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Effects of the synthetic progestin levonorgestrel on zebrafish (*Danio rerio*) reproduction

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Contents

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Abstract

Pharmaceuticals have emerged as a new important class of environmental contaminants. Special concern has been raised about steroidal hormones, which are present in the aquatic environment in many countries in the western world. Most attention has been given to estrogenic steroidal hormones, which are well known to cause endocrine and reproductive disruption in fish at the low concentrations in which they are found in the aquatic environment. Synthetic progestins are used in contraception, and are the compounds chiefly responsible for the contraceptive effect. Progestins are, like estrogens, not efficiently removed by sewage treatment plants (STP:s) and are also wide-spread contaminants in the aquatic environment. Two previous studies have confirmed the suspicion that progestins cause an inhibition of reproduction in fish similar to their effect in humans, and do so at concentrations found in the environment. In this study, the effects of the synthetic progestin levonorgestrel on the reproduction of zebrafish (*Danio rerio*) were examined. Adult zebrafish were semi-statically exposed to levonorgestrel concentrations of 8.1, 90.4, 158.8 and 654.2 ng L^{-1} for 21 days. Levonorgestrel did not cause any statistically significant impairment of reproduction. The results might however indicate that levonorgestrel can cause inhibition of zebrafish fecundity (egg production) similar to that reported in previous studies, although not at environmentally relevant concentrations. In the group exposed to the highest levonorgestrel concentration, there was a trend of decreased gene expression of the reproductive hormones FSH (follicle stimulating hormone) and LH (luteinizing hormone) in the brain. A trend in the opposite direction was observed in the gene expression of GnRH (gonadotropin releasing hormone). These results give an indication that the mechanism behind the previously reported reproductive toxicity of synthetic progestins might occur at the level of neuroendocrine control of reproduction in the brain.

Keywords: zebrafish, levonorgestrel, progestins, reproduction, fecundity, gene expression, FSH, LH, GnRH

Populärvetenskaplig sammanfattning:

P-piller i våra vattendrag kan störa fortplantningen hos fiskar

Det har under de senaste åren uppmärksammats att produktion och användning av läkemedel leder till att dessa substanser förorenar vattenmiljön. Läkemedel är gjorda för att påverka biologiska organismer och är så potenta att de påverkar djurlivet vid de låga halter som finns i miljön. Exempelvis innehåller P-piller hormoner som passerar ut i urinen och kommer ut i sjöar och vattendrag, där de påverkar fiskar på samma sätt som de påverkar människor, och kan ge allvarlig störning av fiskars fortplantningsförmåga.

P-piller är kemiska preventivmedel som används av kvinnor i mycket stor utsträckning i västvärlden. P-piller minskar risken för att en kvinna ska bli gravid genom att förhindra ägglossning och göra förhållandena i livmodern olämpliga för att ett befruktat ägg ska kunna fastna. Detta orsakas av att p-piller innehåller syntetiska varianter av könshormonet progesteron. Detta hormon produceras naturligt av kvinnor under menstruationscykeln, men i allra störst mängd hos gravida kvinnor. P-piller innehåller ofta även syntetiskt östrogen, men det är huvudsakligen de progesteronliknande ämnena, även kallade progestiner, som förhindrar graviditet.

Progestiner och andra syntetiska hormoner går relativt oförändrade genom kroppen och kommer ut i urinen. Reningsverk har visat sig vara ineffektiva när det gäller att rena bort dessa ämnen från avloppsvatten, och då användningen av p-piller är så hög har detta lett till att man har hittat progestiner och andra syntetiska hormoner i sjöar och vattendrag i flera europeiska länder inklusive Sverige, och även i Nordamerika.

Trots de uppenbara skillnaderna, är människor och fiskar häpnadsväckande lika varandra på fysiologisk och cellulär nivå. Därför är forskare oroliga för att ämnen från p-piller som förorenar sjöar och vattendrag ska påverka fiskar på liknande sätt som de påverkar människor, och att fiskar kan få problem med fortplantningen. Det har sedan flera år varit

känt att de östrogener som finns i p-piller kan påverka fiskar genom att minska honors äggläggning samt att ”feminisera” hanar, dvs göra att hanar börjar likna honor och då får nedsatt fertilitet. Under det senaste året har forskare visat att även progestiner påverkar fiskars fortplantning negativt vid de halter som finns ute i naturen.

I min studie utsatte jag zebrafiskar, en av de vanligaste fiskararterna vid djurförsök, för olika halter av progestinen levonorgestrel, en vanlig ingrediens i p-piller och s.k dagen-efterpiller. Mina resultat gav en indikation på att levonorgestrel kan minska äggläggning även hos zebrafisk, vilket har visats hos två andra fiskarter tidigare. Mina resultat gav även en indikation på varför fiskarna lägger färre ägg när de utsätts för levonorgestrel. Detta genom att jag undersökte uttrycket av vissa gener i fiskarnas hjärnor. I varje cell i kroppen finns DNA, som är som en ritning som innehåller all information om vad cellen ska göra och vad den ska producera. En gen är en del av detta DNA som kodar för något speciellt som cellen ska producera, exempelvis ett hormon. Genom att mäta hur mycket en gen uttrycks får man ett mått på hur mycket av den produkt som genen kodar för som produceras. I hjärnan mätte jag uttrycket av tre hormoner som är involverade i fortplantning. Resultaten var inte statistiskt säkerställda, men visade på en viss trend av påverkan av uttrycket av dessa hormoner, vilket tillsammans med resultaten från tidigare studier ger en indikation på att levonorgestrel och andra progestiner kan störa fiskars fortplantning genom att påverka produktionen av viktiga hormoner i hjärnan.

Introduction

Pharmaceuticals in human and veterinary medicine are widely and increasingly used compounds. In Sweden there are approximately 1200 active substances used in human medicine and in the European Union about 3000 (Andersson *et al*. 2006, Christen *et al*. 2010). The most commonly used human pharmaceuticals are anti-inflammatory drugs, antibiotics, beta-blockers, hypolipidemics, steroids and steroid-related hormones (Christen *et al*. 2010). In western countries, the yearly amounts used of these compounds are in the hundreds of tons (Christen *et al*. 2010). During the last decade, pharmaceutical compounds have emerged as an important new group of environmental contaminants. Advances in the field of analytical chemistry have allowed the detection of a wide range of different pharmaceutical compounds in the aquatic environment. Municipal and hospital wastewater are the major sources of pharmaceuticals to the aquatic environment (Vuillet *et al*. 2009, Christen *et al*. 2010). Pharmaceuticals are present in wastewater effluents in the range of ng L^{-1} to µg L^{-1} and in the ng L^{-1} range in surface waters in both Europe and North America (Kolpin *et al*. 2002, Petrovic *et al*. 2002, Kolodziej *et al*. 2003, Andersson *et al*. 2006, Fernandez *et al*. 2007, Viglino *et al*. 2008, Vuillet *et al*. 2009, Fick *et al*. 2010). This wide-spread contamination of wastewater and surface waters indicates that the removal process of many pharmaceuticals by sewage treatment plants (STPs) is far too inefficient. Pharmaceuticals differ from many other environmental contaminants in that they are specially designed to target biological systems, and do so at very low concentrations. As many molecular and metabolic pharmaceutical targets are highly conserved between humans and other animals, human pharmaceutical compounds in the environment may affect identical or similar targets in other vertebrates and invertebrates (Christen *et al*. 2010).

There are a number of reports of pharmaceutical residues having caused adverse effects to wildlife. Most attention has been given to the synthetic hormone 17α-ethinylestradiol, which has had adverse effects on reproduction and caused hormonal disturbances in aquatic organisms (Routledge *et al*. 1998, Petrovic *et al*. 2002, Hutchinson *et al*. 2003, Lange *et al*. 2009). In Pakistan, veterinary use of the anti-inflammatory drug diclofenac

has lead to near extinction of several vulture species, which have died from renal failure after being exposed to the drug from feeding on cattle carcasses (Oaks *et al*. 2004). There is also concern about the development of bacterial resistance to human and veterinary antibiotics, which are released in large amounts into the environment from hospitals, STP:s and animal husbandry (Kümmerer 2003, Larsson *et al*. 2007).

Progestins are a group of steroidal compounds with the common ability to induce "progestational" changes in the mammalian uterus, shifting the endometrium from the estrogen-induced proliferative state to the secretory state (Stanczyk 2002). Progestins are in the literature also referred to as progestagens, progestogens, gestagens and gestogens. There are a number of different synthetic progestins used in pharmaceutical products. These can be divided into two smaller groups based on their molecular structure and from which "natural" compound they are derived. One group contains the progestins that are derived from progesterone, the endogenous human progestin. This group contains for example medroxyprogesterone, megestrol, trimegestone and drospirenone (Sitruk-Ware 2004). The other group is the progestins that are derived from testosterone, and contains for example levonorgestrel, norethindrone, desogestrel and dienogest (Sitruk-Ware 2004). Progestins act through progestin receptors, but many of them also have substantial affinities for the androgen-, glucocorticoid-, mineralocorticoid- and estrogen receptors (Sitruk-Ware 2004). Progestins therefore constitute a very diverse group, pharmacodynamically speaking, and can exert many different combinations of progestogenic, anti-gonadotropic, (anti)androgenic, (anti)estrogenic, glucocorticoid and anti-mineralocorticoid effects (Schindler *et al*. 2008).

As pharmaceuticals, progestins are used mainly in contraception and hormone replacement therapy, but also in cancer- and endometriosis treatment (www.fass.se). In contraception, progestins are used either as a single component or in combination with an estrogenic compound. It is however the progestin that has the major contraceptive effect, while the estrogenic compound is added mainly to stabilize the endometrium in order to achieve better bleeding regularity (Erkkola & Landgren 2005). The contraceptive mechanism of progestins is not fully understood. It seems however that it consists of four

major parts: Inhibition of ovulation by suppression of midcycle peaks of LH and FSH, production of a thick mucus plug in the cervix, inhibition of progesterone receptor synthesis in the endometrium making it unsuitable for implantation, and reduction of motility and ciliary action in the fallopian tubes (Erkkola & Landgren 2005).

Progesterone is the main natural progestin in humans and other mammals, and is essential for normal reproductive function in both males and females. It is produced by females mainly by the corpus luteum during the second part of the estrous cycle, and causes swelling and secretory development of the endometrium making it suitable for implantation of the fertilized egg (Guyton & Hall 1996). Development of mammary lobules and alveoli is also promoted by progesterone (Guyton & Hall 1996). It exerts its effects in females by binding to two types of intracellular progesterone receptors (Coneely *et a*l. 2002). Present in the female genital tract, progesterone also affects sperm, causing hypermotility and the acrosome reaction which are essential for fertilization (Baldi *et al*. 2009). This activation of sperm is thought to be mediated by a membrane-bound progesterone receptor (Baldi *et al*. 2009).

In teleost fish, progestins have a number of key functions in reproduction. The main endogenous progestins in fish are 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DP) and 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P) (Pinter & Thomas 1997 a, Nagahama & Yamashita 2008). In females, 17α,20β-DP is responsible for stimulation of oocyte final maturation, which precedes ovulation and is a prerequisite for fertilization (Nagahama & Yamashita 2008). The developing oocyte is arrested in meiotic prophase I, and increases in size mainly by accumulating lipids, vitamins and vitellogenins (Lubzens *et al*. 2010). Vitellogenins is a class of proteins which are synthesized in the liver under the influence of 17β-estradiol produced by the granulosa layer of follicular cells surrounding the oocyte (Lubzens *et al*. 2010). When the vitellogenic period, during which the oocyte acquires all the nutrients needed for the development of an embryo, comes to its end the follicular cells become competent to produce 17α,20β-DP under the influence of LH (Nagahama & Yamashita 2008). In this context 17α,20β-DP is often called maturation-inducing hormone (MIH), since it causes germinal vesicle breakdown and

resumption and completion of meiosis, followed by ovulation (Lubzens *et al*. 2010). In some species, progestins seem to be involved directly in ovulation, and in other indirectly (Pinter & Thomas 1997 a, Nagahama & Yamashita 2008). In males, progestins are responsible for the stimulation of spermiation and sperm motility (Ueda *et al*. 1985, Tubbs & Thomas 2009). In both sexes, progestins, in particular 17α,20β-DP, seem to be indispensible factors in the initiation of meiosis (Miura *et al*. 2006, Miura *et al*. 2007). Fish progestins exert their actions by binding to both nuclear and membrane-bound progestin receptors, but oocyte final maturation, which is by far the most well studied process involving fish progestins, seems to be governed mainly by membrane-bound progestin receptors (Nagahama & Yamashita 2008, Lubzens *et al*. 2010). Yet another function of progestins in fish is as reproductive pheromones. 17α,20β-DP is used as a reproductive pheromone by a large number of species, and in goldfish (*Carassius auratus*), the most well-studied model, it has been shown that 17α,20β-DP released by females at ovulation increases LH levels and milt production in males (Stacey & Sorensen 2005).

Levonorgestrel (CAS number 797-63-7) is a synthetic progestin used in contraceptive pills, emergency contraceptive pills and contraceptive implants (Fig.1). The systemic name of levonorgestrel is 17β-hydroxy-17α-ethinyl-13β-ethyl-4-gonen-3-one (Edgren & Stanczyk 1999). It has a molecular weight of 312.4 g mol^{-1}, a log K_{OW} of 3.5 and a water solubility of 1.33 mg L^{-1} at pH 6.8 (Zeilinger *et al*. 2009, Fick *et al*. 2010). Levonorgestrel was developed in 1972 and is still one of the most widely used progestins in contraception (Erkkola & Landgren 2005). In Sweden, levonorgestrel is marketed under the brand names Jadelle®, Mirena, Neovletta®, NorLevo, Postinor, Trinordiol®, Trionetta® and Triregol (www.fass.se). Levonorgestrel is generally considered to have a bioavailability of 100 % and a half-life in humans between 10 and 13 hours (Stanczyk 2002). Once in plasma, 47.5% of levonorgestrel is bound to sex hormone-binding globulin (SHBG), 50% to albumin and only 2.5% is unbound (Schindler *et al*. 2008). Levonorgestrel is both a very strong progesterone agonist and a substantial androgen agonist, with binding affinities for the human progesterone- and androgen receptors of 323 and 58% of those of the natural ligands, respectively (Sitruk-Ware 2004).

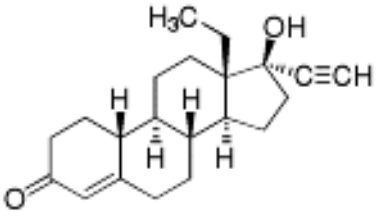

Figure 1: The molecular structure of levonorgestrel (www.fass.se).

Several studies have identified levonorgestrel and other progestins in the aquatic environment. Levonorgestrel has been measured in STP effluents in Sweden, Spain, France and Canada at concentrations ranging from 1 to 30 ng L^{-1} (Petrovic *et al*. 2002, Fernandez *et al*. 2007, Viglino *et al*. 2008, Vuillet *et al*. 2009, Fick *et al*. 2010). Other progestins such as norethindrone, medroxyprogesterone and progesterone have been measured in STP effluents in Sweden, France, Spain, Canada and the U.S. at concentrations ranging from 1.5 to 53 ng L^{-1} (Kolpin *et al*. 2002, Petrovic *et al*. 2002, Kolodziej *et al*. 2003, Andersson *et al*. 2006, Fernandez *et al*. 2007,Viglino *et al*. 2008, Vuillet *et al*. 2009). It is unlikely that aquatic organisms would ever be exposed to pure STP effluent water due to dilution, but some recipient streams and rivers are actually dominated by STP effluent water (Brooks *et al*. (2006). Levonorgestrel, norethindrone and progesterone have been identified in surface waters in the aforementioned countries at concentrations ranging from 3 to 110 ng L^{-1} (Kolpin *et al*. 2002, Petrovic *et al*. 2002, Andersson *et al*. 2006, Viglino *et al*. 2008, Vuillet *et al*. 2009). When such data is available, STP effluent concentrations of progestins are in the same range and sometimes even higher than the influent concentrations (Petrovic *et al*. 2002). The presence of progestins in both STP effluent water and surface water indicates that the removal process in STPs is inefficient, and taken together with the widespread use of these compounds this fact raises concern about possible ecotoxicological effects. It is also highly likely that many progestins will be present in water simultaneously, giving a higher total progestin concentration.

The actual exposure of fish and other aquatic organisms can be estimated using the bioconcentration factor (BCF) of these substances. Normally, the BCF can quite accurately be calculated from the $\log K_{OW}$ of a substance by using the equation by

Fitzsimmons *et al*. (2001). This is however not always the case. In a recent study, Fick *et al*. (2010) exposed juvenile rainbow trout (*Oncorhynchus mykiss*) to STP effluent water with a measured levonorgestrel concentration of 1 ng/L for 14 days. The plasma concentration of levonorgestrel in the fish was measured to 12 ng mL^{-1}, which gives a BCF for levonorgestrel of 12000. This greatly exceeds the predicted BCF of 46 (Fick *et al*. 2010). A possible explanation for this is given by Miguel-Queralt & Hammond (2008) who have shown that the high uptake of sex steroids is mediated by binding to SHBG, which is present in high levels in the gills. In their study they showed that steroid hormones were rapidly taken up from the ambient water via the gills, and the speed at which this occurred was generally proportional to the steroids' binding affinity for SHBG (Miguel-Queralt & Hammond 2008). Fick *et al*. (2010) and Miguel-Queralt & Hammond (2008) propose that binding to SHBG is a mechanism by which fish sequester steroid hormones entering the gills, and prevent them from returning to the ambient water. As levonorgestrel has a substantial binding affinity for SHBG (52% of that of 5α-dihydrotestosterone), exceeding that of the natural fish androgen, 11-ketotestosterone (28% of that of 5α-dihydrotestosterone), this seems a plausible mechanism for the much higher than expected uptake of levonorgestrel by fish (Miguel-Queralt & Hammond 2008, Fick *et al*. 2010). The level of bioaccumulation that levonorgestrel shows, combined with its pharmacological potency, means that exposures of environmental levels can lead to plasma concentrations in fish which exceed the human therapeutic concentration of 2.4 ng L^{-1} (Endrikat *et al*. 2002). This is probably true also for other progestins such as norethindrone, which is less potent (human therapeutic concentration 4.0 ng L^{-1}) and has a lower binding affinity for SHBG (30% of that of 5α-dihydrotestosterone), but is found in somewhat higher concentrations in the aquatic environment than levonorgestrel (Kolpin *et al*. 2002, Petrovic *et al*. 2002, Stanczyk 2002, Miguel-Queralt & Hammond 2008, Vuillet *et al*. 2009).

The possibility of these plasma progestin concentrations in fish causing some form of endocrine disruption seems very real considering the degree of conservation of molecular progestin targets between humans and fish. Zebrafish progesterone- and androgen receptors have 62 and 69% sequence identity with their human counterparts, respectively

(Christen *et al*. 2010). Besides causing endocrine disruption by hormonal action, synthetic progestins might also cause adverse effects to wild fish by acting as pheromones. As described earlier, female fish of many different species use the natural progestin 17α,20β-DP as a pheromone to induce biochemical and behavioral mating responses in males (Stacey & Sorensen 2005). Synthetic progestins might mimic the natural pheromone as Sorensen *et al*. (1990) showed that there is a strong correlation between structural similarity to 17α,20β-DP and pheromonal responses in goldfish. Pheromonal responses at inappropriate times, especially in seasonally breeding species, might be energetically costly, decrease feeding activity and increase the risk of predation (Defraipont & Sorensen 1993).

Zeilinger *et al*. (2009) and Paulos *et al*. (2010) have previously reported that levonorgestrel and norethindrone impair and decrease reproduction in fathead minnow (*Pimephales promelas*) and japanese medaka (*Oryzias latipes*), and this at lowest concentrations of 0.8 ng L^{-1} to 22 ng L^{-1}.These two studies show that at least for levonorgestrel and norethindrone, environmental levels of synthetic progestins can significantly impair fish reproduction. The purpose of this study was to further investigate the effect of a synthetic progestin on fish reproduction. Effects on fecundity (egg production) were studied, this time on a third fish species, zebrafish (*Danio rerio*). New to this study was the investigation of the effects of a synthetic progestin on the expression of certain genes involved in reproduction, to get information on the mechanism behind the previously reported impairment of reproduction in fish by synthetic progestins.

Materials and methods

General experimental setup

Adult zebrafish were subjected to a 21-day reproductive assay with semi-static aqueous exposure to levonorgestrel. The experiment consisted of a 21-day pre-exposure period, followed by 21 days of exposure. The exposure was performed at five different concentrations, including a solvent control. The choice to use only a solvent control and no tap water control was due to practical and ethical reasons. Due to the very low water solubility of levonorgestrel, methanol was chosen as the carrier solvent. A 6-week pilot study was performed in order to decide the range of concentrations. However due to technical problems, this pilot study did not yield any applicable results. The limited time span of this degree project together with the lack of results from the pilot study led to a concentration range being chosen more arbitrarily, with nominal concentrations of levonorgestrel of 10 ng L^{-1}, 100 ng L^{-1}, 1 µg L^{-1} and 10 µg L^{-1}. The wide range of these concentrations was to ensure a biological response with the possibility of observing dose-response relationships, even if the recovery rate would be as low as 1%. The reproductive effects of levonorgestrel were investigated by measuring female fecundity and also by determining the transcription levels of the reproductive hormones FSH (follicle stimulating hormone), LH (luteinizing hormone) and GnRH (gonadotropin releasing hormone) in the brain. Again, time limitations led to gene expression being measured only in the solvent control and 10 µg L^{-1} groups.

Experimental design

Ten-litre glass aquaria were used as test vessels. These were filled with 8 L Cu-free tap water which was aerated via plastic tubes connected to air stones, and kept at 25 ± 0.5°C by heaters. The light regimen was 12:12 h dark/light. Mean dissolved oxygen in the aquaria was 8.4 mg L^{-1} throughout the test. A stainless steel grid was placed in the bottom of each aquarium to induce mating. The grid was elevated ca. 0.5 cm from the bottom so the eggs could fall through and be protected from being eaten by the fish. Five male and five female zebrafish were randomly allocated to each aquarium. Only adult fish with no visible abnormalities were used. The fish were fed Tropical Excel Food Medium

(Aquatic Nature®) *ad libitum* once a day. Each aquarium was randomly assigned one of the five exposure concentrations. Levonorgestrel (HPLC grade, >98% purity) was obtained from Sigma-Aldrich (Steinheim, Germany). Four test solutions of levonorgestrel in methanol (Merck, Darmstadt, Germany) with concentrations of 0.1, 1, 10 and 100 µg ml^{-1} were prepared by dilution of a pre-analyzed levonorgestrel/methanol stock solution with a measured concentration of 100 µg ml^{-1}, kindly provided by Jerker Fick, Department of Chemistry, Umeå University. Mortalities during the test were noted and dead fish immediately removed.

Levonorgestrel exposure

Semi-static exposure renewal was conducted each day, concurrently with 3 L of water being exchanged. One-litre glass jars were first filled with 500 ml water and then dosed with 300 µl of levonorgestrel/methanol solution, or 300 µl of methanol for the solvent control aquaria. The maximum methanol concentration in all aquaria was therefore 100 µl L^{-1} (0.01%). The jars were then filled with an additional 500 ml of water, poured into the aquaria, followed by immediate adding of another 2 L of water. This three-fold mixing was to allow for an even distribution of levonorgestrel in the aquaria.

Chemical analysis

To determine the actual levonorgestrel concentrations, pentaplicate 10-ml water samples were taken from each aquarium on day 1, 7, 14 and 21 of exposure. Chemical extraction and analyses were performed by Jerker Fick, Department of Chemistry, Umeå University. Water samples were filtered (0.45 µm filters), and 1 ml was injected into an in-line SPE column coupled to a liquid chromatography-tandem mass spectrometry system, with medroxyprogesterone used as an internal standard. Samples were quantified using the internal standard method with 3-5 calibration points. The limit of quantification (LOQ) was 0.5 ng L^{-1}.

Measurement of fecundity

Fecundity was determined daily in each aquarium by measuring egg production, both during the pre-exposure and exposure periods. The steel grids were lifted and fixed in one

end by a hook, trapping the fish on the other side. Eggs and debris such as feces and uneaten food was collected from the bottoms of the aquaria by suction through a glass pipette. After being cleaned from most of the debris, the eggs were manually counted in a petri dish divided into 8 sections.

Tissue sample collection and RNA isolation

The day after the exposure period had ended, all fish were killed by decapitation and dissected. Brain, liver and gonads of each fish were snap frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using the Aurum™ Total RNA Fatty and Fibrous Tissue kit (Catalog # 732-6830) from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) as described by the manufacturer. In brief, each tissue sample was homogenized in 1 ml of PureZOL™ RNA Isolation Reagent, followed by addition of a series of reagents, eluents and centrifugation steps. The only deviation from the manufacturer's instructions was in the final elution step where RNA was eluted from the RNA binding column by adding 40 µl of nuclease-free water instead of elution buffer. This was done in order to avoid differences in ion concentrations in cDNA synthesis and real-time qPCR. Each isolated RNA sample was subjected to gel electrophoresis in order to detect possible RNA degradation. The isolated RNA was quantified spectrophotometrically at 260 nm using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). RNA purity and solvent contamination was evaluated by absorbance ratios at 260/280 nm and 260/230 nm, respectively.

Reverse transcription

Synthesis of cDNA was performed using 700 ng RNA from each sample and the iScript™ cDNA Synthesis kit (Catalog # 170-8891, Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the instructions by the manufacturer. Briefly, RNA was incubated together with 5x iScript Reaction Mix (containing oligo (dT) and random hexamer primers) and iScript Reverse Transcriptase for 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. Prior to real-time qPCR, each cDNA sample was diluted 1:25 with nuclease-free water.

Real-time polymerase chain reaction

Quantitative real-time PCR was conducted on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) using the iQ™ SYBR® Green Supermix kit (Catalog # 170-8880, Bio-Rad Laboratories Inc., Hercules, CA, USA). Primer sequences for the genes investigated were found in the published literature, where they had been used successfully (Hoffman & Oris 2006, McCurley & Callard 2008, Tello *et al*. 2008). Primer nucleotide sequences and product sizes for all genes are provided in Table 1. Cycling profiles for each gene were retrieved from the same published literature as the primer sequences. The cycling profile for FSH-β and LH-β consisted of 50°C for 2 min, 95°C for 2 min and 45 cycles at 55°C for 30 s and 72°C for 30 s, with a total primer concentration of 900 nM. The sGnRH cycling profile was 50°C for 2 min, 95°C for 2 min and 45 cycles at 95°C for 15 s and 57°C for 45 s, with a total primer concentration of 200 nM. For EF1-α, cycling consisted of 95°C for 10 min and 40 cycles at 95°C for 15 s and 62°C for 60 s, with a total primer concentration of 250 nM. qPCR validation consisted of a melt curve analysis being performed for each primer, ensuring that the correct products were amplified.

Table 1: Primer nucleotide sequences and product size of all genes used in quantitative real-time PCR

Primer	Sequence(5´-3´)	Target gene	Size(bp)	Reference
FSHβ F	TGAGCGCAGAATCAGAATG	*FSHβ*	105	Hoffman & Oris(2006)
FSHβ R	AGGCTGTGGTGTCGATTGT			
LHβ F	TTGGCTGGAAATGGTGTCT	*LHβ*	110	Hoffman & Oris(2006)
LHβ R	TCCACCGATACCGTCTCAT			
sGnRH F	AAGGTTGTTGGTCCAGTTGTTGCT	*salmon GnRH*	226	Tello *et al*. (2008)
sGnRH R	CAAACCTTCAGCATCCACCTCATTCA			
EF1-α F	CAACCCCAAGGCTCTCAAATC	*EF1-α*	358	McCurley & Callard(2008)
EF1-α R	AGCGACCAAGAGGAGGGTAGGT			

F = Forward, R = Reverse

Calculation of relative gene expression

The ratios of mRNA expression were calculated using the same equation as Hoffman & Oris (2006), with EF1-α (elongation factor 1 alpha) as the internal control gene (Eq. (1)).

$$\frac{1}{E_{(target)}^{Ct(target)} \Big/ E_{(EF1-\alpha)}^{Ct(EF1-\alpha)}} \quad (1)$$

$E_{(target)}$ and $E_{(EF1-\alpha)}$ represent mean reaction efficiencies, calculated using the LinRegPCR software, for the genes investigated (FSHβ, LHβ and sGnRH) and the internal control gene, respectively. The cycle threshold value (Ct) is the number of PCR cycles it takes for the amplification curve of each sample (log-converted using the Rotor Gene 6000 application software) to cross a threshold line manually set at the exponential phase of the amplification curves. The mean expression ratios of each sex in the exposed group were then normalized to the mean ratios of the corresponding sex in the control group and reported as a fold change relative to the control.

Statistical analysis

The effect of levonorgestrel on the expression of FSH-β, LH-β and sGnRH was determined by testing for statistically significant changes relative to controls using GraphPad Prism® version 5.01 (GraphPad Software Inc., CA, USA). Differences were analyzed using an unpaired t-test, and considered significant if $P<0.05$. In the cases where variances were significantly different, Welch's correction for unequal variances was used.

Results

Chemical analysis results

Measured concentrations of levonorgestrel in the aquaria differed from the nominal (Table 2). In the two lowest exposure groups, the recovery rate was around 80-90%. The second highest and highest exposure groups however, showed recovery rates of only 16 and 6.5%, respectively. The measured concentrations were used in presentation and interpretation of the results.

Table 2: Mean measured concentrations ± standard deviation (n=5) of levonorgestrel in the five concentration groups on day 1 of exposure.

Nominal concentration (ng L^{-1})	Measured concentration (ng L^{-1})
0	N.D.
10	8.1 ± 8.0
100	90.4 ± 5.5
1000	158.8 ± 16.6
10000	654.2 ± 12.3

N.D. = Not detected

Mortality

No mortalities were recorded in any aquarium during the pre-exposure period. However during the exposure period, one female and three males died in one of the 90.4 ng L^{-1} aquaria. These mortalities were not however considered to be related to levonorgestrel exposure, as all fish in this aquarium showed symptoms of fungal infection. This aquarium was excluded from subsequent calculations.

Female number

During dissection, it was discovered that one female in the 8.1 ng L^{-1} group and two in the 654.2 ng L^{-1} group were in fact males which at the time of aquarium allocation had been mistaken for females. This was accounted for in subsequent calculations.

Fecundity

Fish in all aquaria displayed the normal cyclic egg laying pattern for the entirety of the test, with 2-3 days between peaks. Egg number data was compiled for each aquarium and the mean was calculated for the three aquaria in each group. The means were then standardized for the number of females in each group. Fig. 2 shows the cumulative egg production per female in the control and different exposure groups, during both the pre-exposure and exposure periods. Egg production showed consistency and similarity between all groups during the pre-exposure period. During the exposure period, egg production in the control and 8.1 ng L^{-1} groups was quite similar compared with the egg production during the pre-exposure period (85 and 88%, respectively). At higher levonorgestrel concentrations however, there was an apparent decline in egg production. This decline was most pronounced at the highest concentration of 654.2 ng L^{-1}, where egg production during the exposure period was only 29% of that during pre-exposure. The declines did not show a clear dose-dependency, since egg production in the 90.4 ng L^{-1} group showed a larger decline than in the 158.8 ng L^{-1} group (60 versus 79% of that of pre-exposure, respectively).

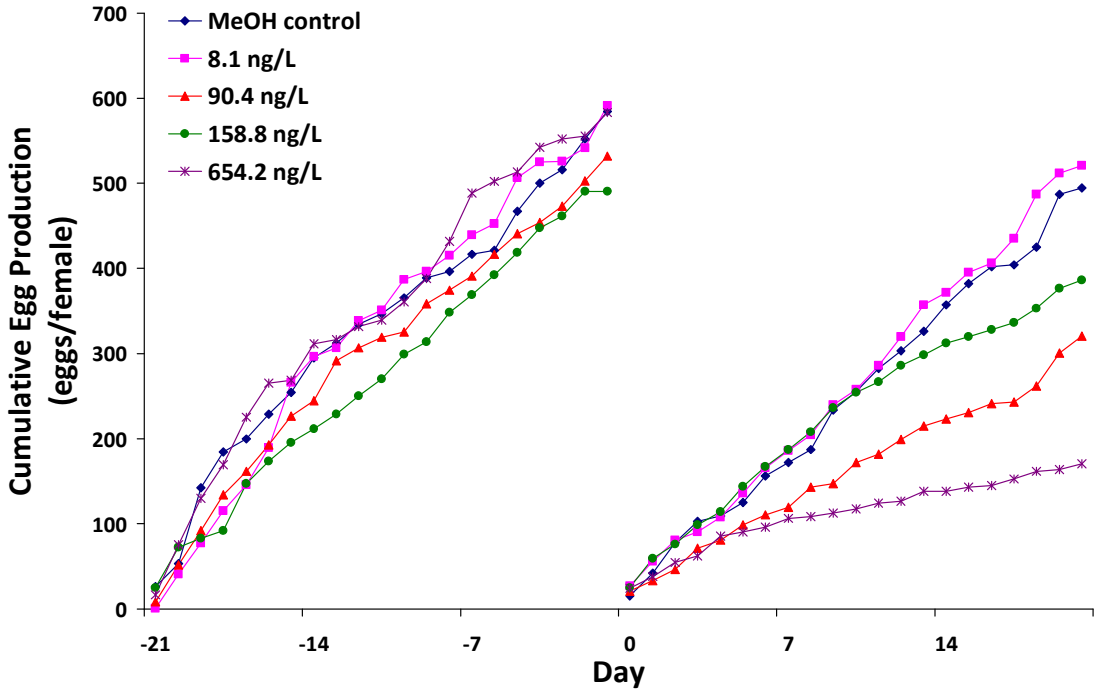

Figure 2: Cumulative egg production per female during a 21-day pre-exposure and a 21-day exposure period when adult zebrafish were exposed to levonorgestrel. The points represent the number of eggs laid in each group standardized to the number of females in that group. Each group consisted of three replicate aquaria. The total number of females in each group was n=15 in methanol control, n=14 in 8.1 ng L^{-1}, n=15 in 158.8 ng L^{-1} and n=13 in 654.2 ng L^{-1}.

Gene expression

Comparison of the relative mRNA expression of FSH-β, LH-β and sGnRH in the brain between the methanol control and 654.2 ng L^{-1} groups showed no statistically significant effects ($P > 0.05$, unpaired t-test) of levonorgestrel exposure at this concentration, neither in males nor females. Two trends could be noted however. The numerical value of mean expression of FSH-β was lower in the exposed group compared to the control group, about 1.7 times lower in males and 2.4 times lower in females (Fig. 3a). The same was noted for the mean expression of LH-β, where the numerical value also was lower in the exposed group, and was 4.7 times lower in males and 2.4 times lower in females (Fig. 3b). The variances in the expression of both these genes were however very high, which explains the lack of significance in spite of the large mean fold changes. The mean expression of sGnRH showed a trend in the opposite direction of FSH-β and LH-β, with the numerical mean value in the exposed group being 1.2-fold higher in exposed males and 1.4-fold higher in exposed females compared with the controls (Fig. 3c). The variances in mean sGnRH expression were similar to those of FSH-β and LH-β in the exposed group, but were small in the control group. The difference in sGnRH expression in females was close to the chosen significance limit ($P=0.052$, unpaired t-test).

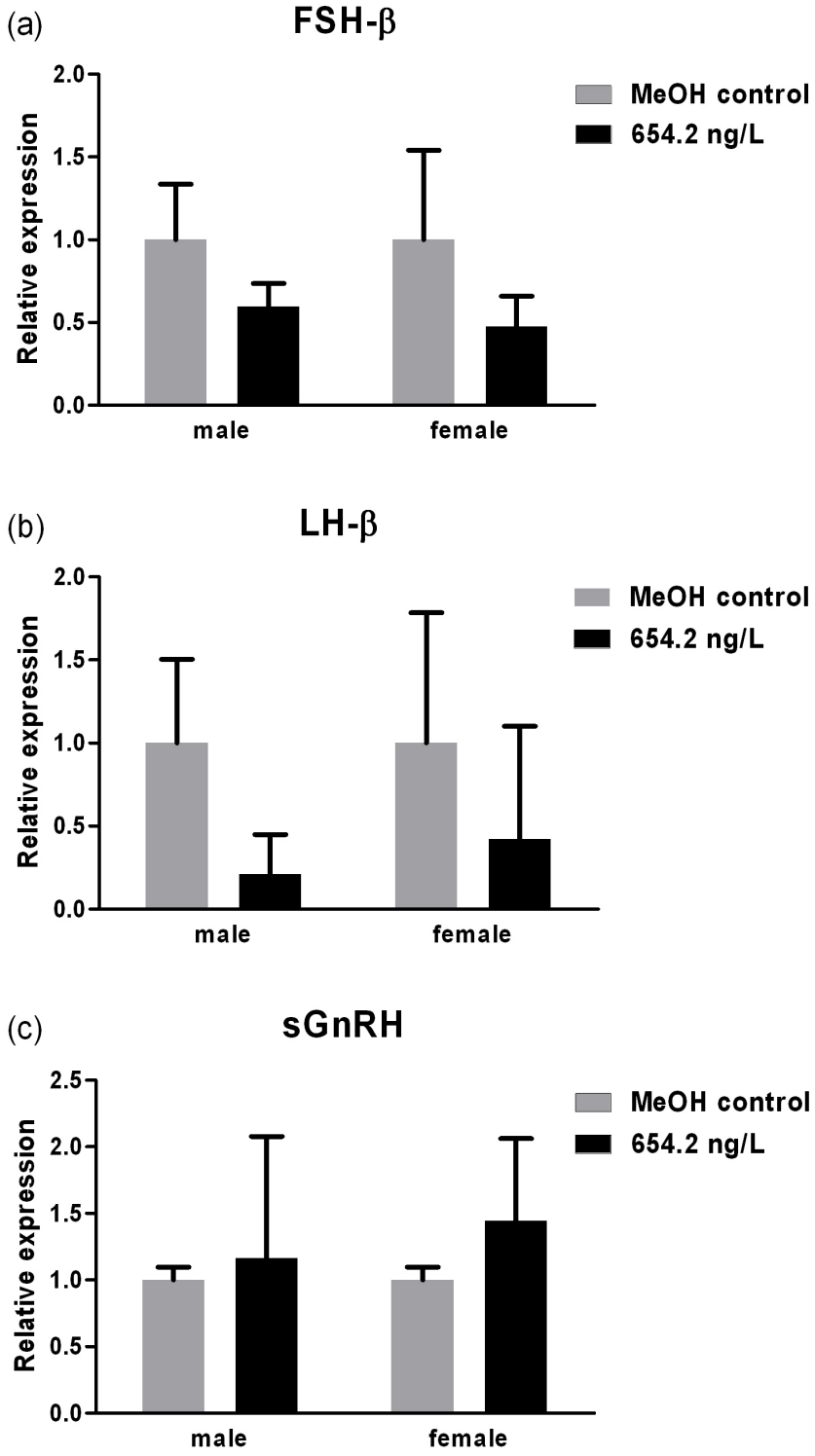

Figure 3: Relative mRNA expression (mean + S.E.) of FSH-β (a), LH-β (b) and sGnRH (c) in brain of male and female zebrafish after 21 days of exposure to 0.01% methanol (n=11 for males and females) or 654.2 ng levonorgestrel L^{-1} (n=11 for males and n=9 for females). Relative expression was calculated according to Eq. (1) using EF1-α as the internal control gene.

Discussion

This study examined the reproductive effects of levonorgestrel in the zebrafish with respect to fecundity and gene expression in the brain. The concentrations of levonorgestrel adopted were for the most part higher than concentrations found in STP effluents and surface waters. Only the lowest concentration (8.1 ng L^{-1}) can be considered to be of environmental relevance.

The results indicate an inhibition of egg production in the three highest exposure groups. Why egg production was lower in the 158.8 ng L^{-1} group than in the 90.4 ng L^{-1} cannot be explained, should the effect be due to levonorgestrel exposure. The difference in measured concentration between these two groups is however quite small. No statistical tests were performed on the egg production data. The results from the six-week pilot study indicated that nominal and measured concentrations could differ substantially. This led to more concentrations being used in the present study, and a limited number of test aquaria therefore led to only three replicates for each concentrations being used, giving a low statistical power with respect to fecundity. The possible inhibition of egg production did not seem to be due to any decrease in spawning frequency. This might suggest that the possible effect of levonorgestrel is not related to any change in breeding behavior. The indication of a decrease in fecundity observed in the present study is consistent with results from previous studies, albeit at much higher concentrations.

Only two studies have previously described the long-term effects of exogenously administered synthetic progestins in fish. In the study by Zeilinger *et al*. (2009), fathead minnows were exposed to levonorgestrel or drospirenone for 21 days. This study showed that levonorgestrel caused reduced fecundity even at the lowest tested concentration of 0.8 ng L^{-1}. At the higher concentrations of 3.3 and 29.6 ng L^{-1}, levonorgestrel also caused masculinization of females with the development of male secondary characteristics. Males displayed a lack of interest for their spawning tiles as well as aggressive behavior towards the females. Gonad histopathology at the highest concentration showed in females an increased percentage of maturing oocytes and atretic follicles compared to the

control and in males an increase in the number of mature spermatids and testis size, but a decrease in the number of spermatocysts. Drospirenone caused similar effects as levonorgestrel, only at the much higher concentration of 6.5 µg L^{-1}. Masculinization of females was absent, as could be expected from drospirenone´s slight anti-androgenic activity (Elger *et al*. 2003). In the other study, Paulos *et al*. (2010) examined the reproductive effects of another progestin, norethindrone. Japanese medakas were exposed for 28 days and fathead minnows for 21 days. In the Japanese medaka study, fecundity was impaired at 22 ng L^{-1}, an effect which at the highest concentration of 596 ng L^{-1} was not reversed 7 days after cessation of exposure. In the fathead minnow study, fecundity was reduced at 1.2 and 85 ng L^{-1}, however not at 16 ng L^{-1}. As in the study by Zeilinger *et al*. (2010), masculinization of females occurred at the highest tested concentration of 85 ng L^{-1}. Exposure to norethindrone also affected sex steroid levels, significantly reducing plasma 17β-estradiol in females at 16 ng L^{-1} and plasma 11-ketotestosterone in males at 85 ng L^{-1}.

The present study is the first where the effects of a synthetic progestin on gene expression have been investigated. No significant effects of levonorgestrel were found on the expression of FSH-β, LH-β and sGnRH in the brain. This would normally lead to the conclusion that the mechanism of action of the reproductive toxicity of levonorgestrel is independent of the expression of these genes. However, the consistent trend that could be seen in the expression of these genes, a higher expression of sGnRH and a lower expression of FSH-β and LH-β in both sexes of exposed fish compared with the controls, corresponds with and might explain the decrease in sex steroids in both sexes of fathead minnow reported by Paulos *et al*. (2010). The gonadotropins FSH and LH stimulate gonadal growth, production of eggs and sperm, and production of sex steroids in fish of both sexes (Clelland & Peng 2009). Therefore the possible reduced expression of gonadotropins observed in the present study would theoretically lead to decreased levels of 17β-estradiol and 11-ketotestosterone as observed by Paulos *et al*. (2010). It should however also be noted that the decrease in sex steroid levels found by Paulos and co-workers might be due to norethindrone, which has a relatively high affinity for SHBG, replacing the endogenous sex steroids from their binding sites on SHBG, thus increasing

their “free” unbound, fraction in plasma and thereby increasing clearance rate. FSH and LH are produced in the pituitary under the influence of GnRH and are controlled by negative feedback from sex steroids (Borg 1994). As levonorgestrel has substantial androgenic properties, a decrease in FHS and LH expression might be due to negative feedback by levonorgestrel, acting on androgen receptors in the pituitary. It is possible that the androgenic effect could contribute considerably to the reproductive impairment caused by levonorgestrel and other progestins. In the study by Zeilinger *et al*. (2009), levonorgestrel was a thousand times more potent than the weak anti-androgen drospirenone, even though the binding affinity of levonorgestrel to the human progesterone receptor is only five times higher (Elger *et al*. 2003). The difference in potency could of course also be due to differences in binding affinities to SHBG, affecting BCF:s and thus uptake and exposure. Binding affinity to SHBG has unfortunately not been determined for drospirenone. The results from the studies by Paulos *et al*. (2010) and Zeilinger *et al*. (2009) fit quite well with the hypothesis that the androgenic properties of synthetic progestins are a major cause of the reproductive impairment caused by said compounds. Paulos *et al*. (2010) showed that norethisterone impaired fathead minnow reproduction at 1.2 ng L^{-1}, and Zeilinger *et al*. (2009) showed that levonorgestrel did this at 0.8 ng L^{-1}. Norethisterone has about three times lower affinity for the human progesterone receptor than levonorgestrel, but similar binding affinity for the androgen receptor (55 compared to 58% of that of the natural ligand) (Sitruk-Ware 2004). The binding affinity of norethisterone to SHBG is quite similar to that of levonorgestrel (30 compared to 52% of that of 5α-dihydrotestosterone, respectively) so a large difference in uptake is not likely (Miguel-Queralt & Hammond 2008). It therefore seems likely that the difference in potency between levonorgestrel, norethindrone and drospirenone is mainly due to the fact that levonorgestrel and norethindrone are androgenic, while drospirenone is not. Paulos *et al*. (2010) themselves propose that it is the androgenic properties of norethindrone that are chiefly responsible for its impairment of fish reproduction. In their article they refer to findings by Pinter & Thomas (1997 b) and Thomas & Das (1997) who have shown that norethisterone and levonogestrel have less than 1% of the binding affinity for nuclear and membrane-bound progestin receptors in spotted seatrout (*Cynoscion nebulosus*) compared to the natural

fish progestin 17α,20β-DP. Thus it may be that fish progestin receptors have very different substrate specificities compared with progestin receptors in other vertebrates, and that synthetic progestins affect these receptors in fish only to a very small extent. It is anyhow evident that synthetic androgens such as 17-β-trenbolone and methyltestosterone can cause adverse effects on fish reproduction similar to those observed by synthetic progestins, and this at similar concentrations of 4.5 to 100 ng L^{-1} (Ankley *et al*. 2003, Andersen 2006, Korsgaard 2006, Miracle *et al*. 2006).

It is difficult to explain the observed trend in the present study of an increase in the expression of GnRH, especially with a simultaneous trend of decreased expression of FSH and LH. GnRH is produced in hypothalamic neurons and in fish acts directly on pituitary cells, stimulating expression of FSH and LH, both *in vivo* and *in vitro* (Borg 1994, Lin & Ge 2009). Why would FSH and LH expression be decreased if the expression of GnRH was increased? The truth of the matter is that the neuroendocrine control of fish reproduction is very complex and at the same time poorly understood. GnRH, as well as FSH and LH, can be both stimulated and repressed by sex steroids depending on species, developmental stage and season (Lin & Ge 2009, Zohar *et al*. 2010). GnRH is also released in a pulsatile manner in many vertebrates, and changes in pulse frequency can dramatically alter gonadotropin secretion (Burger *et al*. 2004). Exogenous alteration of GnRH pulse frequency by xenobiotics might therefore affect gonadotropin secretion in an unexpected manner. The progestin activity of levonorgestrel might provide a possible explanation to the trends observed. Mathews *et al*. (2002) showed that a 24h-treatment with 17α,20β-DP caused an inhibition of LH release in response to LHRH (luteinizing hormone releasing hormone, a synthetic analog to GnRH) in atlantic croaker (*Micropogonias undulates*). The presence of membrane-bound progestin receptors in the zebrafish pituitary shown by Hanna & Zhu (2009) suggests a direct effect of progestins on pituitary hormone release, which might account for the modulation of the GnRH response. Mathews et al. (2002) did however also observe a clear inhibitory effect of 17α,20β-DP on pituitary GnRH levels. A study in humans has shown that androgens can inhibit the negative feedback by progesterone on GnRH production (Sullivan & Moenter 2005). If this effect is present also in fish, it might be so

that the androgenic effect of levonorgestrel diminishes the negative feedback by its progestin activity.

It is difficult to explain the low recovery rate in the two highest exposure groups shown by the chemical analysis results. Precipitation can clearly not be the explanation as the water solubility of levonorgestrel is 1.33 mg L^{-1}, more than a thousand times higher than even the highest nominal concentration used in this study. Adhesion to glass surfaces of aquaria might be the reason for a low recovery rate, but the question still remains as to why the recovery rate was so dramatically different between the two lowest and the two highest exposure groups.

The methanol concentration in the aquaria never exceeded 0.01%, the maximum limit of carrier solvents recommended by the OECD (2000) for chronic testing. Oehlmann *et al*. (2009) have previously reported that a methanol concentration as low as 0.01‰ for 20 days can decrease sperm motility in zebrafish. The authors however argue that this effect is due to methanol decreasing the level of oxidative stress in the seminiferous tubules and sperm, which is needed in the final stages of sperm maturation and activation. Effects on sperm were not investigated in the present study, so possible effects of methanol are not considered to be of any major relevance to the results presented.

This is the third study that has examined the reproductive effects of a synthetic progestin in fish. No statistically significant effects of levonorgestrel were observed. However this study indicated a decrease in egg production in the three highest exposure groups, consequent with the results from previous studies. The concentrations employed were however too high to be considered of environmental relevance. Of more importance are however the results from the study of gene expression. Though not statistically significant, clear trends in the expression of brain FSH, LH and GnRH suggest that the mechanism of action of the reproductive toxicity of synthetic progestins might be in the HPG (hypothalamus-pituitary-gonadal) axis, where progestins exert negative feedback on the production of reproductive hormones. This warrants further, more targeted studies, in which to find more clear evidence of this possible mechanism of action. Other genes in

the brain, such as aromatase, could be of interest. The use of progestin- and androgen antagonists might also reveal whether synthetic progestins exert their toxicity mainly via progestin- or androgen receptors, or a combination of both. It is also of great importance to further investigate the apparent problem with achieving proximity to nominal test concentrations in this type of semi-static exposure. This to allow fewer concentrations being used, but with a higher number of replicates, ensuring a high enough statistical power needed for testing of effects on fecundity in fish breeding in groups.

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