Effects of the UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) on expression of genes involved in hormonal pathways in fathead minnows (*Pimephales promelas*) and link to vitellogenin induction and histology

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ABSTRACT

UV-filters are increasingly used in cosmetics and in the protection of materials against UV-irradiation, and ultimately they reach aquatic systems. The lipophilic UV-filter 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC) belongs to one of the most frequently used UV-filters and accumulates in aquatic animals. Despite its ubiquitous presence in water and biota, very little is known about its potential hormonal effects on aquatic organisms. In our study, we evaluated the effects of measured water concentration of 5.4, 37.5, 244.5 and 394 µg/L EHMC on the expression of genes involved in hormonal pathways in the liver, testis and brain of male and female fathead minnows (*Pimephales promelas*). We compare the transcription profile with the plasma vitellogenin (VTG) content, secondary sex characteristics, and gonad histology. Transcripts of the androgen receptor (*ar*) were significantly down-regulated in the liver of females at 37.5, 244.5 µg/L and 394 µg/L EHMC. Additionally, the 3β-hydroxysteroid dehydrogenase (*3β-HSD*) transcript was significantly decreased in the liver of males at 37.5, 244.5 and 394 µg/L EHMC, and at 244.5 and 394 µg/L EHMC in females. The expressional changes were tissue-specific in most cases, being most significant in the liver. Vitellogenin plasma concentration was significantly increased at 244.5 µg/L EHMC in males. EHMC induced significant histological changes in testes and ovaries at 394 µg/L. Testes displayed a decrease in spermatocytes, and ovaries a decrease in previtellogenic oocytes. The induction of VTG plasma concentration and the histological changes in gonads suggest an estrogenic and/or antiandrogenic activity of EHMC. On the other hand, the gene expression profile shows an antiestrogenic (e.g.: down-regulation of *esr1*) activity of EHMC. In conclusion, our data demonstrate that EHMC displays low but multiple hormonal activities in fish.

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1. Introduction

UV-absorbing compounds (UV-filters) are increasingly used in personal care products in particular to protect the human skin from direct exposure to harmful UV-radiation from sunlight. There are two types of UV-filters, physical filters such as titanium dioxide and zinc oxide, which mainly scatter and reflect UV-light, or organic compounds absorbing UV-light. UV-filters are widely used in cosmetics (lipsticks, shampoos, creams, fragrances, skin lotions, hair sprays) and in the UV-protection of numerous materials and products (Balmer et al., 2005; Fent et al., 2010a). Currently, 28 UV-filters are registered in the European Union (Schlumpf et al., 2008). UV-filters enter directly into surface water via recreational activities or indirectly via wastewater, which was found to be the dominant source in rivers (Balmer et al., 2005; Fent et al., 2010). Many UV-filters used in sunscreens are lipophilic and can accumulate in biota, which has been demonstrated for 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC; formerly octylmethoxycinnamate, OMC) (Fent et al., 2010a).

Recent findings demonstrate that several UV-filters may have hormonal activity in mammals (Schlumpf et al., 2008) and in fish (Kunz et al., 2006a,b; Coronado et al., 2008; Weisbrod et al., 2007; Fent et al., 2008; Zucchi et al., 2011). They may negatively affect corals (Danovaro et al., 2008) or the reproduction of *Daphnia magna* (Fent et al., 2010b). Hormonal activity *in vitro* of benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-4 (BP-4), 3-benzylidene camphor (3-BC) and ethyl-4-aminobenzoate (Et-PABA) has also been shown. In addition to estrogenic activity (Schreurs et al., 2002; Inui et al., 2003; Kunz et al., 2006a; Coronado

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et al., 2008), 3-BC, BP-2 and BP-3 impair fertility and reproduction in fish (Kunz et al., 2006b; Weisbrod et al., 2007; Fent et al., 2008; Coronado et al., 2008). Despite these few studies, ecotoxicological consequences of UV-filters in personal care products are fairly unknown and need to be further investigated.

EHMC is one of the most widely used UV-filters in topically applied sunscreens. Environmental concentrations between 0.01 and 0.1 μg/L were reported in treated, and up to 19 μg/L in untreated municipal wastewater (Balmer et al., 2005). Lower concentrations occur in lakes, rivers (Straub, 2002; Balmer et al., 2005; Fent et al., 2010), and coastal seawater, where up to 390 ng/L was detected (Langford and Thomas, 2008). EHMC occurred even at very remote environments such as the Pacific Ocean (Polynesia) (Goksoyr et al., 2009). Furthermore, concentrations of 0.26–5.61 μg/L were reported in untreated drinking water (Loraine and Pettigrove, 2006). EHMC is very lipophilic (log K_{ow} of 6.1, Balmer et al., 2005) and accumulates in aquatic biota of different trophic levels with concentrations of up to 340 ng/g lipids in cormorants (Fent et al., 2010).

Despite its widespread presence in the environment and its accumulation tendency, little is known about environmental risks of EHMC. Multiple hormonal activities were shown *in vitro* including antiestrogenic, antiandrogenic and weak androgenic activity (Kunz and Fent, 2006). High concentrations of EHMC (9.87 mg/L) led to induction of vitellogenin (VTG) in male medaka (Inui et al., 2003), whereas no VTG induction was observed in fathead minnows at lower concentrations (Kunz et al., 2006a). Therefore, there is a need for a better understanding of potential environmental risks associated with EHMC contamination, also because the mode of action of EHMC is unknown.

The goals of this study are two-fold. First, molecular effects of EHMC on gene expression are evaluated by applying a targeted gene approach. We focus on seven transcripts involved in hormonal pathways and steroidogenesis in three different organs (liver, gonads and brain), both in male and female fish. Second, we determine whether gene expression alterations are linked to physiological effects. Thus, we compare plasma concentrations of VTG, secondary sex characteristics and the histology of gonads with gene expression changes.

2. Materials and methods

2.1. Chemicals

2-Ethyl-hexyl-4-trimethoxycinnamate (EHMC, CAS No. 5466-77-3) and 3-(4-methylbenzylidene)-camphor (4MBC; CAS No. 36861-47-9) were purchased from Merck (Glattbrugg, Switzerland), ethanol (EtOH), methanol and dichloromethane in HPLC grade from J.T, Baker (Stehelin AG, Basel, Switzerland) and formic acid from Sigma Aldrich (Fluka AG, Buchs, Switzerland), Cremophor RH40 from BASF (BASF Chem Trade GmbH, Ludwigshafen, Germany), paraffin tissuewax, xylol, UltraClear and haematoxylin from Medite, Nunningen (Switzerland) and eosin from Carl Zeiss AG, Feldbach (Switzerland).

Stock solutions of EHMC were prepared by dissolving the compound in ethanol at a concentration of 20 g/L. Subsequently 1:1 dilutions of the stock solutions were prepared in 20% Cremophor RH40 (dissolved in nanopure water) to obtain a concentration 10 g/L EHMC. Working solutions were stored in the dark at 4 °C between uses.

2.2. Maintenance of fish

Sexually mature fathead minnows (*Pimephales promelas*) were obtained from Osage Catfisheries Inc., Osage Beach, MO/USA. Fish were acclimatized in 300 L culture tank for 1 month prior to the experiment. The fish were held in reconstituted tap water with a total hardness of 125 mg/L as CaCO$_3$ and a conductivity of 270 μS/cm. The water temperature was held constant at 25 ± 1 °C with the photoperiod was set at 16:8 h light/dark. Fish were fed twice daily with brine shrimp (*Artemia salina*) and white mosquito larvae.

2.3. Experimental design

The experimental design was similar as in previous studies (Kunz et al., 2006b; Weisbrod et al., 2007). The experimental setup consisted of four replicate tanks of each water controls, solvent controls (1:1 dilution of EtOH and 20% Cremophor RH40) and four nominal EHMC concentrations (30, 300, 1000 and 3000 μg/L). At the beginning of the experiment, four female and two male fish per group were randomly selected and assigned to well-aerated 20 L replicate stainless steel tanks at 25 ± 1 °C and a photoperiod at 16:8 h light/dark. A 21 d pre-exposure period was performed in aquaria containing a stainless steel funnel as a spawning substrate. This was done in order to establish the reproductive capacity of unexposed fish and to provide tank-specific baseline data for statistical comparison once the exposure with EHMC was initiated. After the 21 d pre-exposure, fish were exposed for 14 d to nominal values of 30, 300, 1000 and 3000 μg/L EHMC, respectively. During exposure survival, reproductive behavior, secondary sex characteristics and fecundity (in terms of cumulative number of spawned eggs per day) were determined. Eggs attached to the spawning substrate were removed and counted daily. Due to the very low fecundity, data on egg laying were not valid and therefore not included in our study.

A static-renewal procedure was used during the pre-exposure and exposure period. Thereby food remains and feces were removed after 48 h by siphoning two thirds of the water and replaced by new exposure water containing the appropriate EHMC or solvent concentrations. This water renewal procedure was chosen to minimize handling stress and disturbances for the fish. The quality of the exposure water was continuously monitored by determining oxygen concentration (>70%), the pH-value (6.7–7.2) and the temperature (25 ± 1 °C) (OECD 229).

Mortality and abnormal behavior were recorded daily. At the end of the exposure experiment (day 14), all fish were anaesthetized in clove oil solution (Fluka AG, Buchs, Switzerland). Individual length and weight of fish (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males) were measured in order to assess the condition factor (CF = weight (g)/length (mm) × 100). To determine plasma concentration of VTG, blood was collected from the caudal vein of male and female fish (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males) using a heparinised capillary tube (Kabe Labortechnik GmbH, Nümbrecht-Elsenroth, Germany) and transferred into a labeled Eppendorf® tube. Plasma was then separated from the blood by centrifugation (full-speed for 2 min) and the plasma was stored at −80 °C until analysis.

After sampling of the blood, gonads of female and male fish (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males) were removed and weighted in order to assess the gonadosomatic index (GSI = 100 × gonad weight/body weight). Gonads, liver and brain from female and male fish were immediately excised and stored in RNA*later* (Qiagen, Basel, Switzerland) at −80 °C for determining mRNA content of selected genes by quantitative reverse-transcription real-time polymerase chain reaction (qRT-PCR). For analysis of the secondary sex characteristics, nuptial tubercles are counted in male fish, and classified according to the OECD guideline 229.

Table 1

Primer sequences for quantitative real-time PCR analysis and sources: *18S rRNA* (18S ribosomal RNA), *ar* (androgen receptor), *cyp19a1a* (cytochrome P450, family 19, subfamily A, polypeptide 1a), *cyp19a1b* (cytochrome P450, family 19, subfamily A, polypeptide 1b), *esr1* (estrogen receptor), and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*), *ptgds* (prostaglandin D2 synthase) and *vtg1* (vitellogenin 1).

Gene	Sequence accession #	dir	Sequence	Product size (bp)
18S rRNA[a]	Y855349.1	fw	aaa cgg cta cca cat cca ag	116
		rv	tta cag ggc ctc gaa aga ga	
ar[b]	AY727529	fw	gtg cca tgc gct tcc aa	150
		rv	ctg acc ttt gtg ggc aag ga	
cyp19a1a[b]	AF288755	fw	gga gag ctg agc gct gag a	58
		rv	gga gcc gcg atc aac atc t	
cyp19a1b[b]	AJ277866	fw	gga cgt ttc caa tag act ctt cct aa	72
		rv	ata gcg atg gat ctt tat cag caa	
esr1[b]	AY727528	fw	aac tca tct ttg ctc agg atc tca	64
		rv	agc cat ccc ctc gac aca t	
3β-HSD[c]	BC045457	fw	atg aga tgc cct acc caa aga c	76
		rv	ccc ttt acc ttt gtg cca ttg	
ptgds[b]	UniGene Ppr.12268	fw	ttg gac acc ggc atc ctt	80
		rv	ttt aag aga ccc tca ggc atc tg	
vtg1[b]	AF130354	fw	gct gca gag gcc att tct aag a	68
		rv	agc att gcc cag aac ttt cag	

[a] Data source: Wintz et al. (2006).
[b] Data source: Dorts et al. (2009).
[c] Data source: Garcia-Reyero et al. (2009).

2.4. qRT-PCR analysis

Total RNA was extracted from the brain, liver and gonads using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations were determined spectrophotometrically using a NanoDrop ND-1000 UV-VIS Spectrophotometer at 260 nm.

1 μg of total RNA template was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche Diagnostics, Basel, Switzerland) and deoxynucleoside triphosphate (Sigma–Aldrich, Buchs, Switzerland). The reaction mixture was incubated for 5 min at 70 °C, 1 h at 37 °C, and the reaction was stopped by heating at 95 °C for 5 min.

cDNA was used as a template to perform qPCR using SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Basel, Switzerland).

We selected genes with focus on hormonal activity of UV-filters (Kunz and Fent, 2006; Kunz et al., 2006a; Weisbrod et al., 2007; Coronado et al., 2008; Zucchi et al., 2011) and on those altered by 17α-ethynylestradiol (EE2) (Hoffmann et al., 2006). Expressional changes on the estrogen receptor alpha (*esr1*), vitellogenin 1 (*vtg1*), and prostaglandin D2 synthase (*ptgds*) (Hoffmann et al., 2006; Dorts et al., 2009) were selected for estrogenic/antiestrogenic activity, mRNA changes on the androgen receptor (*ar*) for effect on the androgenic/antiandrogenic pathway. Effects on steroidogenesis were evaluated by determination of transcripts of P450 aromatases (*cyp19a1a* and *cyp19a1b*) and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*).

Gene-specific primers for 18S ribosomal RNA (*18S rRNA*), androgen receptor (*ar*), cytochrome P450, family 19, subfamily A, polypeptide 1a (*cyp19a1a*), cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1b*), estrogen receptor *alpha* (*esr1*), and 3β-hydroxysteroid-dehydrogenase (*3β-HSD*), *ptgds* (prostaglandin D2 synthase) and *vtg1* (vitellogenin 1) were obtained from fathead minnow published primers sequences (Table 1). The *18S rRNA* was selected in this study as reference gene for normalization, because their expression profile did not vary either under experimental conditions or in different analysed tissues.

Real time PCR amplification was performed on a Biorad CFX96 RealTime PCR Detection System (Biorad, Reinach, Switzerland) under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 61 °C for 60 s followed by a melting curve analysis post run.

For calculation of mRNA expression levels, normalization was performed against the reference gene *18S rRNA* (*18S*). The relative linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Transcriptional alterations of the different genes are expressed as fold change (log 2).

2.5. Vitellogenin analysis

For quantification of VTG a commercially available, homologous enzyme-linked immunosorbent assay (ELISA) for fathead minnow VTG from Biosense Laboratories (Biosense, Bergen, Norway) was used. The assay was performed according to the manufacture's protocol.

2.6. Histological analysis of testes and ovaries

Testes and ovaries of one male and one female fish per replicate tank were examined histologically to reveal changes induced by EHMC exposure. Whole animals were fixed in neutral buffered formalin (Roth, Arlesheim, Switzerland), dehydrated in graded ethanol and xylene, paraffinized in a tissue processor (Gewebeentwässerungsautomat TPC 15 Duo, Medite, Nunningen, Switzerland) and finally embedded in paraffin wax. Sections were taken along the long axis of the gonads at 5 μm intervals, in a serial step fashion. Two serial sections were collected from 3 steps equally spaced between the leading edge of the tissue and the midline of the gonad, for a total of 6 tissue sections/sample.

Cross sections of 5 μm thickness were stained with haematoxylin and eosin. Obtained sections were microscopically examined to determine the reproductive condition of fish. Pictures of testes and ovary were made using Olympus BX41 microscope (Olympus Schweiz AG, Volketswil, Switzerland, 40× magnification).

The ovaries were evaluated based upon relative frequencies of primary oocytes, previtellogenic, vitellogenic oocytes and atretic follicles. Frequencies were evaluated by counting the different stages in three randomly selected fields of vision/female. For each male, pictures of three randomly selected fields of vision were taken and the areas of the different stages (spermatogonia, spermato-

Table 2

Nominal and measured concentrations of EHMC in exposure waters at the day of the substance addition (day 1, 3, 7, 10), 24 h after the addition (day 4, 8, 11) and 48 h after the addition (day 5, 9, 12) during the 14 d exposure. Arithmetic means ± standard deviations of 3 replicates.

Nominal Concentration	Exposure waters						
	Measured concentration (µg/L)						
	0 h	% of nominal	24 h	% of nominal	48 h	% of nominal	Arithmetic Mean
30 µg/L	14.6 ± 0.7	48.7	1.55 ± 1.6	5.1	0.19 ± 0.2	0.6	5.4
300 µg/L	97.97 ± 80	32.7	12.85 ± 12.6	4.3	1.64 ± 1.9	0.5	37.5
1000 µg/L	675.91 ± 116.8	67.6	55.85 ± 54	5.6	1.77 ± 1.3	0.2	244.5
3000 µg/L	1165.17 ± 1.3	38.8	14.95 ± 18.6	0.5	2.42 ± 3.7	0.1	394.2

cytes and spramatides) were measured using the software ImageJ (Abramoff et al., 2004).

2.7. Chemical analysis

To determine actual EHMC concentrations during exposure different amounts of aquaria water were taken during the experiment prior to water renewal (0 h) and after 48 h exposure from randomly selected replicate tanks. Aliquots of 250 mL were taken at the lower EHMC concentrations (30 and 300 µg/L) and the controls, and 25 mL for the higher EHMC concentrations (1000 and 3000 µg/L) for EHMC analysis. Water sampling took place on days 1–3, 7–9 and 10–12, respectively. The water samples were stored in the dark at −20 °C until analysis by HPLC. Extraction of water samples and chemical analysis was performed according to Kunz et al. (2006b). To control for the influence of Cremophor RH40 on the EHMC stability, a control experiment including three replicate tanks without fish was performed with 1000 µg/L EHMC over 48 h.

2.8. Data analysis and statistics

The data were illustrated graphically with GraphPad® Prism 5 (GraphPad Software, San Diego, CA, USA). Data distribution for normality was assessed with the Kolmogorov–Smirnov test and variance homogeneity with the Bartlett test. Differences between treatments were assessed by analysis of variance followed by a Tukey test (Bartelett test $p > 0.05$) to compare treatment means with respective controls. Results are given as means ± standard error of means. Differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. EHMC concentration and gross effects

To determine actual EHMC concentrations in exposure water, samples were analysed by HPLC-DAD at 0 h, 24 h and 48 h. The EHMC concentrations were much lower than nominal at the beginning (0 h), and they decreased further at 24 h and 48 h (Table 2). The arithmetic means at these time points are taken as exposure concentrations, as we assume that arithmetic means are best estimates.

To evaluate the role of Cremophor RH40 as a solubilizing agent, a control experiment was performed in tanks lacking fish with nominal concentration of 1000 µg/L EHMC using EtOH as a solvent, and a 1:1 dilution of EHMC in 20% Cremophor RH40. The data show that Cremophor RH40 was not responsible for the drop of the EHMC concentration in the tank as it was even more pronounced with EtOH, nor is this drop due to metabolism by fish. The median of EHMC concentration using Cremophor RH40 as solvent were 280 and 420 µg/L, respectively, in the two replicates, immediately after giving EHMC into tank water.

No significant differences in survival, body length and in the gonadosomatic index (Table 3; Figs. 1 and 2) occurred in any of the EHMC-exposed fish compared to control fish.

Data on expressional changes, VTG and histology from control groups and from the four different EHMC exposure treatments are depicted in the graphs (Fig. 1–6 and in supplementary material, Figs. S1) as water (water control), solvent control (Crem/EtOH), environmentally realistic concentration, 5.4 µg/L (E), low concentration, 37.5 µg/L (L), medium concentration, 244.5 µg/L (M) and high concentration, 394 µg/L (H).

3.2. Alterations in gene expression

There were no significant differences between the solvent control and the water control in any of the transcripts. Therefore, only the qRT-PCR data relative to the solvent control are shown in Fig. 3(A–D).

The expression pattern in males showed an overall tendency (albeit not significant) for an up-regulation of the esr1 transcript mostly in the brain (Fig. 3A). However, in the liver of females esr1 expression was down-regulated in a concentration-dependent manner, being significant at 394 µg/L EHMC. As observed in males, esr1 mRNA showed a slight increase (albeit not significant) in the brain of females (Fig. 3A). In the liver of both males and females vtg1 mRNA showed an overall up-regulation trend (although not significant).

In female fish, the expression of the androgen receptor (ar) was significantly down-regulated in the liver by 37.5, 244.5 and 394 µg/L EHMC, as well as in the ovary, although not significantly (Fig. 3C). In the liver of males the transcription of ar tended to be down-regulated in a concentration-dependent manner (Fig. 3C). A significant inhibition of 3β-HSD transcript was noted in the liver of
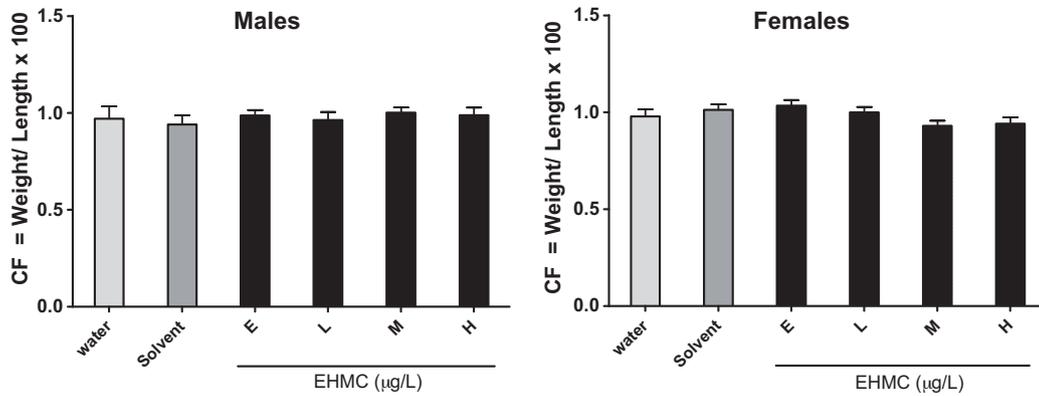
Table 3

Body weight, length and survival of exposed fish after 14 d of exposure ($n = 24$, 16 females and 8 males per treatment).

	Exposure (µg/L)	Survival (%)	Male		Female	
			Body weight (g)	Body length (mm)	Body weight (g)	Body length (mm)
Controls	Water	100	3.09 ± 1.2	67.6 ± 6	1.47 ± 0.3	53.3 ± 2.9
	Solvent	100	3.57 ± 0.5	72.5 ± 3.9	1.70 ± 0.3	55.1 ± 3.6
EHMC	5.4	100	2.88 ± 0.4	66.3 ± 4.2	1.62 ± 0.2	53.9 ± 2.3
	37.5	96	2.99 ± 0.1	67.9 ± 2.1	1.54 ± 0.3	53.5 ± 4.1
	244.5	96	2.86 ± 0.6	65.5 ± 4.5	1.64 ± 0.3	55.9 ± 3.5
	394.2	100	2.72 ± 0.4	65.0 ± 3.0	1.51 ± 0.3	54.3 ± 3.0

Fig. 1. Condition factor (CF) of female and male fathead minnows. Controls (grey bars) and fish exposed to increasing EHMC concentrations (black bars). Values are means ± SEM (solvent control: 12 females and 6 males; water control and EHMC treatments: 16 females and 8 males). No significant differences occurred.

male fish at 37.5, 244.5 and 394 µg/L EHMC, and at 244.5 µg/L and 394 µg/L EHMC in female fish (Fig. 3D). Furthermore, a tendency of 3β-HSD mRNA down-regulation was noted at all concentrations in the ovary (Fig. 3D).

No significant mRNA alterations in any of the tissues of male and female fish were noted for *cyp19a1a*, *cyp19a1b* and *ptgds* transcripts, thus they were in the range of inter-individual variability (supplementary material, Fig. S1).

In summary, even if some transcripts altered by EHMC displayed a tendency, the observed alterations lacked statistical significance in most cases. Nevertheless, significant down-regulation occurred in the liver for transcripts involved in hormonal pathway such as *esr1*, *ar*, and 3β-HSD. Some gene expression profiles varied among tissues, and in some cases, between male and female fish.

3.3. Plasma vitellogenin and secondary sex characteristics

The plasma VTG concentration in male was significantly increased by 224.5 µg/L EHMC (mean concentration: 13.06 µg/mL) as compared to water (mean concentration: 0.37 µg/ml) and solvent control (mean concentration: 0.22 µg/mL). The observed increase in plasma VTG in the 394 µg/L EHMC dose group (mean concentration: 4.95 µg/mL, Fig. 4A) lacked statistical significance. VTG plasma concentrations in female fish did not significantly change (supplementary material, Fig. S2).

No significant alterations in the number and score of nuptial tubercles of EHMC-exposed males as compared to control fish were noted (supplementary material, Fig. S3).

3.4. Histology of testis and ovary

We analysed the histology of testis and ovary of fish from the water and solvent controls, and in all EHMC groups. In the testis of males exposed to the highest EHMC concentration, alterations in the frequency of cells at different stages of spermatogenesis (spermatocytes and spermatides) occurred (Fig. 5A). These testes were characterized by a significant decrease in spermatocytes compared to solvent control fish and an increase in mature spermatides. The EHMC-induced changes on the histology of testis are shown in Fig. 6.

In females exposed to EHMC, there was also a visible alteration in the frequency of cells at the different stages of oogenesis (primary oocytes, previtellogenic, vitellogenic oocytes and atretic follicles) (Fig. 5B). A reduced number of primary oocytes occurred in the ovary of females exposed to the highest EHMC concentration. On the other hand, a significant increase in vitellogenic oocytes was noted compared to the solvent control. The changes induced by EHMC in the ovary are depicted in Fig. 7.

4. Discussion

EHMC is one of the most frequently used UVB-filter in sunscreen formulations. Previously, we showed that this UV-filter has multiple hormonal activities *in vitro* (Kunz and Fent, 2006). Estrogenic activity has been reported at high concentrations in male medaka EHMC (Inui et al., 2003), while no significant VTG induction was observed in juvenile fathead minnows exposed to low aqueous concentrations (Kunz and Fent, 2006).
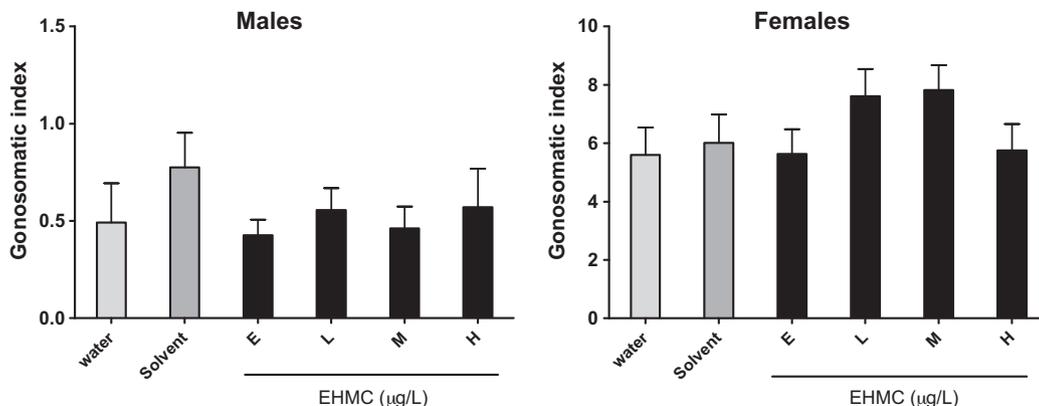

Fig. 2. Gonosomatic index (GSI) of male and female fathead minnows. Controls (grey bars) and fish exposed to increasing EHMC concentrations (black bars). Values are means ± SEM (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males). No significant differences occurred.
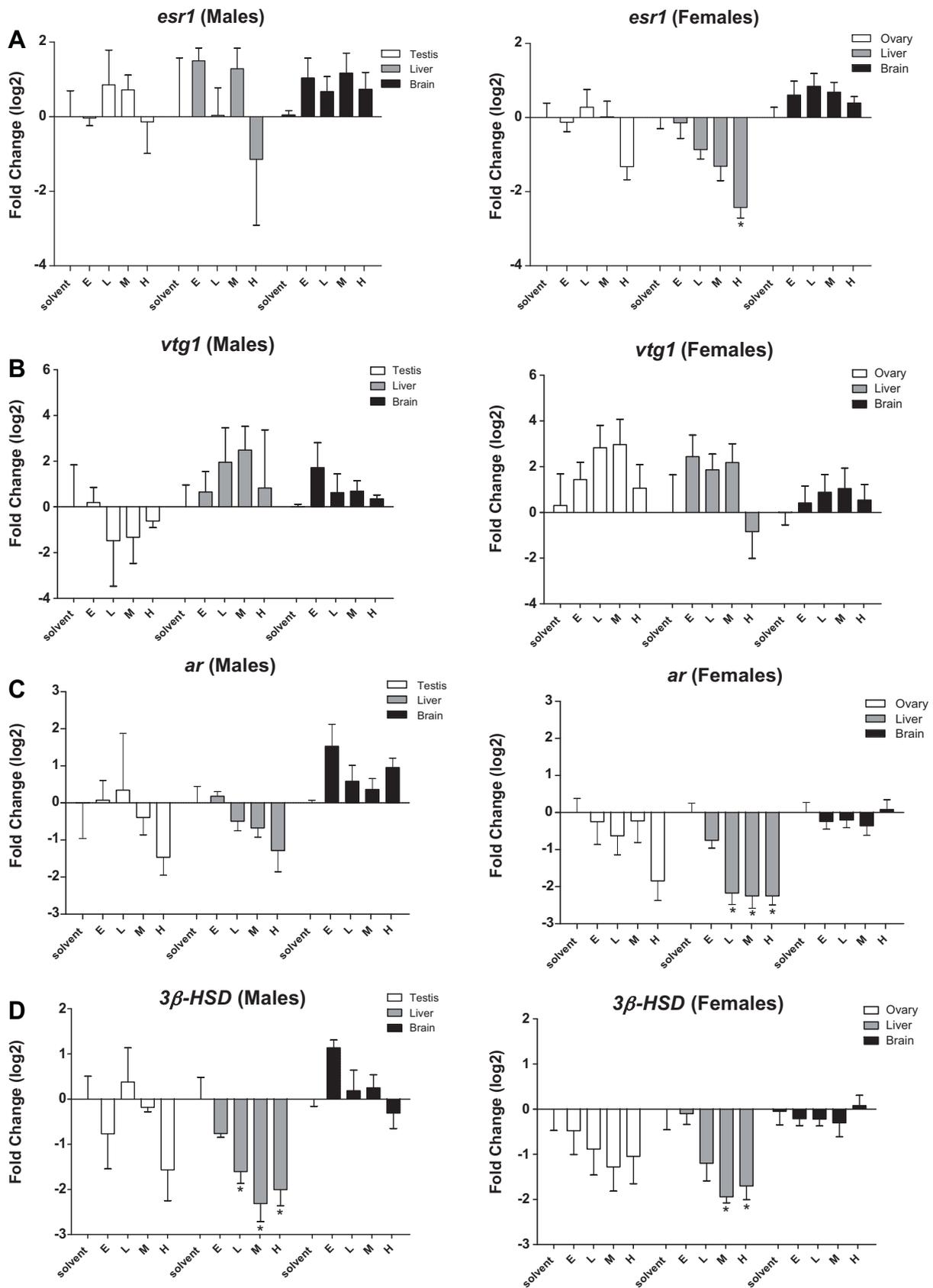
Fig. 3. Relative gene expression of *esr1* (A), *vtg1* (B) *ar* (C) and *3β-HSD* (D) in liver, brain and gonads of male and female fathead minnows after EHMC exposure. Data in the graphs are represented as follows: solvent (Crem/EtOH), *E* (environmental concentration: 5.4 µg/L), *L* (low concentration: 37.5 µg/L), *M* (medium concentration: 244.5 µg/L) and *H* (high concentration: 394 µg/L). Relative transcript abundance was quantified by real-time reverse transcription PCR; the fold changes (log 2) in mRNA abundance as compared to solvent controls (solvent control: 9 females and 3 males; water control and EHMC treatments: 12 females and 4 males) were determined using $2^{-\Delta\Delta CT}$ method. Results are given as the mean value ± SEM. Asterisks indicate significantly altered expression compared to controls (*$p < 0.05$).
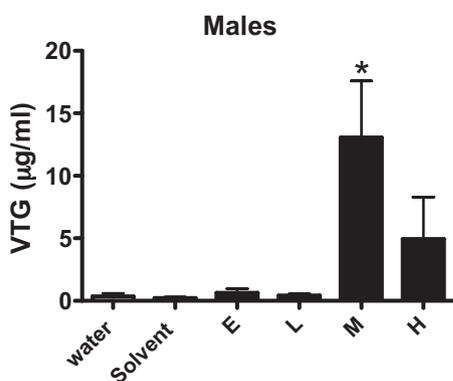
Males

Fig. 4. Concentration of plasma vitellogenin in male fathead minnows of water and solvent control, and fish exposed to EHMC concentrations (black bars). Data in the graph are represented as follows: water (water control), solvent control (Crem/EtOH), E (environmental concentration: 5.4 μg/L), L (low concentration: 37.5 μg/L), M (medium concentration: 244.5 μg/L) and H (high concentration: 394 μg/L). Results are given as mean value ± SEM (solvent control: 6 males; water control and EHMC treatments: 8 males). Asterisk denotes significant induction of vitellogenin in males exposed to 244.5 μg/L as compared to control groups ($^*p < 0.05$).

In our present study we demonstrate that EHMC displays endocrine activity in adult fathead minnows (*Pimephales promelas*) based on data on alterations in gene expression, VTG plasma concentrations and gonad histology. The activity of EHMC seems not high, and the toxicological profile seems complex due to the multiple hormonal activities exhibiting different net effects in expressional profiles and associated physiological effects.

4.1. Water analysis

The measured EHMC concentrations were significantly lower than the nominal concentration and decreased further during exposure. The decrease was due to several factors including adsorption of highly lipophilic EHMC (log K_{ow} 6.1, Balmer et al., 2005) to surfaces such as tank walls, spawning substrate etc., as well as uptake into fish and eggs. The low concentration of EHMC (as compared to nominal values) at the beginning and the further decrease during exposure is partly due to the use of a solubilizing agent (in the present study Cremophor RH40), and to a minor extent, to dimerization of EHMC (Broadbent et al., 1996). The control experiment (data not shown) demonstrated that Cremophor RH40 itself does not led to the EHMC decrease observed during the exposure time (0–48 h); an immediate drop in EHMC concentration occurred with both solvents, Cremophor RH40 and EtOH. Therefore, our exposure was rather pulsative than constant, and consequently, exposure
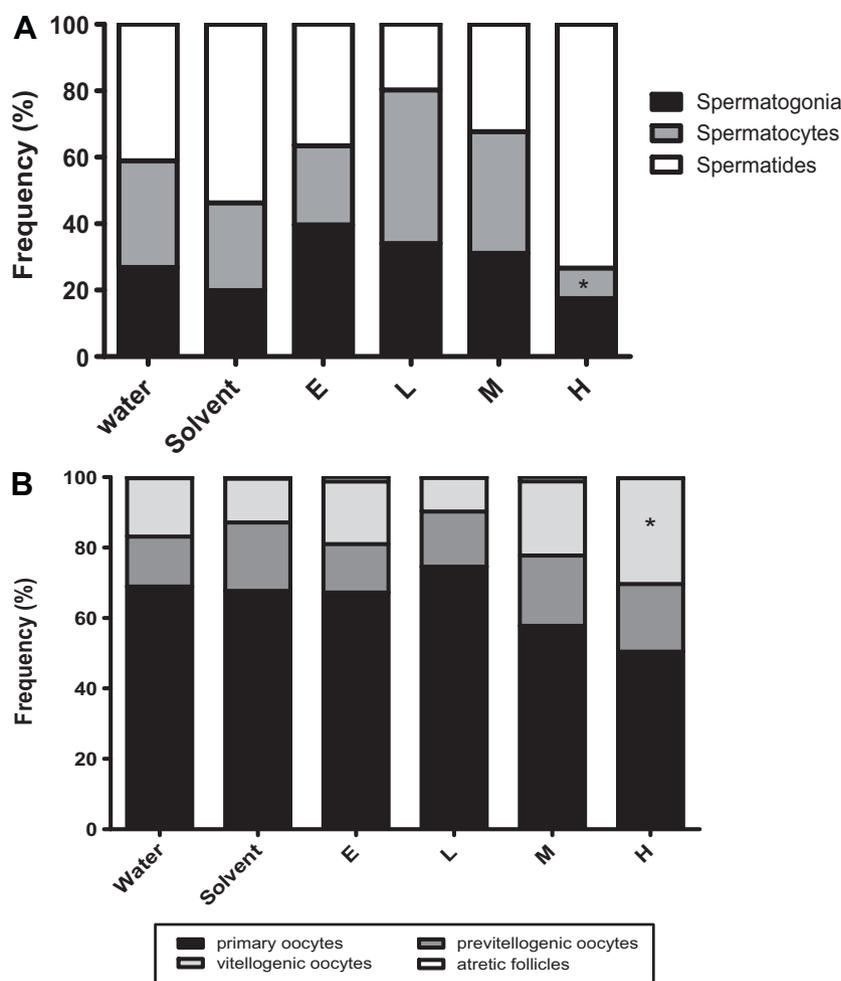

Fig. 5. Relative percentage of cells at the different stages of (A) spermatogenesis in testis of male, and (B) oogenesis in ovary of female fathead minnow after EHMC exposure determined in three randomly selected areas per fish. Data in the graphs are represented as follows: water (water control), solvent (Crem/EtOH), E (environmental concentration: 5.4 μg/L), L (low concentration: 37.5 μg/L), M (medium concentration: 244.5 μg/L) and H (high concentration: 394 μg/L). Results are given as means ± SEM (solvent control: 3 females and 3 males; water control and EHMC treatments: 4 females and 4 males). Asterisks denote significant difference from solvent control at $p \leq 0.05$.
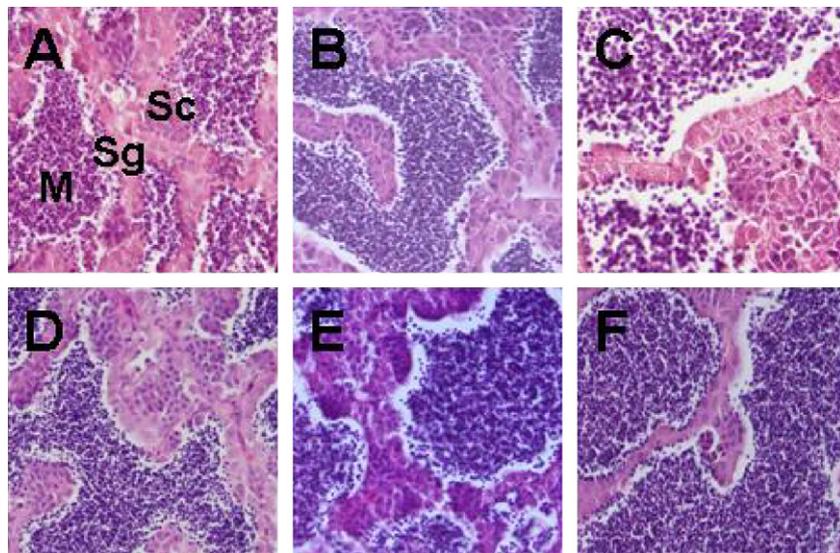
Fig. 6. Histological sections of typical seminiferous tubules in testis of males ($n = 3$ in solvent control, $n = 4$ in water control and EHMC treatments). (A) Water control, (B) solvent control, (C) fish exposed to 5.4 μg/L EHMC, (D) fish exposed to 37.5 μg/L EHMC, (E) fish exposed to 244.5 μg/L EHMC and (F) fish exposed to 394 μg/L EHMC. Visible are the increase in spermatides and the decrease in spermatocytes. Spermatogonia (Sg), spermatocytes (Sc) and spermatides (mature sperms M) are marked.

concentrations were much lower than under a constant EHMC exposure. This control experiment also shows that metabolism is not the reason for the drop in EHMC.

The measured EHMC concentrations of 5.4 and 37.5 μg/L are in the range of levels found in wastewater, but are higher than those found in coastal waters (390 ng/L) (Langford and Thomas, 2008). Maximal wastewater concentrations were 19 μg/L in Switzerland (Balmer et al., 2005), and 1.7 μg/L in Spain (Rodil et al., 2009). In a lake with swimming activity EHMC levels reached up to 3.0 μg/L (Rodil et al., 2009).

4.2. Gene expression analysis

Exposure of female fathead minnows to 394 μg/L EHMC led to a significant decrease in the expression of *esr1* in the liver. The modulation of this sex-steroid receptor is in line with previous UV-filter studies (Schlumpf et al., 2008), where often an inhibition of ERα

was observed, in particular by 4-MBC. The down-regulation of *esr1* in the liver suggests an antiestrogenic activity of EHMC, which is in accordance to previous *in vitro* data (Kunz and Fent, 2006). In contrast to our findings, Inui et al. (2003) reported increases of mRNA expression of *esr1*, in male medaka (*Oryzias latipes*) after exposure to very high EHMC levels (9.87 mg/L). Additionally, *vtg* mRNA was up-regulated (although not significantly) in male liver at 244.5 μg/L EHMC. The lack of statistical significance in the induction of *vtg* in male liver may partly be due to different time-course of induction and stability of mRNA as compared to the VTG protein. The transient EHMC concentration may have resulted in a transient *vtg* mRNA induction.

In our study EHMC significantly inhibited the expression of *ar* in female liver at 37.5 μg/L, which is in line with data obtained *in vitro* in recombinant yeast (Kunz and Fent, 2006). In male medaka, antiandrogenic activity was not observed (Inui et al., 2003), which is apparently consistent with our observation in males. In com-
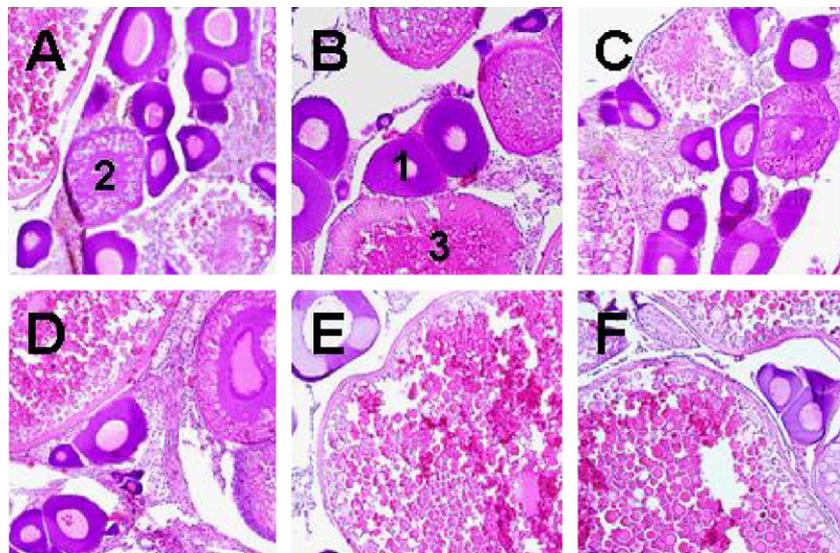

Fig. 7. Histological section of a typical ovary ($n = 3$ in solvent control, $n = 4$ in water control and EHMC treatments). (A) Water control, (B) solvent control, (C) fish exposed to 5.4 μg/L EHMC, (D) fish exposed to 37.5 μg/L EHMC, (E) fish exposed to 244.5 μg/L EHMC and (F) fish exposed to 394 μg/L EHMC. The decrease in primary oocytes and the increase in vitellogenic oocytes is visible. (1) primary oocytes, (2) previtellogenic oocytes and (3) vitellogenic oocytes.

parison, the estrogenic EE2 results in down-regulation of the *ar* transcript (Filby et al., 2007), whereas the antiandrogenic flutamide results in a strong down-regulation in hepatic *ar* mRNA. Based on these data an antiandrogenic activity of EHMC cannot be ruled out in the liver of females, but seems not to occur in males.

EHMC exposure also affected the mRNA expression of an important enzyme involved in steroidogenesis. 3β-Hydroxysteroid-dehydrogenase (3β-HSD) is involved in the conversion of pregnenolone to progesterone (Arukwe et al., 2008). In our study, a significant down-regulation of *3β-HSD* transcripts was observed in the liver (Fig. 4D). In the ovaries, there was also a tendency for down-regulation. The inhibition of *3β-HSD* may result in imbalance of steroid hormones, and in turn, reproductive dysfunction in fish (Villeneuve et al., 2008).

Potential changes in hormones may also in part be a reason for the observed histological changes in testes and ovaries of EHMC-exposed fish (Figs. 5–7). The fact that exposure of fish to E2 or EE2 (Govoroun et al., 2001; Baron et al., 2005) led to inhibition of *3β-HSD* mRNA suggests that EHMC exhibits an estrogenic activity in the liver, and to a lesser extent in ovaries.

In summary, our data indicate that EHMC displays multiple hormonal activities, including antiestrogenic activity (down-regulation of *esr1*), estrogenic activity (down-regulation of *3β-HSD*), and antiandrogenic activity (down-regulation of *ar*). However, significant alterations were observed in the liver of male and female fish only.

4.3. Plasma vitellogenin

EHMC led to VTG induction in male fathead minnows, which was significant at 244.5 μg/L EHMC. Additionally, *vtg* mRNA is induced at this concentration (although not statistically significantly). The lack of statistical significance in the induction of *vtg* in male liver may partly be due to different time-course of induction and stability of mRNA versus protein. The transient EHMC exposure (due to immediate decrease of EHMC after water-renewal) may have affected the transcriptional level, but less the VTG protein, which is more stable. Statistical significance may also be lacking, because only 4 males per group were analysed for *vtg* mRNA induction, which is in contrast to 8 males for VTG plasma protein determination. Induction of plasma VTG as a biomarker for estrogenic compounds (Sumpter and Jobling, 1995) was demonstrated for many environmental chemicals (e.g. Jobling et al., 1998; Sohoni et al., 2001). VTG induction is associated with adverse effects on fertility and reproduction (Sumpter and Johnson, 2005; Tyler et al., 1998). We previously showed that estrogenic UV-filters including benzophenone-1, benzophenone-2 and 3-benzylidene camphor led to VTG induction (Kunz et al., 2006b; Kunz and Fent, 2009), which was paralleled by adverse effects on fertility and reproduction (Kunz et al., 2006b; Weisbrod et al., 2007). VTG induction and associated reduced fertility was also shown for benzophenone-3 in medaka (Coronado et al., 2008). Very high EHMC concentrations of 9.87 mg/L resulted in *vtg* mRNA induction in medaka (Inui et al., 2003). Data of our present study also suggest an estrogenic activity of EHMC in fathead minnows, but it remains open, whether this is based on direct interaction of the compound with estrogen receptors, and/or indirectly by the action on enzymes involved in steroidogenesis.

4.4. Histology

EHMC induced effects on gonad histology of male and female fish. Males exposed to 394 μg/L EHMC displayed significant alterations in the frequencies of different spermatogenic stages in testes, as compared to control males. Spermatogenesis appeared to be inhibited, as testes were characterized by enlarged areas of mature

sperms and a reduced presence of spermatocytes. Spermatogonia apparently did not undergo any further differentiation into spermatocytes. Similarly, in fish exposed to other UV-filters, namely 3-BC (Kunz et al., 2006b) and BP-2 (Weisbrod et al., 2007), an inhibition of testicular development was also shown. Similar to EHMC, an increase in the frequency of spermatides and a decreased frequency of spermatocytes were observed. The histological effects in the testes are also analogous to E2 and EE2, showing inhibition of testicular development, depending on the dose (Gimeno et al., 1998; Miles-Richardson et al., 1999a; Pawlowski et al., 2004). Analogously, an inhibition of testicular development was reported for fish exposed to the weak estrogen receptor agonist 4-nonylphenol, which led to a significant reduction of fecundity (Harries et al., 2000; Miles-Richardson et al., 1999b) and a significant necrosis of sperm cells and spermatozoa (Miles-Richardson et al., 1999b). These data lead to the conclusion that the histological effects of EHMC in the testis indicate an overall estrogenic or antiandrogenic effect.

EHMC exposure also affected ovaries in female fathead minnows, where a significant increase in vitellogenic oocytes was observed at 394 μg/L EHMC. Different effects were found after exposure of fathead minnow to 3-BC (Kunz et al., 2006b), BP-2 (Weisbrod et al., 2007) and additional compounds (e.g. Leino et al., 2005). 3-BC and BP-2 and high concentrations of weak estrogen receptor agonists such as methoxychlor also resulted in increased follicular atresia (Ankley et al., 2001). Therefore, EHMC is concluded to exhibit a different activity on the ovary than the previously analysed UV-filters 3-BC and BP-2.

In summary, the data on gonad histology suggest an estrogenic or antiandrogenic effect of EHMC in testis. This may be related to the effect of EHMC on either steroid hormone receptors or steroidogenesis, or a combination of both (Jensen et al., 2001).

5. Conclusions

Induction of VTG and alterations in the histology of testes indicate an estrogenic and/or antiandrogenic activity of EHMC. The observed changes in gonad histology suggest a negative interference with maturation of sperms (significantly more mature spermatides and significant less immature spermatocytes in seminiferous tubules) and oocytes (significantly more vitellogenic and less primary oocytes). The targeted gene analysis showed that EHMC displays multiple hormonal activities including estrogenic (down-regulation of *3β-HSD*), antiestrogenic (down-regulation of *esr1*), and antiandrogenic activity (down-regulation of *ar* in the liver of females). These expressional changes are supported by our previous *in vitro* study showing multiple hormonal activities of EHMC (Kunz and Fent, 2006). Additionally, the data demonstrate that the toxicological profile of EHMC seems complex due to the multiple hormonal activities. Most sensitive expressional changes of EHMC were observed at concentrations in the range of those found in municipal wastewater (Balmer et al., 2005), or in most contaminated surface waters (Rodil et al., 2009; Straub, 2002; Balmer et al., 2005; Fent et al., 2010; Langford and Thomas, 2008). Furthermore, EHMC accumulates in all trophic levels (Fent et al., 2010), and may contribute to the additive action of UV-filters (Kunz and Fent, 2009). Forthcoming studies are needed to investigate the effects of EHMC on fertility and reproduction of fish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.01.013.

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