1 **Reproductive and transcriptional effects of the antiandrogenic**
2 **progestin chlormadinone acetate in zebrafish (*Danio rerio*)**

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Abstract

Chlormadinone acetate (CMA) is a frequently used progestin with antiandrogenic activity in humans. Residues may enter the aquatic environment but potential adverse effects in fish are unknown. While the associated work focused on effects of CMA *in vitro* and in zebrafish eleuthero-embryos, the present study reports on reproductive and transcriptional effects in adult female and male zebrafish (*Danio rerio*).

We performed a reproductive study using breeding groups of zebrafish. After 15 days of pre-exposure, we exposed zebrafish to different measured concentrations between 6.4 and 53,745 ng/L CMA for 21 days and counted produced eggs daily to determine fecundity. Additionally, transcriptional effects of CMA in brains, livers, and gonads were analyzed. CMA induced a slight but statistically not significant reduction in fecundity at 65 ng/L and 53,745 ng/L. Furthermore, we observed differential expression for gene transcripts of steroid hormone receptors, genes related to the hypothalamic-pituitary-gonadal axis, and steroidogenesis. In particular, we found significant decrease of transcript levels of vitellogenin (*vtg1*) in ovaries and liver, and of *cyp2k7* in the liver of males, as well as a significant increase of transcripts of the progesterone receptor (*pgr*) in testes, and *cyp2k1* in liver of females. The observed effects were weaker than those of other very potent progestins, which is probably related to the lack of interaction of CMA with the zebrafish progesterone receptor.

Key words: Chlormadinone acetate; progestins; reproductive effects; zebrafish; endocrine disrupters

Capsule: Progestin chlormadinone acetate show low effects on reproduction of fish

1. Introduction

Progestins are used in oral contraception as well as in medicine. Besides bearing mainly progestogenic activity, they may exhibit (anti)estrogenic, (anti)mineralocorticoid, (anti)androgenic, and (anti)glucocorticoid activities in humans (Schindler et al., 2008). In mammals and fish, progestins mainly bind to nuclear progesterone receptors (PGR α and PGR β) and mediate effects through transcriptional changes (Kastner et al., 1990; Thomas et al., 2004). Unlike some other fish which have more than one PGR isoform, zebrafish has only one PGR (Chen et al., 2010; Y. B. Zhao et al., 2015c).

Human use of progestins is larger than that of estrogens and androgens (Fent, 2015). Progesterone (P4) and progestins enter the environment from human and animal excretion (urine/feces) and will eventually enter surface water via wastewater treatment works. Likewise, agriculture can be significant sources, particularly for large scale livestock operations in countries where progestins are used as growth promotors (Fent, 2015). Progestins excreted from livestock can runoff to surrounding rivers and lakes. When detected, levels of P4, norethisterone, levonorgestrel, medroxyprogesterone acetate, and megestrol acetate, usually were in the low ng/L range in treated wastewater and surface water (Fent, 2015; Kumar et al., 2015).

Exposure to synthetic progestins (levonorgestrel, norethisterone, gestodene) at ng/L levels has been shown to cause reproductive dysfunction in fish (Runnalls et al., 2013; Zeilinger et al., 2009; Paulos et al., 2010). Similarly, adverse reproductive effects were caused at higher concentrations ($\mu\text{g/L}$ range) of other progestins (drospirenone, (Runnalls et al., 2013; Zeilinger et al., 2009; Y. B. Zhao et al., 2015a), desogestrel (Runnalls et al., 2013), dydrogesterone (Y. B. Zhao et al., 2015b), megestrol acetate (Han et al., 2014), and P4 (DeQuattro et al., 2012). Some progestins also interfere with male reproduction by altering testicular differentiation and development (Kroupova et al., 2014; Svensson et al., 2014). Levonorgestrel (Kroupova et al., 2014; Svensson et al., 2014, 2013), drospirenone (Zucchi et al., 2014), P4 (Blüthgen et al., 2013b; DeQuattro et al., 2012; Garcia-Reyero et al., 2013; Y. B. Zhao et al., 2015a; Zucchi et al., 2013), dydrogesterone (Y. B. Zhao et al., 2015b), and medroxyprogesterone acetate (Y. B. Zhao et al., 2015b) have been shown to cause changes in transcriptional expression of nuclear steroid receptors and steroidogenesis enzymes. In addition to transcriptional alterations, exposure to levonorgestrel (Kroupova et al., 2014), norethisterone (Paulos et al., 2010), desogestrel (Runnalls et al., 2013), megestrol acetate (Han et al., 2014), and gestodene (Runnalls et al., 2013) alters sex hormone levels in adult fish. P4 reduces vitellogenin in female zebrafish (Zucchi et al., 2013). In general, changes in the transcriptional level occurred at lower concentrations than physiological effects including alterations in hormone and vitellogenin blood levels, altered gonad histology, and decreased fecundity (Fent, 2015).

Chlormadinone acetate (CMA) is used in hormone replacement therapy, and in combination with 17 α -ethinylestradiol (EE2), in oral contraceptive pills (Druckmann, 2009). CMA is structurally related to P4 (17 α -hydroxy-derivative) (Besse and Garric, 2009). As a potent PGR agonist and androgen receptor (AR) antagonist in humans it exhibits antiandrogenic effects via different pathways, including blocking of the AR and inhibiting transactivation, competitive inhibition of the enzyme 5 α -reductase (converts testosterone to 5 α -dihydrotestosterone [DHT]), and suppression of gonadotropin secretion, thereby reducing production of androgens (Raudrant & Rabe 2003). In zebrafish eleuthero-embryos (*Danio rerio*), we found a down-regulation of AR and PGR (Siegenthaler et al., 2016). However, effects of CMA on adult fish and their reproduction are currently unknown.

The ligand binding domain of fish PGR differs from that of humans, and consequently, its binding affinity to P4 and synthetic progestins is also different (Ellestad et al. 2014; Bain, Kumar, et al. 2015). In our previous study we examined the binding of CMA to and transactivation of nuclear steroid receptors in recombinant cell systems expressing either human or fish receptors (Siegenthaler et al., 2016). CMA did not interact with the fish progesterone receptor *in vitro* and showed no progestogenic activity in zebrafish eleuthero-embryos, but bound to the human progesterone receptor. The aim of the present study was to assess endocrine effects of CMA in adult fish, in particular on fecundity. We also related reproductive effects with transcriptional effects of a series of key target genes for endocrine disruption to elucidate the potential mode of action. Moreover, CMA as frequently used progestin is a potential contaminant in the aquatic system, but has not yet been investigated for adverse effects in fish. Therefore, the aim of our study is to support risk assessment of CMA and progestins in general.

2. Materials and methods

2.1 Chemicals

17 α -ethinylestradiol (EE2) (European Pharmacopoeia Reference Standard, CAS no. 57-63-6), chlormadinone acetate (CMA) (European Pharmacopoeia Reference Standard, CAS no. 302-22-7, purity $\geq$ 98%), dimethylsulfoxide (DMSO) (purity $\geq$ 99.5%, CAS no. 67-68-5), and ethanol (purity $\geq$ 99.8%, CAS no. 64-17-5) were purchased from Sigma-Aldrich, Fluka AG, Buchs, Switzerland. CaCl₂*2H₂O (purity $\geq$ 99%), KCl (purity $\geq$ 99%), MgSO₄*7H₂O (purity $\geq$ 99%), NaHCO₃ (purity $\geq$ 99.5%) were obtained from Carl Roth GmbH & Co., Karlsruhe, Germany.

For RNA extraction with TRIzol, chloroform (purity $\geq$ 99%, CAS no. 67-66-3) was obtained from J.T. Baker® Chemicals, isopropanol (Isopropyl alcohol, ChromAR, CAS no. 67-63-0) from Macron Fine Chemicals™, TRIzol® Reagent from Ambion by Life Technologies, Carlsbad, CA, USA. The GeneJET RNA Purification Kit was purchased from ThermoFisher Scientific, Reinach, Switzerland. M-MLV reverse transcriptase and M-MLV 5x reaction buffer were purchased from Promega Duebendorf, Switzerland. The random hexamer mix and the FastStart Universal SYBR Green Master were obtained from Roche Diagnostics, Basel, Switzerland. The dNTP was obtained from Sigma-Aldrich, Fluka AG, Buchs, Switzerland.

Progesterone-d9 used as internal standard (IS) for chemical analysis was purchased from Sigma-Aldrich (Fluka AG, Buchs, Switzerland). All solvents used for chemical analysis were of reagent grade or higher. Formic acid, ammonium formate, methanol (MeOH), acetonitrile (ACN) and ethyl acetate (EtOAc) (HPLC grade) were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade Milli-Q water was obtained by Barnstead™ Smart2Pure™ from Thermo Scientific (Waltham, USA). For solid-phase extraction Oasis HLB cartridges (500 mg / 6 mL) were purchased from Waters (Baden-Dättwil, Switzerland). Chromatographic analysis was performed using an XSelect CSH C18 column 2.5 μ m, 3.0 x 100 mm acquired from Waters (Waters Corp., Milford, MA).

2.2 Zebrafish

Adult zebrafish maintenance was performed as previously described (Blüthgen et al., 2013a; Y. B. Zhao et al., 2015b; Zucchi et al., 2014). In brief, we obtained adult zebrafish (*D. rerio*), the inbred-strain ABxTU, from the Friedrich Miescher Institute (Basel, Switzerland) and kept them in big fish culture tanks (300 L water tanks with 240 L water) containing reconstituted de-ionized water (CaCl₂*2H₂O [147.0 g/L], KCl [2.9 g/L], MgSO₄*7H₂O [61.6 g/L], NaHCO₃ [32.4 g/L]) with a conductivity of 470 to 480 μ S/cm and a temperature of 27 $\pm$ 1 °C. We exchanged the water and checked the water parameters (nitrite, nitrate, pH, oxygen concentration) once a week. We fed the fish twice daily with TetraMin XL

flakes (Tetra[®]) and twice per week with frozen white mosquito larvae, brine shrimps (*Artemia salina*), and *Daphnia magna*. The light-dark cycle was 14:10 h.

2.3 Flow through system conditions

We used the same parameters for the flow through system as previously described (Y. B. Zhao et al., 2015b). We used Flow Control Devices (DVS 70F, Péquitech, Fuellinsdorf, Switzerland) to regulate the flow of reconstituted water into glass mixing chambers, equipped with magnetic stirrers. The CMA and solvent control supply solution was dosed into 1 L mixing chambers of these groups, by means of a computer controlled dispenser (SED 6000 series Dispenser, Péquitech, Fuellinsdorf, Switzerland) resulting in nominal exposure concentrations of 10, 100, 1,000, 10,000, 100,000 ng/L. Due to low toxicity and for examining a large concentration range of CMA (that has not been studied before), we included a very high and pharmacological concentration to determine dose-response relationships. Each tank was supplied from the mixing chambers via Teflon tubing using gravity driven splitting units (NFlex, Péquitech, Fuellinsdorf, Switzerland) with a flow rate of 200 ml every 14 min and 24 s.

2.4 Experimental design

The entire experiment (pre-exposure and exposure) was performed in 10 L stainless steel tanks in a continuous flow through system, which ensured a complete exchange of reconstituted water every 12 h. Adult zebrafish (5 months old) were selected from the culture tank and randomly placed into 10 L stainless steel tanks in well-aerated water. Water parameters (pH, oxygen concentration, nitrite, nitrate) were measured regularly to ensure water quality, and water temperature was held at 27±1 °C. The photoperiod was set to 14 h light to 10 h dark. The experimental setup consisted of a solvent control (0.01% DMSO), and five concentrations of CMA. Each treatment consisted of three replicates and each replicate contained five male and five female fish as breeding pairs. Every tank was equipped with a spawning tray consisting of a stainless steel tray (265 x 185 x 30 mm) covered with a stainless steel grid (mesh size: 2.5 mm) and artificial weed was attached to the center of the grid to provide spawning substrate for the fish.

We chose five different CMA concentrations based on previous studies on reproductive effects of progestins (DeQuattro et al., 2012; Paulos et al., 2010; Runnalls et al., 2013; Zeilinger et al., 2009; Y. B. Zhao et al., 2015b), and to compare with effects in eleuthero-embryos (Siegenthaler et al., 2016). The lowest concentration was assumed as environmentally relevant, and the highest doses were pharmacological doses (Schindler et al., 2008). The highest concentration did not cause toxicity to the fish.

After an acclimatization phase of 7 days (including spawning trays), the experiment with the adult zebrafish started with a pre-exposure period of 15 days to establish the baseline fecundity of each tank (spawning group), followed by 1 day of equilibration when chemical dosing started, and finally 21 days of exposure. During the entire experiment, appearance, mortality, and abnormal behavior of fish were recorded daily, and fish were fed twice per day with TetraMin XL flakes (Tetra®). The study was conducted according to OECD Guideline 229/230 (OECD, 2009a, 2009b). Every morning before feeding, we removed the spawning trays from the tanks, collected the eggs, and transferred them to Petri dishes for counting. The cleaned spawning trays were then put back into the tanks. During the last week of exposure, we inspected the eggs under a stereomicroscope (Stemi DV4, Carl Zeiss AG, Feldbach, Switzerland) to determine the fertilization rate.

At the end of exposure, we euthanized the fish using KoiMed Sleep (ingredient phenoxy ethanol, 2 mL/L water, KOI&BONSAI Zimmermann, Buehlertann, Germany). We measured wet weight (g) and length (cm) to calculate the condition factor. The condition factor is a measure for general health (ratio of weight to length) and is calculated as body wet weight (g) / [body length (mm)]³ × 100. Three fish of each gender from each of the three replicates were immediately dissected. Brains, livers, and gonads of three males were pooled (n = 3 samples per tissue and treatment), transferred to RNeasy (Qiagen, Basel, Switzerland) and stored at -20 °C for later molecular analysis. For females, ovaries were collected from individual female and weighed to assess the gonadosomatic index (GSI = gonad weight (g)/ body weight (g) × 100). For RNA analysis, ovaries, brains, and livers of three fish were pooled and transferred to RNeasy (n = 3 samples per tissue and treatment). Pooling was necessary because of the small sample size and varying extraction efficiencies. Sampling took place between 9 a.m. and 2 p.m. and lasted about 5 hours.

2.5 RNA isolation and RT-qPCR

Total RNA of gonads of adult zebrafish was extracted using TRIzol reagent according to the manufacturer's instructions. Total RNA of brains and livers from adult zebrafish was extracted using the GeneJET RNA Purification Kit (ThermoFisher Scientific, Reinach, Switzerland). RNA concentrations and quality were spectrophotometrically determined using a NanoDrop 1000 spectrophotometer at 260 nm. Purified RNA was stored at -80 °C.

2.6 RT-qPCR analysis

For cDNA synthesis, 1 µg of RNA and RNase-free water (total volume of 14 µL) were incubated with 1 µL of random hexamers at 70 °C for 5 min. Then, 10 µL of a mastermix solution, consisting of 5 µL M-MLV 5x reaction buffer, 3.35 µL RNase-free water, 1.25 µL dNTPs, and 0.4 µL M-MLV reverse transcriptase, were added to each sample. The samples

were incubated at 37 °C for 50 min (reverse transcription) and subsequently for 5 min at 95 °C to stop the reaction. The cDNA samples were stored at -80 °C.

For RT-qPCR, each sample contained 1 µL of cDNA, 9.5 µL nuclease free water, 2 µL of appropriate primer mix (forward and reverse primer), and 12.5 µL FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland) as recommended by the manufacture's guidelines. The program we used consisted of 10 min at 95 °C for enzyme activation, followed by 40 cycles of transcript amplification (15 s at 95 °C, 60 s at 60 °C, plate read), and then the temperature was increased gradually in 0.5 °C steps every 5 s with plate read for melting curve analysis. All qPCR analyses were performed on BIORAD CFX96™ real-time PCR detection system (BIO-RAD, Switzerland).

We selected genes including hormone receptors, steroidogenic enzymes, circadian rhythm genes and metabolizing enzymes based on mechanistic considerations and previous data (Table 1) (Rossier et al., 2016; Y. B. Zhao et al., 2015b). We chose *cyp2k1* and *cyp2k7* to potentially detect androgenic or antiandrogenic activity (Fetter et al., 2015; Martyniuk et al., 2007). We used the reference gene *β-actin* for normalization of the data. This gene showed the most stable expression, as found in our previous studies (Zhao et al., 2016) and by others (Hua et al., 2016). We evaluated melting curves to ensure specificity of the qPCR products. For analysis of the qPCR data, we did a relative quantification using the $\Delta\Delta C_t$ -method (Livak and Schmittgen, 2001). The mRNA expression levels of target genes were expressed as fold changes (log2) according to the formula $2^{-\Delta\Delta C_t}$, with $\Delta\Delta C_t = (C_t[\text{target gene, treated}] - C_t[\text{reference gene, treated}]) - (C_t[\text{target gene, control}] - C_t[\text{reference gene, control}])$. The primers are given in Table 1.

2.7 Chemical analysis

Sample collection. The nominal concentrations of CMA during exposure ranged between 10 and 100,000 ng/L. During the experiment on day 10 and day 15 of exposure, water samples were collected from each tank and replicates were pooled to one sample. The amount of water samples differed depending on the nominal concentrations (10-400 mL). We also included solvent controls (0.01% DMSO) for analytical analysis. We stored the aqueous samples in glass bottles at -20 °C in the dark until analysis.

For chemical analysis, standards were dissolved in methanol (1 mg/mL) and subsequently diluted to the concentration of 10 ng/µL (stock solution). Working solutions were prepared freshly before each analytical run by diluting stock solution in methanol to concentration of 0.125, 0.05, 0.025, 0.01, 0.005, 0.002 and 0.001 ng/µL. All stock and working solutions were stored at -20 °C in the dark.

Solid-phase extraction. Aqueous samples were stored in glass bottles at -20°C in the dark until analysis (the storage period was approximately two weeks). For samples with nominal concentrations of 1 µg/L, 10 µg/L and 100 µg/L CMA, 1 mL aliquots were centrifuged at 12,000 g and then 400 µL supernatant was collected for direct analysis after spiked with 5 ng internal standard (¹³C-progesterone). For samples with nominal concentrations of 10 ng/L and 100 ng/L CMA, 200 µL aliquots were concentrated with SPE method for analysis. The SPE procedure was carried out using the Thermo Scientific™ Dionex™ AutoTrace™ 280 solid-phase extraction (Thermo Scientific, USA). The SPE cartridge (Oasis HLB, 6 mL, 500 mg) was preconditioned consecutively with 6 mL methanol (MeOH), 10 mL ethyl acetate (EtOAc) and 12 mL Milli-Q water. The water samples filtered with 0.22 µm glass fiber membrane (MACHEREY-NAGEL, Düren, Germany) were loaded onto the SPE cartridges at a flow rate of 5 mL/min after spiking with 5 ng internal standard. The cartridges were subsequently dried with nitrogen gas for 1 h and then eluted with 2x6 mL EtOAc and 2x6 mL acetonitrile/EtOAc (1:1, v/v). The eluates were then dried under a gentle nitrogen stream. Dried samples were re-dissolved in 400 µL Milli-Q water: methanol 50:50, filtered through a 0.22 µm membrane filter (Infochroma AG, Zug, Switzerland) into a 2 mL amber glass vial for instrumental analysis.

Liquid chromatography - tandem mass spectrometry (HPLC-MS/MS). The target compounds were analyzed by an Agilent 1200 series ultra-high performance liquid chromatograph (Agilent, USA) coupled to an Agilent 6410 triple quadrupole mass spectrometer (UHPLC-MS/MS). The chromatographic separation was performed on XSelect CSH C18 column 2.5 µm, 3.0 x100 mm, acquired from Waters (Waters Corp., Milford, MA). The column oven temperature was maintained at 40 °C, and the injection volume was 5.0 to 100 µL. The mobile phase consisted of (A) Milli-Q water containing 0.1 mM ammonium formate, and 0.1% formic acid (v/v), and (B) MeOH. The gradient program of the mobile phase was 40% B at 0 min, held for 1 min, increased to 70% B in 5 min, then increased to 80% B in 4 min, hold for 1 min and increased to 80% B in 0.5 min and held for 2 min at a flow rate of 0.6 mL/min.

The MS analysis was performed in the positive ion mode and the operating conditions were: gas temperature, 340 °C; gas flow, 13 mL/min; nebulizer pressure, 40 psi; and capillary voltage, 4,900 V. Mass spectrometer analyses were done in the multiple reaction monitoring (MRM) mode as shown in the supplementary material (Table S1). Quantification was performed using the isotopic dilution method and calibration curves were made freshly before each analytical run. Instrumental and procedural blanks were also included in each analytical run to check for potential contaminations.

2.8 Statistical analysis and graphical illustration

For statistical analysis and graphical illustration of our data, we used Prism ver. 6.00 (GraphPad Software, La Jolla, CA, U.S.A., www.graphpad.com). We designed a hierarchical clustering map using the MultiExperimental Viewer v4.9 (<http://www.tm4.org/mev.html>; Dana-Farber Cancer Institute, Boston, MA, U.S.A.). We analyzed RT-qPCR data, condition factor, and gonadosomatic index using one-way analysis of variance (one-way ANOVA) with subsequent post-hoc Bonferroni test for multiple comparison of differences between treatment and respective control. We analyzed significance of differences in the reproductive performance (number of eggs/female/day, spawning frequency, and fertilization rate) using two-way analysis of variance (two-way ANOVA) followed by Bonferroni post-hoc test for multiple comparison of differences between treatment and respective control, and differences between pre-exposure and exposure within the same treatment group. Results are shown as mean $\pm$ standard error of mean (SEM), unless otherwise indicated. We set the significance level α to 0.05 (95% confidence interval).

3. Results

3.1 Chemical analysis

Measured CMA concentrations in different dose groups were between 6.4 and 53745 ng/L (Table 2). They were 46-35% lower than nominal but fairly consistent at the two time points sampled. The lower than nominal concentrations have already been observed in our previous experiments with other progestins (Y. B. Zhao et al., 2015b, 2015a, Zucchi et al., 2014, 2013) and are probably related to the apparatus and sorption to tubing fish, and fish debris. Furthermore, some (bio)transformation of CMA to chlormadinone may account for the decrease.

3.2 Reproduction

Three-week exposure to 6.4-53,745 ng/L CMA did not affect length, weight or condition factor in either sex of zebrafish (Figure S1), and did not lead to any compound-related mortality or behavioral alterations. The gonadosomatic index of females, was not altered (Figure S2).

During the 15-day pre-exposure period, egg production was relatively consistent and similar across all groups. The 6.4 ng/L dose group showed a slightly reduced egg production during pre-exposure and CMA exposure compared to the other groups, but this change was statistically not significant (Figure 1).

The number of eggs per female per week (assuming seven days a week) showed no significant changes in any of the treatment groups (Figure 2A). However, when the number

of eggs per female per day during pre-exposure and exposure were compared, a significant decrease in egg production occurred in the 65 ng/L and 53,745 ng/L dose groups (Figure 2B). The spawning frequency tended to decrease across all treatment groups (Figure 2C). The fertilization rate was around 85% for all groups indicating no influence of CMA on fertilization (Figure 2D).

3.3 Transcriptional responses

We selected a number of genes belonging to different endocrine signaling pathways based on previous studies (Blüthgen et al., 2013b; Y. B. Zhao et al., 2015b; Zucchi et al., 2014) and analyzed the relative expression of these genes in the brains, livers, and gonads of exposed adult fish compared to solvent control. Detailed graphs of gene expression data are supplied in the SM (Figures S3-S5).

In the brains, CMA exposure did not lead to any significant changes in steroid receptor transcripts (*esr1*, *ar*, *pgr*, *gr*) in females (Figure 3). In males, transcript levels of *esr1* and *pgr* showed a slight tendency for down-regulation that was, however, only significant for *esr1* at 65 and 54,000 ng/L CMA. The *ar* transcript was slightly up-regulated at 65 and 551 ng/L CMA in males. The vitellogenin transcript (*vtg1*) was strongly induced ($\log_2 = 9.2$) at the highest CMA concentration in females. Gonadotropin gene expression (*fshb* and *lhb*) levels in brain showed no statistically significant alterations, although larger alterations occurred for *lhb* than *fshb*, and a tendency of up-regulation in females (Figure S3). A progesterone receptor-mediated direct stimulatory effect on *fshb* mRNA was described in the pituitary of male zebrafish (Wang et al. 2016).

In the livers, CMA exposure did not alter steroid receptor transcripts (*er2b*, *ar*, *pgr*, *gr*) but significantly lowered the *ar* transcript in females at 53,745 ng/L CMA (Figure 4). The transcript of *cyp2k1* was induced in females at all doses, while the *cyp2k7* transcript was significantly down-regulated in a dose-dependent manner in males. Furthermore, down-regulation of *vtg1* occurred in males that was, however, only significant at 53,745 ng/L due to high variation among replicates of control fish. In females, *vtg1* was significantly down-regulated at the highest concentration.

In gonads, *esr1*, *ar*, and *gr* transcript levels did not significantly change, but the *pgr* transcript was induced in testes upon CMA exposure of 65 ng/L and higher (Figure 5). Relative transcript levels of *hsd11b2*, *hsd17b3*, *cyp11a* and *cyp19a* remained unchanged, while *cyp2k7* transcripts showed a tendency for down-regulation in testes, albeit not significant. The *vtg1* transcript was down-regulated at all concentrations in ovaries, being significant at 551 ng/L CMA. Although down-regulation of *cyp19a* in females (Figure S5) is statistically not significant, there is an interesting correspondence between *cyp19a* down-

regulation and *vtg1* down-regulation in females, suggesting an antiestrogenic activity of CMA.

The heat map in Figure S6 shows that overall down-regulations occurred for *vtg1* in livers and gonads of both genders. Furthermore, in the livers of males the *cyp2k1* and *cyp2k7* transcripts were down-regulated, while in females they were up-regulated.

4. Discussion

Previously, we showed that CMA did not show progestogenic activity but displayed antiandrogenic, antiglucocorticoid and some antiestrogenic activities with Murray-Darling rainbowfish (*Melanotaenia fluviatilis*) receptors (Siegenthaler et al., 2017). Furthermore, in zebrafish eleuthero-embryos, CMA led to slight down-regulation of the *ar* and some other transcripts including *pgr* and *lhb* at 144 hpf. Due to the lack of interaction with the progesterone receptor and weak transcriptional effects in embryos, we hypothesized that CMA leads to little effects in adult zebrafish on fecundity and transcriptional alterations of genes involved in antiandrogenic and antiestrogenic activity.

4.1 Reproduction

We found only very little adverse effects of CMA on fecundity; there were no significant effects on egg production in treatment groups compared to the control. However, when comparing egg production of each individual treatment group before and during exposure, we noted a significant decrease in egg production at 65 and 53,745 ng/L. In reproductive studies, when analyzing cumulative mean egg production, it is important to compare the egg production curve slopes not only to the control group during exposure but also to the curve slope during pre-exposure of the same group to correct for variable baseline fecundity. Each tank contained five males and five females as a spawning group. Egg production may vary among female zebrafish. Despite random selection and distribution of male and female adult fish, it can happen that one spawning group produces more or fewer eggs than average. Before and during exposure with CMA, the 6.4 ng/L CMA group showed reduced fecundity compared to the other groups. Additionally, during the time of our experiment and among all treatment groups, we observed a general trend towards decreased egg production, especially in spawning frequency.

In our previous studies, spawning frequency was more consistent (Blüthgen et al., 2013a; Y. B. Zhao et al., 2015b). In the present experiment, fish spawned about every second day. In addition, replicates varied considerably as evident in the graphs. Furthermore, there might be differences in spawning frequency and fecundity between

different strains of zebrafish. All these reasons might have contributed to the weak reproductive effects, which did not show a dose-response relationship.

Overall, CMA had only a minor effect on fecundity as compared to other more potent progestins. Similar to our present study, megestrol acetate did not significantly decrease egg production after 21 days (Hua et al., 2016). However, the authors observed a trend towards decreased egg production and suggested that the decreased egg production would have become more significant after prolonged exposure. Some progestins have been shown to adversely affect egg production up to reproductive failure. Strong adverse effects on egg production have been reported at 0.8 ng/L levonorgestrel (Zeilinger et al., 2009), 1 ng/L gestodene (Runnalls et al., 2013), 1 ng/L norethisterone (Paulos et al., 2010). Others decreased fecundity at higher concentrations, including P4 at 10 ng/L (DeQuattro et al., 2012), dydrogesterone at 1,263 ng/L (Y. B. Zhao et al., 2015b), drospirenone at 6,500 ng/L (Zeilinger et al., 2009) and desogestrel at 10,000 ng/L (Runnalls et al., 2013). CMA seems to be by far the least potent synthetic progestin tested.

Our reproduction experiment has also some limitations as only three replicates per treatment were included due to technical constraints (number of aquaria in the flow-through system and manpower). Furthermore, the baseline fecundity showed variability between breeding pairs, which is quite common in zebrafish but limits statistical significance. To confirm our present results, a study with greater number of replicates would be needed.

4.2 Transcriptional responses

Transcriptional effects of CMA in different organs of adult fish were minor in the magnitude of mRNA changes, similar to in eleuthero-embryos (Siegenthaler et al., 2016). The most consistent effect was down-regulation of *vtg1*. At all concentrations, *vtg1* was significantly down-regulated in ovaries and livers of female but not male fish. Down-regulation of *vtg1* transcripts in livers of fish has been shown for other progestins, such as levonorgestrel (Svensson et al., 2013) and progesterone (Zucchi et al., 2013) at concentrations of ≥ 40 ng/L and ≥ 3.5 ng/L, respectively. Progestins seem to exhibit antiestrogenic activity in fish, as well as in mammals (Schindler et al., 2008). Increased levels of the follicle-stimulating hormone (FSH) lead to increased synthesis of estrogens, and increased plasma levels of estrogens activate vitellogenesis (Nagahama and Yamashita, 2008). Progestins are thought to inhibit gonadotropin secretion (luteinizing hormone and FSH) in humans (Raudrant and Rabe, 2003), however, a recent publication indicated that $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one (DHP), a major progestin in fish, increases the expression of *fshb* mRNA in the pituitary of male zebrafish (Wang et al. 2016). Reduced levels of FSH might lead to decreased plasma levels of estrogens, and hence vitellogenesis. Down-regulation of *vtg1* was found in response to two model antiestrogens (Sun et al., 2010). This might also be the mode of

450 action of CMA, in particular as antiestrogenic activity was found *in vitro* and in eleuthero-
451 embryos (Siegenthaler et al., 2016), but support for this hypothesis should be obtained in
452 forthcoming studies by analysis of plasma concentrations of vitellogenin and steroid
453 hormones.

454 In the brains, transcript levels of *esr1* and *ar* were slightly down-regulated, the latter
455 also in the liver of female fish. As in eleuthero-embryos (Siegenthaler et al. 2017),
456 down-regulation of *ar* may suggest an antiandrogenic effect, while down-regulation of *esr1*
457 suggest an antiestrogenic activity. The relevance of these transcriptional alterations is not
458 well understood in the brain. Mechanistically, down-regulation of *esr1* and *ar* is probably
459 mediated by antagonistic action of CMA to the respective hormone receptors, as previously
460 shown (Siegenthaler et al. 2017). Similarly, a down-regulation of *esr1* was previously found
461 in the brain by medroxyprogesterone acetate and dydrogesterone (Zhao et al. 2015b), and in
462 the liver of zebrafish exposed to progesterone (P4) and drospirenone (DRS) (Zhao et al.
463 2015a). The high induction of *vtg1* in the highest CMA concentration is remarkable. This is in
464 contrast to other progestins, such as P4 and DRS that led to a down-regulation of *vtg1* in the
465 brain of zebrafish (Zucchi et al. 2013, 2014), or in the liver (Zhao et al. 2015a). In case of
466 dydrogesterone and medroxyprogesterone acetate, no alteration of *vtg1* was found (Zhao et
467 al. 2015b). The mode of action for *vtg1* regulation in zebrafish brain is not yet recognized, while
468 obviously more pathways are involved in, besides the regulation by estrogen receptors, and no
469 general effect of progestins on this regulation can be found.

470 We observed increased levels of *pgr* transcripts in testes of fish exposed to CMA,
471 similar to observations in zebrafish eleuthero-embryos exposed to P4 and levonorgestrel (Y.-
472 Q. Liang et al., 2015a; Zucchi et al., 2012). In contrast, the *pgr* transcript showed no
473 statistically significant changes in the brains of adult males and in embryos. Transcriptional
474 responses can differ from one tissue to another and between embryos and adults as
475 previously found for benzophenone-4, a UV-absorber (Zucchi et al., 2011).

476 In the livers of male fish, we observed a significant and dose-related down-regulation
477 of *cyp2k7* transcripts, whereas in females, we found a trend towards up-regulation of *cyp2k7*,
478 albeit not significant. In a previous study, we observed the similar phenomenon. The effect
479 on this transcript is the most consistent one of all transcripts. Up-regulation of *cyp2k7* in
480 females and down-regulation in males were observed for medroxyprogesterone acetate and
481 dydrogesterone (Y. B. Zhao et al., 2015b). As seen in male livers, a tendency for down-
482 regulation of *cyp2k7* occurred in testes and ovaries. The enzyme *cyp2k7* is mainly expressed
483 in the liver but its substrates have not yet been identified. It is potentially involved in
484 biotransformation and metabolism of androgenic steroids due to its strong up-regulation upon
485 exposure to androgens. Moreover, *cyp2k7* expression is potentially mediated via AR (Fetter
486 et al., 2015; Hoffmann et al., 2008). Down-regulation of *cyp2k7* transcripts in males might

therefore suggest antiandrogenic activity of CMA. Therefore, we suggested that *cyp2k7* might also be involved in the metabolism of progestins; however, further studies are needed to clarify this possibility. The significance of our observation is that CMA acted similarly as other progestins on *cyp2k7* expression, as for instance, dydrogesterone and medroxyprogesterone acetate (Zhao et al. 2015b). The transcription of this gene is to be likely regulated by the androgen receptor, and hence, androgenic or antiandrogenic compounds lead to up- or down-regulation of *cyp2k7*, respectively. Expressional changes of *cyp2k7* may therefore serve as an androgenic or antiandrogenic biomarker in zebrafish, similarly as shown previously with *cyp2k22* (synonym for *cyp2k7*) (Fetter et al. 2015).

In the livers of female fish, we observed an up-regulation of *cyp2k1* transcripts. Induction of *cyp2k1* has previously been observed in livers of female fish exposed to EE2 (Martyniuk et al., 2007) and the weak estrogen nonylphenol (Thibaut, 2002). *Cyp2k1* is possibly involved in the phase I metabolism of estrogens in the liver, and possibly, also CMA may potentially be metabolized by this enzyme. Alternatively, CMA may have led to increased levels of estrogens via antiandrogenic activity.

While our data clearly indicate transcriptional effects on several key genes in the brain (*vtg1*), liver (*cyp2k7*), and gonads (*pgr*) that are probably related to the mode of action of CMA, transcriptional analyses have also certain limitations. They reflect the response at one point in time, and changes on mRNA level do not necessarily translate into physiological level. Transcriptional analyses are therefore ideally accompanied by analyses of physiological parameters (Fent and Sumpter, 2011). Thus, here we combined the reproductive outcome with transcription analysis to link transcriptional changes of HPG axis genes with fecundity and the gonado-somatic index, however, additional parameters such as plasma hormone levels would add to the effect assessment. Nevertheless, our gene expression analyses were useful to detect the modes of action of CMA.

Expressional changes are also influenced by circadian rhythm, as shown for metabolism enzymes (Zhao and Fent, 2016) or steroidogenesis enzymes (Di Rosa et al., 2016). Previously, we also showed that progestins interfere with the regulation of key genes of the circadian rhythm (Zhao et al., 2016). Therefore, it is important to control for the time-related effects on gene expression by keeping sampling time short and sampling of different groups randomly. In our study, sampling lasted about 5 hours and we took care that the different dose-groups were sampled randomly for not producing false positive reactions (for instance by first sampling control groups and sequentially groups with increasing concentrations).

4.3 Possible reasons for observed effects *in vivo*

The observed effects of CMA were generally weaker than those of some previously investigated progestins, including levonorgestrel or norethindrone (for review Fent 2015). CMA has pleiotropic effects in humans and is used as a pharmaceutical (Druckmann, 2009). Its effects have also been demonstrated in mammals and various cell lines (Murakoshi et al., 2000; Poulin et al., 1991; Raudrant and Rabe, 2003; Térouanne et al., 2002). We tested the binding affinity of CMA for human PGR (CMA three times less potent than P4) and human AR (CMA 80 times less potent than DHT), and the transactivation of the receptors (Siegenthaler et al., 2016). Levonorgestrel and gestodene have a much higher affinity for both receptors (five and ten times more potent than P4 and three and four times less potent than DHT, respectively) (Runnalls et al., 2013). CMA is a less potent progestin in humans and higher doses are needed to exert the same effects. The lower potency of CMA compared to these other progestins has been found here in fish.

Additionally, pharmacodynamics may differ between fish and humans. In fact, two groups showed differential binding of progestins to fish PGR and AR. Binding of several synthetic and endogenous progestins to fathead minnow nuclear PGR and AR showed that P4 is less potent in activating PGR than the fish endogenous progestins (DHP, 20 β -S), and only one of the synthetic progestins (drospirenone) was capable of PGR transactivation (Ellestad et al., 2014). In contrast, almost all progestins were strong AR agonists (some more potent than DHT). Binding of several progestins was tested in Murray-Darling rainbowfish AR (two subtypes) and PGR (Bain et al., 2015), and all 19-nortestosterone derivatives tested potently activated AR but none of them activated PGR. Effects observed in fish after progestin exposure seem to be mediated by interaction with AR rather than PGR (contrary to humans where PGR is the major target). In our associated work, we demonstrated that CMA does not transactivate rainbowfish PGR but exhibits antiandrogenic and antiglucocorticoid activity (Siegenthaler et al., 2016). The lack of AR and PGR agonism in fish might be the reason for the observed weak to zero effects; that is if CMA interacts similarly with zebrafish receptors.

In addition, there could also be differences in the pharmacokinetics of CMA between humans and fish leading to differential sensitivities. In humans, oral bioavailability of CMA is nearly 100%; it accumulates in the fat tissue and is excreted rather slowly. CMA also has active metabolites in humans (Schindler et al., 2008). Fish take up progestins into the blood via their gills through special transporter proteins or through passive diffusion through membranes. After sulfonation or glucuronidation and by organic anion transporter polypeptides (OATPs), fish can take up steroid hormones into their cells (James, 2011). However, there are no data on the distribution and metabolism of CMA in fish. Presumably, CMA is not efficiently taken up or is rapidly metabolized and excreted before fish are affected.

4.3 Ecological relevance

Environmental levels of progestins have been poorly assessed and only a fraction of all existing progestins has been investigated. This impedes a proper ecotoxicological risk assessment. CMA has not yet been measured in the environment. We expect the environmental concentrations of CMA to be in the range of a few ng/L as other progestins investigated so far (Fent 2015).

In adult zebrafish, the lowest observed effect levels (LOELs) for transcriptional changes were 6.4 ng/L CMA (significant up-regulation of *cyp2k1* in livers) in females and 65 ng/L CMA (significant up-regulation of *ar* in brains) in male fish. Assuming the decrease in fecundity at 65 ng/L was coincidental, the LOEL for reproductive effects was 53,745 ng/L CMA. Assuming environmental CMA concentrations of less than 10 ng/L, our LOEL for transcriptional effects in females are environmentally relevant. The LOELs for reproductive and transcriptional effects in males seem above environmental levels. Further studies on physiological consequences of the observed transcriptional changes should be conducted to determine the risk posed to females. But considering the strength and inconsistency of our observations, adult fish do not seem to be at a high risk for hazardous effects by CMA. However, further studies are needed to confirm effect levels and environmental concentrations of CMA.

5. Conclusions

We provided first insights into the reproductive and transcriptional effects of the synthetic progestin CMA in adult zebrafish. CMA led to a significant but very weak reduction of egg production in adult zebrafish at 65 ng/L and 53,745 ng/L CMA. We observed several significant changes in gene expression in brains, livers, and gonads of adult zebrafish. Differentially expressed genes included *vtg1*, *pgr*, and *cyp2k7*, which may be related to antiestrogenic, progestogenic, and antiandrogenic activities. Compared to other progestins, CMA exerted weak effects, which may be related to the weak binding to zebrafish receptors. Further ecotoxicological studies are needed to enlighten the mode of action of CMA and possibly confirm the weak activity in fish and other aquatic organisms.

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Tables

Table 1. Primer sequences for quantitative real-time PCR (RT-qPCR) analyses and sources.

Gene	Gene Bank No.	Sense Primer (5'-3')	Antisense Primer (5'-3')	Product size (bp)
<i>ar</i> ^a	NM_0010831	CACTACGGAGCCCTCACTTGCG GA	GCCCTGAACTGCTCCGACCTC	237
<i>cyp11a</i> ^b	AF527755.1	GAGGGGTGGACTCGGTTACTT	GCAATACGAGCGGCTGAGAT	109
<i>cyp17</i> ^b	AY281362.1	CTGCTCTGTTTAAGCCTGTTCTC	GCTGGCACAAATCCATTCATC	80
<i>cyp19a</i> ^c	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92
<i>cyp19b</i> ^c	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94
<i>cyp2k1</i> ^d	—	GGCAGAAAGAGAAGCGAAGAG	CAAGGCTAAGGGCAGTTTTG	—
<i>cyp2k7</i> ^e	AF487990.1	CGTCAGACCAGCTGTGATGT	TGTCAGGTGTTTCCCACTCA	115
<i>esr1</i> ^f	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163
<i>esr2b</i> ^g	NM_174862.3	CGCTCGGCATGGACAAC	CCCATGCGGTGGAGAGTAAT	80
<i>fshb</i> ^h	NM_205624	GCTGTGCACTACCAACATCTC	GTGACGCAGCTCCACATT	61
<i>gr</i> ⁱ	EF567112	ACAGCTTCTTCCAGCCTCAG	CCGGTGTCTCCTGTTTGAT	116
<i>hsd11b2</i> ^j	NM_212720	TGCTGCTGGCTGTACTTCAC	TGCATCCAACCTTCTTGCTG	123
<i>hsd17b3</i> ^d	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121
<i>lhb</i> ^h	AY714132	GAGACGGTATCGGTGGA AAA	AACAGTCGGGCAGGTTAATG	178
<i>pgi</i> ^k	NM_001166335	GGGCCACTCATGTCTCGTCTA	TCTCCACTCTGAAAATATGTG GACTTT	96
<i>vtg</i> ^h	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94
<i>β-actin</i> ^l	AF057040.1	TCTGGCATCACACCTTCTACAAT	TGTTGGCTTTGGGATTACAG	97

Data sources: a (Hossain et al., 2008), b (Y.-Q. Liang et al., 2015b), c (Arukwe et al., 2008), d (Martyniuk et al., 2007), e (Hoffmann et al., 2008), f (Martyniuk et al., 2007), g (Chandrasekar et al., 2010), h (Hoffmann et al., 2006), i (Alsop and Vijayan, 2008), k (Chen et al., 2010), l (Y. Q. Liang et al., 2015).

Table 2. Mean measured exposure concentrations in exposure water during 21 days exposure.

Nominal concentrations (ng/L)	Measured (mean*) (ng/L)	concentration	Accuracy measured – nominal concentration (%)
Solvent control (0.01% DMSO)	< LOQ [†]	-	-
CMA 10	6.4		64
CMA 100	65		65
CMA 1,000	551		55
CMA 10,000	6,166		62
CMA 100,000	53,745		54

LOQ[†] = limit of quantification. * Mean is referred to two samples taken at different days for all samples.

Figure captions

Figure 1. Cumulative mean number of eggs per female after two weeks of pre-exposure followed by three weeks of exposure to solvent control (0.01% DMSO) and different concentrations of CMA. Pre-exposure period is indicated as -15 to -1 days followed by the exposure period indicated as 1 to 21 days. Each dot represents the mean values of three replicate tanks per day and treatment.

Figure 2. Reproductive effects in zebrafish after exposure to different concentrations of CMA. (A) Number of eggs per female per day after two weeks of pre-exposure followed by three weeks of exposure to solvent control (0.01% DMSO) and different concentrations of CMA. Data are shown as mean $\pm$ SEM of three replicate tanks per week and treatment. (B) Average number of eggs per female per day after two weeks of pre-exposure followed by three weeks of exposure to solvent control (0.01% DMSO) and different concentrations of CMA. Each bar represents the mean $\pm$ SEM of three replicate tanks per treatment, comparing pre-exposure and exposure. Asterisks indicate significant different number of eggs per female and day. * p-value < 0.05, ** p-value < 0.01. (C) Spawning frequency of groups of zebrafish, i.e., how often the breeding groups spawned per week, assuming seven days a week. Spawning frequencies are shown for two weeks of pre-exposure followed by three weeks of exposure to solvent control (0.01% DMSO) and different concentrations of CMA. Each bar represents the mean value $\pm$ SEM of three replicate tanks per treatment and week. (D) Fertilization rate of eggs originating from adult zebrafish exposure to solvent control (0.01% DMSO) and different concentrations of CMA. The data are shown as mean $\pm$ SEM of three replicate tanks per treatment, expressed in percentages.

Figure 3. Relative transcript levels in brains of adult zebrafish males (blue) and females (red) after 21 days of exposure to different concentrations of CMA. (A) Hierarchical clustering (HAC) of transcriptional responses in males (M) and females (F). (B) Relative gene expressions of progesterone (*pgr*), androgen (*ar*), estrogen receptor (*esr1*), and vitellogenin (*vtg1*) are shown. Significant transcriptional changes compared to solvent control (0.01% DMSO) are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean $\pm$ SEM (n = 3 per treatment and tissue and gender).

Figure 4. Relative transcript levels in livers of adult zebrafish males (blue) and females (red) after 21 days of exposure to different concentrations of CMA. (A) Hierarchical clustering (HAC) of transcriptional responses in males (M) and females (F). (B) Relative gene expression of progesterone (*pgr*) and androgen receptor (*ar*), *cyp2k1*, *cyp2k7*, and vitellogenin (*vtg1*) are shown. Significant transcriptional changes compared to solvent control (0.01% DMSO) are indicated with asterisks. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001. Data are shown as mean $\pm$ SEM (n = 3 per treatment and tissue and gender).

Figure 5. Relative transcript levels in gonads of adult zebrafish males (testes, blue) and females (ovaries, red) after 21 days of exposure to different concentrations of CMA. (A) Hierarchical clustering (HAC) of transcriptional responses in males (M) and females (F). (B) Relative gene expression of progesterone (*pgr*) and androgen receptor (*ar*), *cyp2k7*, and vitellogenin (*vtg1*) are shown. Significant transcriptional changes compared to solvent control (0.01% DMSO) are indicated with asterisks. * p-value < 0.05. Data are shown as mean $\pm$ SEM (n = 3 per treatment and tissue and gender).

875 **Figure 1**

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Figure 2

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Figure 3

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