yes	A weekly measurement of at least two tank replicates was conducted.	yes

Sexual development and reproduction capability represent main apical endpoints with regard to the identification of potential endocrine acting substances. Reproduction is characterised by fecundity (egg numbers) and fertilisation success. In the ZEOGRT studies, differences between egg numbers derived from the parental generation compared to the filial generation were observed, e.g. 64 (+/- 11) eggs per female and day (mean +/- standard deviation) were found for F0 in the study with Dienogest, while a mean egg number of 31 (+/- 9) eggs per female and day was recorded for F1. This difference is obviously linked to the age and developmental stage of the animals. While the F0 generation is prepared from adult cultured fish, the F1 fishes developed during the course of the study. However, both performances are well in line with the acceptance criteria as mentioned in the respective OECD guideline 229 (OECD 2012), requesting at least 10 eggs per female and day. A continuous spawning performance of the fish groups was observed for both studies, with low variability in the data sets which allowed a robust statistical evaluation. The parameter sex ratio is a critical one for zebrafish, and might be impeded by high between-replicate variability. In the presented data sets, F_1 sex ratios of 60.9 (+/- 10.7) %males (mean value +/- standard deviation; study with Dienogest) and 43.3 (+/-7.6) %males (mean value +/- standard deviation; study with Dexamethasone) were calculated for controls. Both control means were in line with the acceptance criteria for zebrafish sex ratio, i.e. 30-70% of respective sex (males or females). However, the data expressed bias for one fish sex. Genetic variability of zebrafish cultures is discussed to be a reason for variability. To meet this issue, the ZEOGRT was adapted in that way, that fertilised eggs collected to prepare the F_1 generation are sampled and pooled over two successive days. This procedure should ensure, that the source of eggs is e.g. not limited to one female fish and thus genetic mixture is increased. Concerns are especially linked to weak acting substances and a high variability of sex ratio may impede the overall decision if a substance acts as endocrine active or not. Further effort will be necessary to identify external environmental and intrinsic factors influencing the sex ratio expression.

The egg-yolk precursor protein Vitellogenin represents an established biomarker in endocrine related fish screening and high-level studies, i.e. OECD 229 and 230 (OECD 2009, OECD 2012), and furthermore OECD 234 (OECD 2011). Initial validation work for the named guidelines was conducted for clearly receptor-linked mode of actions like estrogen- or androgen receptor agonists or antagonists. Current regulatory needs focus on mode of actions beside these, for example effects linked to thyroid disruption or – more general – to substances impacting the steroidgenesis. In this context, Vitellogenin may not always represent the best physiological parameter. Moreover, the discrimination of effects linked to systemic toxicity against potential endocrine mediated effects is not possible by solely standing VTG results. There are several demands to assess if quality of VTG measurements is appropriate to allow a further assessment. This is e.g. a clear gap between male and female VTG concentrations measured for control groups. The VTG data derived from the presented ZEOGRT studies fulfilled those requirement; e.g. male control VTG concentration were recorded at 62.0 +/- 21.5 ng/mL (mean value+/-SD; study with Dienogest, F_0 generation) and 75.4 +/- 18.5 ng/mL (mean value+/-SD; study with Dexamethasone, F_0 generation), while female VTG concentrations were 3-4 orders of magnitude higher at 1.64E+07 +/- 2.58E +06 ng/mL (mean value+/-SD; study with Dienogest, F_0 generation) and 4.89E +07 +/- 2.77E+07 ng/mL (mean value+/-SD; study with Dexamethasone F_0-generation).

The evaluation of VTG measurements can moreover be impeded by high variation of individual measurement results, also in control groups. Even significant efforts to prevent cross-contamination between individual animals of different sex cannot fully prevent the occurrence of single values outside the expected range. As discussed by Wheeler et al. (Wheeler, Valverde-Garcia et al. 2019), this can diminish the identification of potential endocrine acting substances. To prevent significant bias of data set due to single outliers, it was decided for the ZEOGRT

studies presented here to calculate geometric mean values of the VTG concentrations per replicate tank.

The identification of underlying mode of actions is a key issue for the development of tailored risk assessment strategies for the different chemical regulations including pharmaceuticals. The conducted fish studies were shown to be appropriate to identify sensitive apical endpoints. By comparison with tests on other aquatic organisms, it became obvious that fish represent the most sensitive organism group most probable related to the very direct route of exposure via the respiratory organs. It is essential also with regard to the EU definition of Endocrine disruptors to include physiological parameters (Vitellogenin) and also histopathological parameters to assist the identification of underlying mechanisms and to create a plausible link between cause and effect. However, as presented in this report, both were not sufficiently sensitive for the test substances applied.

Fish test protocols like the ZEOGRT allow the collection of appropriate tissue material without extending the number of animals used.

The following sampling regime is proposed:

1. F_0 generation

- Head and tail, preserved during animal preparation. Torso will be saved for histopathological analysis.

2. F_1 generation

- 96 hours old embryos, separately exposed either in the test tank or in a multiwell-plate (20 embryos/replicate)

- 16 fishes from the early life stages phase, i.e. day 35 post fertilisation. These should be the remaining fish after randomised reduction to 20 fishes per replicate tank.

c. Head and tail, preserved during animal preparation. The torso will be saved for histopathological analysis.

3. F_2 generation

- 96 hours old embryos, separately exposed either in the test tank or in a multiwell-plate (20 embryos/replicate)

Within the studies reported, the tissue parts were sampled and stored. However, due to limited budget, further investigations could not be performed by now.

Nevertheless, there are promising methodical approaches which can improve the identification of underlying modes of action.

As the Adverse Outcome Pathway (AOP) concept became an important tool for the assessment of endocrine acting substances, available -omic methods provide valuable information on the molecular initiating event (MIE) and the following Key Events (KE). The collected tissues allow to generate this data for different life stages of exposed fish.

E.g. for the progestins, gene expression analysis can help to discriminate anti-estrogenic or glucocorticoid-like MoA (related to Progesterone analogues) or anti-estrogenic or androgenic MoA (more related to the Nortestosterone-analogues). Beside the described methods for detection of immunosuppressive mechanisms, i.e. LPS or PAMPs challenge, available -omic methods can be used to discriminate the direct impacts from secondary indirect effects due to immune suppression.

5 List of references

Bal, N., A. Kumar, J. Du and D. Nugegoda (2016). "Prednisolone impairs embryonic and posthatching development and shell formation of the freshwater snail, *Physa acuta*." Environmental Toxicology and Chemistry **35**(9): 2339-2348.

Bal, N., A. Kumar, J. Du and D. Nugegoda (2017). "Multigenerational effects of two glucocorticoids (prednisolone and dexamethasone) on life-history parameters of crustacean *Ceriodaphnia dubia* (Cladocera)." Environmental Pollution **225**: 569-578.

Brüggemann, M., Böhmer, W., Teigeler, M. (2023). "Effect studies of new hormone active substances on aquatic snails *Potamopyrgus antipodarum*." UBA-Texte 81/2023, Project No. 118916 (FKZ), FB001144/ENG.

DellaGreca, M., A. Fiorentino, M. Isidori, M. Lavorgna, L. Previtera, M. Rubino and F. Temussi (2004). "Toxicity of prednisolone, dexamethasone and their photochemical derivatives on aquatic organisms." Chemosphere **54**(5): 629-637.

Dunnett, C. W. (1955). "A multiple comparison procedure for comparing several treatments with a control." Journal of the American Statistical Association **50**(272): 1096-1121.

Dunnett, C. W. (1964). "New tables for multiple comparisons with a control." Biometrics **20**(3): 482-491.

EC (2000). "European Commission, Directorate General Health and Consumer Protection: SANCO/3029/99 rev.4 (11/07/2000), 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.".

EEC (2010). "European Union. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276/33."

EMEA and CHMP (2006). "Guideline on the environmental risk assessment of medicinal products for human use. SWP/4447/00 corr 2.".

Essfeld, F., H. Reinwald, G. Salinas, C. Schäfers, E. Eilebrecht and S. Eilebrecht (2022). "Transcriptomic profiling of clobetasol propionate-induced immunosuppression in challenged zebrafish embryos." Ecotoxicology and Environmental Safety **233**: 113346.

Faught, E. and M. M. Vijayan (2019). "Loss of the glucocorticoid receptor in zebrafish improves muscle glucose availability and increases growth." Am J Physiol Endocrinol Metab **316**(6): E1093-e1104.

Herbomel, P., B. Thisse and C. Thisse (1999). "Ontogeny and behaviour of early macrophages in the zebrafish embryo." Development **126**(17): 3735-3745.

Hidasi, A. O., K. J. Groh, M. J. F. Suter and K. Schirmer (2017). "Clobetasol propionate causes immunosuppression in zebrafish (*Danio rerio*) at environmentally relevant concentrations." Ecotoxicology and Environmental Safety **138**: 16-24.

Holbech, H., L. Andersen, G. I. Petersen, B. Korsgaard, K. L. Pedersen and P. Bjerregaard (2001). "Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*)." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **130**(1): 119-131.

Jonckheere, A. (1954). "A test of significance for the relation between *m* rankings and *k* ranked categories." British Journal of Statistical Psychology.

Kugathas, S., T. J. Runnalls and J. P. Sumpter (2013). "Metabolic and Reproductive Effects of Relatively Low Concentrations of Beclomethasone Dipropionate, a Synthetic Glucocorticoid, on Fathead Minnows." Environmental Science & Technology **47**(16): 9487-9495.

LaLone, C. A., D. L. Villeneuve, A. W. Olmstead, E. K. Medlock, M. D. Kahl, K. M. Jensen, E. J. Durhan, E. A. Makynen, C. A. Blanksma, J. E. Cavallin, L. M. Thomas, S. M. Seidl, S. Y. Skolness, L. C. Wehmas, R. D. Johnson and G. T. Ankley (2012). "Effects of a glucocorticoid receptor agonist, dexamethasone, on fathead minnow reproduction, growth, and development." Environmental Toxicology and Chemistry **31**(3): 611-622.

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

Liang, Y.-Q., G.-Y. Huang, S.-S. Liu, J.-L. Zhao, Y.-Y. Yang, X.-W. Chen, F. Tian, Y.-X. Jiang and G.-G. Ying (2015). "Long-term exposure to environmentally relevant concentrations of progesterone and norgestrel affects sex differentiation in zebrafish (*Danio rerio*)." Aquatic Toxicology **160**(Supplement C): 172-179.

Maack, G. and H. Segner (2003). "Morphological development of the gonads in zebrafish." Journal of fish biology **62**(4): 895-906.

Nagel, R. (1986). "Untersuchungen zur Eiproduktion beim Zebrabärbling (*Brachydanio rerio*, Ham.-Buch.)." Journal of Applied Ichthyology **2**(4): 173-181.

Nagel, R. (1998). Der vollständige Life Cycle Test (Complete Life Cycle Test, CLC Test) mit dem Zebrabärbling (Danio rerio, vormals Brachydanio rerio), Entwurf. UBA-Texte 58/98, 166-175.

OECD (2008). "Detailed review paper onfish life-cycle tests. OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 95 (ENV/JM/MONO (2008) 22). Paris, France."

OECD (2009). "Test No. 230: 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2010). "Guidance document on the diagnosis of endocrine-related histopathology in fish gonads. Series on testing and Assessment No. 123." ENV/JM/MONO(2010)14.

OECD (2010). "Test No. 233: Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2011). "*Test No. 234: Fish Sexual Development Test*." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2012). "*Test No. 229: Fish Short Term Reproduction Assay*." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2013). "*Test No. 210: Fish, Early-life Stage Toxicity Test*." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2013). "*Test No. 236: Fish Embryo Acute Toxicity (FET) Test*." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2016). "*Test No. 242: Potamopyrgus antipodarum Reproduction Test*." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Oettel, M., H. Breitbarth, W. Elger, T. Gräser, D. Hübler, G. Kaufmann, C. Moore, V. Patchev, W. Römer and J. Schröder (1999). "The pharmacological profile of dienogest." The European Journal of Contraception & Reproductive Health Care **4**(sup1): 2-13.

Overturf, M. D., C. L. Overturf, D. Baxter, D. N. Hala, L. Constantine, B. Venables and D. B. Huggett (2012). "Early Life-Stage Toxicity of Eight Pharmaceuticals to the Fathead Minnow, *Pimephales promelas*." Archives of Environmental Contamination and Toxicology **62**(3): 455-464.

Runnalls, T. J., N. Beresford, E. Losty, A. P. Scott and J. P. Sumpter (2013). "Several Synthetic Progestins with Different Potencies Adversely Affect Reproduction of Fish." Environmental Science & Technology **47**(4): 2077-2084.

Ryu, B., J. G. Je, Y. J. Jeon and H. W. Yang (2021). "Zebrafish Model for Studying Dexamethasone-Induced Muscle Atrophy and Preventive Effect of Maca (*Lepidium meyenii*)." Cells **10**(11).

Sadoul, B. and M. M. Vijayan (2016). Stress and growth. Fish physiology, Elsevier. **35:** 167-205.

Sitruk-Ware, R. and A. Nath (2010). "The use of newer progestins for contraception." Contraception **82**(5): 410-417.

Svensson, J., J. Fick, I. Brandt and B. Brunström (2013). "The Synthetic Progestin Levonorgestrel Is a Potent Androgen in the Three-Spined Stickleback (*Gasterosteus aculeatus*)." Environmental Science & Technology **47**(4): 2043-2051.

Takahashi, H. (1977). "Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*." Bulletin of the Faculty of Fisheries Hokkaido University **28**(2): 57-65.

Teigeler, M., D. Schaudien, W. Bohmer, R. Lange and C. Schafers (2021). "Effects of the Gestagen Levonorgestrel in a Life Cycle Test with Zebrafish (*Danio rerio*)." Environ Toxicol Chem **41(3): 580-591**.

Terpstra, T. J. (1952). "The asymptotic normality and consistency of Kendall's test against trend, when ties are present in one ranking." Indagationes Mathematicae **14**(3): 327-333.

Wheeler, J. R., P. Valverde-Garcia and M. Crane (2019). "Control performance of fish short term reproduction assays with fathead minnow (*Pimephales promelas*)." Regulatory Toxicology and Pharmacology **108**: 104424.

Williams, D. (1971). "A test for differences between treatment means when several dose levels are compared with a zero dose control." Biometrics: 103-117.

Williams, D. (1972). "The comparison of several dose levels with a zero dose control." Biometrics: 519-531.