Supporting information

Synthetic Progestins Medroxyprogesterone Acetate and Dydrogesterone and Their Binary Mixtures Adversely Affect Reproduction and Lead to Histological and Transcriptional Alterations in Zebrafish (*Danio rerio*)

Yanbin Zhao,[a] Sara Castiglioni,[b] and Karl Fent[a,c]*

[a] University of Applied Sciences and Arts Northwestern Switzerland, School of Life Sciences, Gründenstrasse 40, CH–4132 Muttenz, Switzerland

[b] IRCCS – Istituto di Ricerche Farmacologiche "Mario Negri", Environmental Biomarkers Unit, Department of Environmental Health Sciences, Via La Masa 19, I-20156, Milan, Italy

[c] Swiss Federal Institute of Technology (ETH Zürich), Institute of Biogeochemistry and Pollution Dynamics, Department of Environmental System Sciences, CH–8092 Zürich, Switzerland

*** Corresponding author:**

Karl Fent

Phone: +41 61 467 4571; fax: +41 61 467 47 84

e-mail: karl.fent@fhnw.ch; karl.fent@bluewin.ch

Number of pages: 51

Number of figures: 13

Number of tables: 8

Materials and methods

Chemicals. Medroxyprogesterone 17-acetate (MPA, European Pharmacopoeia (EP) Reference Standard, CAS no.: 71-58-9), dydrogesterone (DDG, European Pharmacopoeia (EP) Reference Standard, CAS no.: 152-62-5) and dimethylsulfoxide (DMSO) (purity ≥ 99.5%) were purchased from Sigma-Aldrich (Fluka AG, Buchs, Switzerland). Medroxyprogesterone (MP) used as internal standard (IS) for chemical analysis was purchased from Sigma Aldrich (Sigma-Aldrich CO., Dorset, UK). All solvents used for chemical analysis were of reagent grade or higher. Acetone and methanol were from Carlo Erba reagents (Italy). Acetonitrile for LC-MS, acetic acid and ammonium hydroxide solution (25%) were from Fluka (Buchs, Switzerland). Water used for sample preparation and HPLC was of HPLC grade and in-house prepared (Nanopure Diamond, Barnstead, Switzerland and MILLI-RO PLUS 90, MILLIPORE, Molshelm, France). For solid-phase extraction, Bakerbond SPE C18 cartridges (200 mg/3 mL) (J. T. Baker, Deventer, Netherlands) were used. Chromatographic analysis was performed using an XTerra MS C18 3.5 μm, 1x100 mm column acquired from Waters (Waters Corp., Milford, MA).

KoiMed Sleep (Ethylene glycol monophenyl ether) was purchased from KOI&BONSAI Zimmermann (Bühlertann, Switzerland). Heparin ammonium salt (100 KU) was obtained from Sigma-Aldrich (Fluka AG, Buchs, Switzerland), and BD Micro-Fine + Innen sterile insulin syringes (0.5 mL, 0.33 mm (29G) × 12.7 mm) from Becton Dickinson (Allschwil, Switzerland). Bouin's solution (HT10132) used for fixation was obtained from Sigma-Aldrich (Fluka AG, Buchs, Switzerland).

Maintenance of Zebrafish. Adult zebrafish maintenance was conducted as previously.[1,2] In brief, adult zebrafish (*Danio rerio*) were obtained from Harlan Laboratories, Inc. (Itingen, Switzerland), transferred to culture tanks (300 L) and acclimatized for more than two months in our laboratory prior to the experiment. Fish were held in reconstituted deionized water (salts: CaCl2×2H_2O 147.0 g/L, KCl 2.9 g/L, $MgSO_4$×7H_2O 61.6g/L, $NaHCO_3$ 32.4 g/L) with a conductivity of 470–480 μS/cm. Water was renewed weekly and held constant at 27±1 °C. The photoperiod was 14:10 h light/dark. Fish were fed twice daily with a combination of

frozen brine shrimps (*A. salina*), white mosquito larvae and *Daphnia magna*. Water parameters, such as nitrate, nitrite and pH, were controlled regularly using Test strips (Easy Test, JBL) and the oxygen concentration was ≥80%.

Flow-through System Conditions. Flow Control Devices (DVS 70F, Pequitec, Switzerland) were used to regulate the flow of reconstituted water into glass mixing chambers, equipped with magnetic stirrers. The progestins and solvent control supply solution was dosed into 1L mixing chambers of these groups, by means of a computer controlled dispenser (SED 6000 series Dispenser, Pequitec, Switzerland) resulting in nominal exposure concentrations of MPA, DDG and their mixtures. Each tanks was supplied from the mixing chambers through Teflon tubing using gravity driven splitting units (N-Flex, Pequitec, Switzerland) with a flow rate of 200 mL every 14 min and 24 sec and allocated to the different chemical concentrations and solvent control.

RNA Isolation and RT-PCR Analysis. Total RNA were extracted from adult tissues and embryo pools by use of the RNeasy Mini Kit (Qiagen, Basel, Switzerland). The samples were then treated with RNase-free DNase (Qiagen, Basel, Switzerland) with a concentration of DNase at 30 Kunitz units/sample to purify the RNA from DNA contamination, and to subsequently remove DNase and divalent cations from the samples. RNA concentrations and qualities were analyzed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies Inc. Wilmington DE, U.S.); the purity of each sample was between 1.8 and 2.0 (260 nm/280 nm ratio).

Total RNA (1 μg) was reverse-transcribed using the cDNA Synthesis Kit (Promega, Dübendorf, Switzerland). The total volume of 14 μL (RNA + RNase-free water) was incubated with 1 μL of random hexamers (Roche, Switzerland) at 70◦C for 5 min to melt secondary structures within template. A volume of 10 μL of a master-mixsolution, containing 5 μL MMLV 5× reaction buffer, 1.25 μL dNTPs (Sigma–Aldrich, Switzerland), 0.4 μL MMLV reverse transcriptase and 3.35 μL RNase-free water, were added to each sample. The complete reaction mixture was incubated at 37◦C for 50 min, following 5 min at 95◦C to stop the reaction. The cDNA was then stored at −20◦C.

RT-PCR was conducted on BIO-RAD CFX96 Real-Time PCR Detection System (BIO-RAD, Switzerland) using SYBR Green Fluorescence (Roche Diagnostics, Basel, Switzerland) as recommended by the manufacture's guidelines. Two-step real-time PCR profile was used: enzyme activation step at 95◦C (10 min) and 40 cycles of 95◦C (30 s), 58–62◦C (60 s) depending on the target transcript, followed by a melting curve analysis post run (65–95◦C) which confirmed specificity of chosen primers as well as absence of primer dimers. For the primers designed in the present laboratory, the intron/exon boundary-spanning primers were preference to minimize the DNA contamination, when the primers were designed by use of Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Normalization and calculation of expression levels was performed as previously.[1,2] The mRNA expression levels of different target genes are expressed as fold change by use of the delta−delta CT method of relative quantification.

Chemical Analysis. For chemical analysis, standards were dissolved in methanol at concentration of 1 mg/mL and subsequently diluted to 10 ng/μL (stock solution). Working solutions were prepared freshly before each analytical run by diluting stock solution in methanol to concentration of 1, 0.1 and 0.01 ng/μL. All stock and working solutions were stored at -20 °C in the dark.

Four groups of water samples from the adult zebrafish exposure experiment were collected at day 1, 7, 14 and 21 of exposure. At collection, equal amounts of water sample from each replicate were pooled (in total 200 mL for solvent control, 50 ng/L MPA and 50+500 ng/L mixture; 100 mL for 500 ng/L MPA, 500 and 5000 ng/L DDG and 500+5000 ng/L mixture). The nominal concentrations were as follows: MPA alone 50 and 500 ng/L, DDG alone 500 and 5000 ng/L and MPA+DDG mixture at 50+500 and 500+5000 ng/L. The solvent (0.01% DMSO) controls from the exposure experiments and analytical blanks were included within each set of samples.

Four groups of water samples from the embryo experiment were analyzed. At collection, equal amounts of water sample (100 mL) from each replicate were pooled (in total 500 mL per treatment). Two groups were collected at the beginning (T0; G1 and G3) and two after 24 h (T24h; G2 and G4). In embryo exposures, three dose groups with following nominal

concentrations were assessed: MPA alone 5, 50 and 500 ng/L, DDG alone 5, 50 and 500 ng/L and MPA+DDG mixtures 5+5, 50+50 and 500+500 ng/L. Water and solvent (0.01% DMSO) controls from the exposure experiments and analytical blanks were included within each set of samples.

Solid-Phase Extraction. Aqueous samples were stored in polypropylene (PET) bottles at -20°C in the dark until analysis (the maximum storage period was six weeks). Briefly, different aliquots of samples (5-100 mL) were prepared for extraction depending on the concentrations of exposure. . 5 mL aliquots were prepared for samples spiked at 5000 ng/L, 10 mL aliquots were prepared for samples spiked at 500 ng/L, 50 mL aliquots for samples spiked at 50 ng/L, and 100 mL aliquots for samples spiked at 5 ng/L and for control samples (water control and DMSO control). Each aliquot was spiked with 2 ng of IS and the pH was adjusted to 7.0 with 25% ammonium hydroxide. SPE have been performed using an automated system, GX-274 ASPEC (Gilson, Middleton, WI, USA). Cartridges were conditioned before use by washing with 5 mL methanol and 3 mL Milli-Q water. Samples were then passed through the cartridges under vacuum, at a flow rate of 10 mL/min. Cartridges were vacuum dried for 10 minutes and eluted with 3 mL of methanol. The eluates were dried under a gentle nitrogen stream.

Liquid Chromatographic - Tandem Mass Spectrometry (HPLC-MS/MS). Dried samples were re-dissolved in 100 µL of Milli-Q water: methanol 80:20, centrifuged for 2 min at 2500 rpm (Megafuge 1.0, Heraeus Instruments) and transferred into glass vials for instrumental analysis. The HPLC system consisted of a 1200 Series Binary Pump SL and Autosampler (Agilent Technologies, Santa Clara, CA). The MS system was an API 5500 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems – Sciex, Thornhill, Ontario, Canada). The MS analysis parameters are reported in Table S1.

The chromatographic separation was performed by gradient elution using acetic acid 0.1% in Milli-Q water as solvent A and acetonitrile as solvent B at a flow rate of 70 µL/min. The analysis started with 60% of eluent A, followed by a 10-min linear gradient to 70% of eluent B, a 2-min linear gradient to 100% of eluent B, a 2-min isocratic washing step with

eluent B and a 1-min linear gradient to 100% of eluent A, which was finally maintained for 15 minutes to equilibrate the column. The injection volume was 2 µL and the column was kept at room temperature.

The MS analysis was performed in the positive ion mode with a spray voltage (IS) of 5.5 kV and a source temperature of 350°C. Mass spectrometer analyses were done in the multiple reaction monitoring (MRM) mode, measuring the fragmentation products of the protonated pseudo-molecular ions of the investigated compounds (Table S1). The choice of fragmentation products for each substance and the optimization of energy collisions and other instrument parameters were performed in continuous-flow mode using standard solutions at concentration of 100 pg/µL. Quantification has been performed using the isotopic dilution method and calibration curves were made freshly before each analytical run.

Results of chemical analyses of the different groups of water samples are reported in Tables S2-S5.

Table S1. Analyzer conditions for MRM determination of medroxyprogesterone acetate, dydrogesterone and medroxyprogesterone (IS).

	Declustering Potential (DP)	Entrance Potential (EP)	Precursor ion (m/z)	Quantifier transitions (m/z) and collision energy (eV)	Qualifier transitions (m/z) and collision energy (eV)
Dydrogesterone	40	8	313.1	173.1 (29)	159.1 (30) 171.1 (29) 105.1 (50)
Medroxyprogesterone acetate	40	8	387.1	327.1 (19)	123.1 (32) 285.1 (24)
Medroxyprogesterone	40	8	345.2	123.1 (30)	97.1 (30)

Results and discussion

Chemical analysis. MPA and DDG concentrations in the embryo exposures were monitored during the 6 days exposure period by collecting two samples at T 0h and two samples at T 24h (pools of replicates). The concentrations of both chemicals were stable during the experiment conducted in April 2014 (Tables S4 and S5), and confirmed the nominal levels of exposure. During the adult zebrafish exposure experiment, a decrease of nominal concentrations for MPA and DDG has been noted (Table S2 and S3). MPA concentrations were about 14-32% lower than nominal; while DDG concentrations were 67-82% lower than nominal. The similar phenomenon was also demonstrated in our previous studies for DRG and P4.[2] As previously observed, some factors could be responsible for this decrease as for example, adsorption to the flow-through system (tubes), fish, particles and debris in the exposure experiment. The explanations for the differed degrees of decreased concentrations between MPA and DDG were also approached for several factors. The different stabilities during storage and transportation between these two compounds were negligible, since the similar storage and transportation conditions and storage periods (8 April to 21 May 2014 for embryo exposures, and 11 June to 15 July 2014 for adult fish exposures) were conducted for the water samples in both embryo and adult fish exposures. The absorption capacities by fish is less probable due to the similar lipophilicity of the progestins with log Kow values of 4.09 for MPA and 3.45 for DDG.[5] Our best hypothesis to explain this decrease is a more selective absorption for DDG to the flow-through system, particles and fish debris. This may also be associated with a potential lower stability in the exposure system. These factors will be carefully checked in future experiments in order to evaluate and control for them.

Table S2. Medroxyprogesterone acetate and dydrogesterone concentrations in four groups of water samples collected during the adult fish exposure experiment in July 2014 in ng/L.

Nominal Conc.	Conc. at day 1		Conc. at day 7		Conc. at day 14		Conc. at day 21	
DMSO control	<LOQ*		<LOQ*		<LOQ*		<LOQ*	
MPA								
50 ng/L	44.5		44.8		42.3		39.9	
500 ng/L	-		341.0		376		310	
DDG								
500 ng/L	89.9		104.8		57.2		87.4	
5000 ng/L	1270		1450		1210		1120	
Mixtures MPA+DDG	MPA	DDG	MPA	DDG	MPA	DDG	MPA	DDG
50+500 ng/L	44.4	151	38.5	166	39.1	177.0	35.3	87.2
500+5000 ng/L	405	2210	429	1300	461.0	1470.0	432.0	1670.0

*LOQ= limit of quantification.

Table S3. The mean concentrations of medroxyprogesterone acetate and dydrogesterone in the water samples collected during the adult fish exposure experiment. Concentrations are the means of four replication samples in ng/L (raw data are shown in Table S2).

	DMSO control	MPA (50 ng/L)	MPA (500 ng/L)	DDG (500 ng/L)	DDG (5000 ng/L)	Mixture-Low		Mixture-High	
						MPA (50 ng/L)	DDG (500 ng/L)	MPA (500 ng/L)	DDG (5000 ng/L)
Mean (ng/L)	<LOQ*	43	342	89	1263	39	145	432	1663
S.D.	-	2	33	22	255	4	40	23	395
R.S.D.	-	5.3	9.6	25.1	20.2	9.6	27.6	5.3	23.8

*LOQ= limit of quantification.

Table S4. Medroxyprogesterone acetate and dydrogesterone concentrations (ng/L) for the four groups of samples (G1-G2 and G3-G4) collected during the embryo exposure experiment in April 2014.

Nominal Conc.	Conc. at 0 h *G1*		Conc. at 24 h *G2*		Accuracy 0 h –Nominal Conc. (%)		Residual amount at 24 h (%)	
Water Control	<LOQ*		<LOQ*					
DMSO control	<LOQ*		<LOQ*					
DDG								
5 ng/L	5.4		4.69		108		87	
50 ng/L	55.6		52.8		111		95	
500 ng/L	540		525		108		97	
MPA								
5 ng/L	4.84		4.7		97		97	
50 ng/L	41		45.2		82		110	
500 ng/L	416		370		83		89	
Mixtures								
MPA+DDG	MPA	DDG	MPA	DDG	MPA	DDG	MPA	DDG
5 ng/L	4.7	5.5	4.54	4.96	94	110	97	91
50 ng/L	45.8	50.6	46.8	51.2	92	101	102	101
500 ng/L	420	526	382	476	84	105	91	91

Nominal Conc.	Conc. at 0 h *G3*		Conc. at 24 h *G4*		Accuracy 0 h –Nominal Conc. (%)		Residual amount at 24 h (%)	
Water control	<LOQ*		<LOQ*					
DMSO control	<LOQ*		<LOQ*					
DDG								
5 ng/L	4.84		4.41		97		91	
50 ng/L	46.8		45.4		94		97	
500 ng/L	443		376		89		85	
MPA								
5 ng/L	4.13		4.69		83		114	
50 ng/L	33.2		37		66		111	
500 ng/L	329		401		66		122	
Mixtures								
MPA+DDG	MPA	DDG	MPA	DDG	MPA	DDG	MPA	DDG
5 ng/L	3.97	4.02	4.66	3.89	79	80	117	97
50 ng/L	35.6	39.8	40.2	39	71	80	113	98
500 ng/L	369	382	433	352	74	76	117	92

*LOQ= limit of quantification.

Table S5. The mean concentrations of medroxyprogesterone acetate and dydrogesterone in the water samples collected during the embryo exposure experiment. Concentrations are the means of duplicate samples in ng/L (raw data are shown in Table S4).

Nominal Conc.	Conc. at 0 h (Mean)	Conc. at 24 h (Mean)	Accuracy 0 h – Nominal Conc. (%)	Residual amount at 24 h (%)	Conc. during 6 day exposure (Mean)
Water control	<LOQ*	<LOQ*			<LOQ*
DMSO control	<LOQ*	<LOQ*			<LOQ*
DDG					
5 ng/L	5.1	4.5	102	89	4.8
50 ng/L	51.2	49.1	102	96	50.2
500 ng/L	491.5	450.5	98	92	471
MPA					
5 ng/L	4.5	4.7	90	105	4.6
50 ng/L	37.1	41.1	74	111	39.1
500 ng/L	372.5	385.5	75	104	379
Mixtures					

MPA+DDG	MPA	DDG	MPA	DDG	MPA	DDG	MPA	DDG	MPA	DDG
5 ng/L	4.3	4.8	4.6	4.4	87	95	106	93	4.5	4.6
50 ng/L	40.7	45.2	43.5	45.1	81	90	107	100	42.1	45.2
500 ng/L	394.5	454.0	407.5	414.0	79	91	103	91	401	434

*LOQ= limit of quantification.

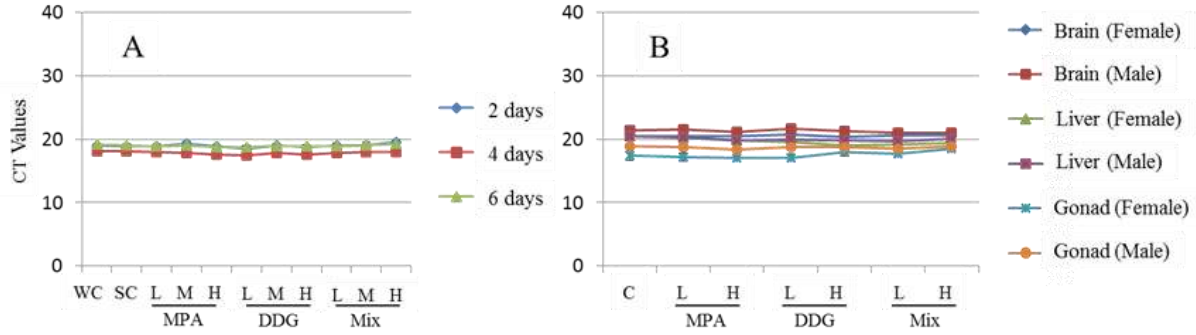
Table S6. Primer sequences for quantitative real-time PCR analysis and sources.

Gene	Gene Bank no.	Sense primer (5'-3')	Antisense primer (5'-3')	Product size (bp)
rpl13α[a]	NM_212784	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC	100
pr[b]	NM_001166335	GGGCCACTCATGTCTCGTCTA	TCTCCACTCTGAAAATATGTGGACTTT	96
ar[c]	NM_001083123	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC	237
er1[d]	NM_152959	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT	163
gr[e]	EF567112	ACAGCTTCTTCCAGCCTCAG	CCGGTGTTCTCCTGTTTGAT	116
mr[e]	EF567113	CCC ATT GAG GAC CAA ATC AC	AGT AGA GCA TTT GGG CGT TG	106
vtg1[f]	AY034146	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT	94
cyp2k7[g]	AF487990.1	CGTCAGACCAGCTGTGATGT	TGTCAGGTGTTTCCCACTCA	115
mprα[h]	NM_183345.1	CGCTCAAGTGCGAACTTTTT	CGTACTTGCCATAGCAGCAG	83
mprβ[h]	AY149120.1	ACGTCAAGCCACAGTACACG	TCCTGATGCACTGGACGATA	87
cyp11a[i]	AF527755.1	GAGGGGTGGACTCGGTTACTT	GCAATACGAGCGGCTGAGAT	109
hsd3b[g]	AY279108	GCAACTCTGGTTTTCCACACTG	CAGCAGGAGCCGTGTAGCTT	102
cyp17[i]	AY281362.1	CTGCTCTGTTTAAGCCTGTTCTC	GCTGGCACAAATCCATTCATC	80
hsd17b3[g]	NM_200364.1	TTCACGGCTGAGGAGTTTG	GGACCCAGGTAGGAATGG	121
cyp11b[e]	NM_001080204	GCTCATGCACATTCTGAGGA	TGTGCTGAAGGTGATTCTCG	115
hsd11b2[e]	NM_212720	TGCTGCTGGCTGTACTTCAC	TGCATCCAACTTCTTTGCTG	123
cyp19a[j]	AF226620	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG	92
lhβ[f]	AY714132	GAGACGGTATCGGTGGAAAA	AACAGTCGGGCAGGTTAATG	178
fshβ[f]	NM_205624	GCTGTCGACTCACCAACATCTC	GTGACGCAGCTCCCACATT	61
cyp19b[j]	AF183908	CGACAGGCCATCAATAACA	CGTCCACAGACAGCTCATC	94
nr1d1[a]	NM_205729	GTGAACAACCAGCTGCAGAA	ACTGTAAGGCCTGGACATGG	125
nr1d2b[k]	NM_131065	GCACCTGGTCTGCCCGA	CGGACCACCAGCACCTCA	207
per1b[k]	NM_212439	CCTCCTGAGTCAGATATCGTAATGG	GCAGCGCACACCTCTTGATAA	324
cry5[a]	NM_131788.1	CATGGAGAGAACGAACTGGG	GTGCAGACAAGCAGCCGAAC	115
ccnb1[l]	ccnb1b	GGTCCACTACCCTCCCTCTC	ATGCTTAGAAAGGCCCTCGT	184
mycb1[m]	NM_200172	TGCGATGATGCGGACTA	TCAGCGTGCAAAGACG	89
cdc20[m]	NM_213080	GGTCATTCAGCAAGGGTG	GTGTCCGCCGAAGGTA	98
ahr1[n]	AF258854	TAGACAGCGATATACAGCAG	TCTCTCCAACACCATTCATG	213
ahr2[n]	NM_131264	ACGGTGAAGCTCTCCCATA	AGTAGGTTTCTCTGGCCAC	228

cyp1a[d]	AF210727	CCTGGGCGGTTGTCTATCTA	TGAGGAATGGTGAAGGGAAG	79
arnt2[o]	AF219989	CACCTTTGGATCACATCTCATTG	TCACCCTCCTTAGACGGACC	85
zp3[p]	NM_131696	GGATGCCTTTAGGTTTCACAAGTT	CCCGATTCTTAGCACTCACAGA	101

Data sources: *a(6); b(7); c(8); d(9); e(10); f(11); g(12); h(13); i(14); j(15); k(16); l(17); m(18); n(19); o(20); p(21)*

Figure S1. Stability analysis of RpL13a gene expressions in different treatments and tissue categories. (A) RpL13a gene expressions in zebrafish embryos in different treatments (B) RpL13a gene expressions in brain, liver and gonad of adult zebrafish in different treatments. Each bar represents the mean value ± S.D. of 4-8 replicates per treatment.



Results

Figure S2. Average number of eggs per female per day after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. Each bar represents the mean value ± S.D. of four replicate tanks per treatment. Asterisks indicate significantly different number of eggs per female per day compared to solvent control (*p < 0.05).

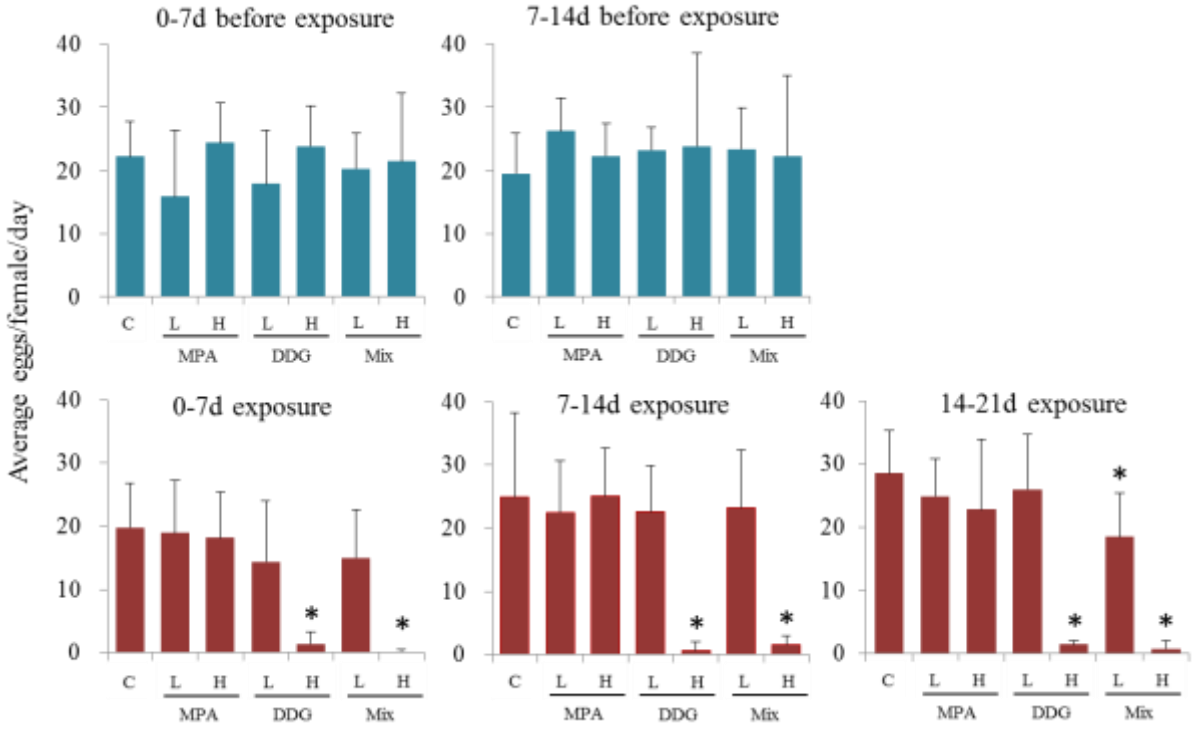


Figure S3. Body length, body weight and condition factor (CF) of adult female and male zebrafish after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. The CF was calculated as body wet weight [mg]/body length [mm] × 100 (n = 16 females and 16 males, respectively, per treatment). Asterisks indicate significantly difference compared to solvent control (*p < 0.05).

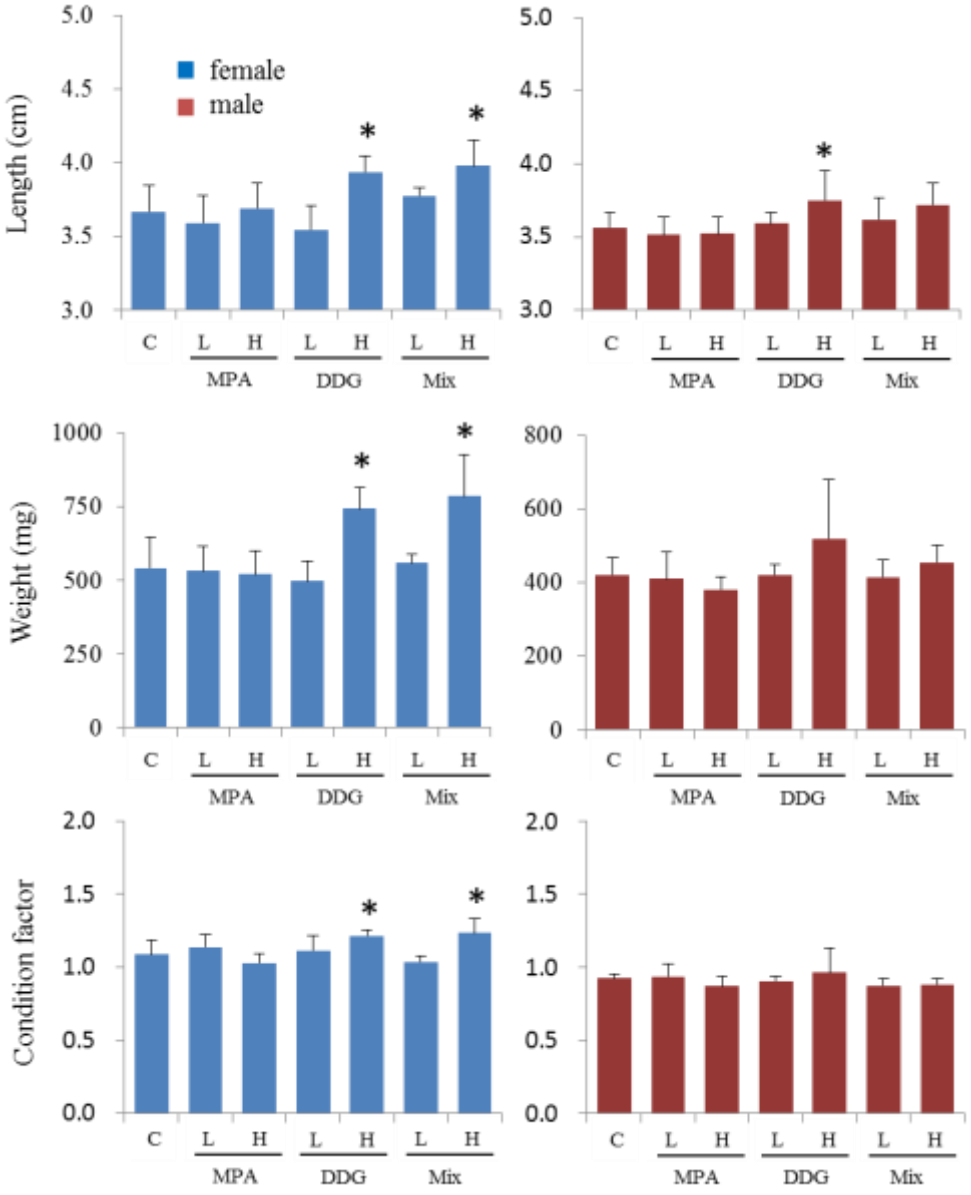


Figure S4. Gonadosomatic index (GSI) and diameter of late vitellogenic oocyte (LV) of adult female zebrafish after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. The GSI was calculated as gonad weight [mg]/body weight [mg] × 100 (n = 16 samples per treatment). n = 32 samples per treatment for the measurement of diameter of late vitellogenic oocyte (LV). Asterisks indicate significantly difference compared to solvent control (*p < 0.05).

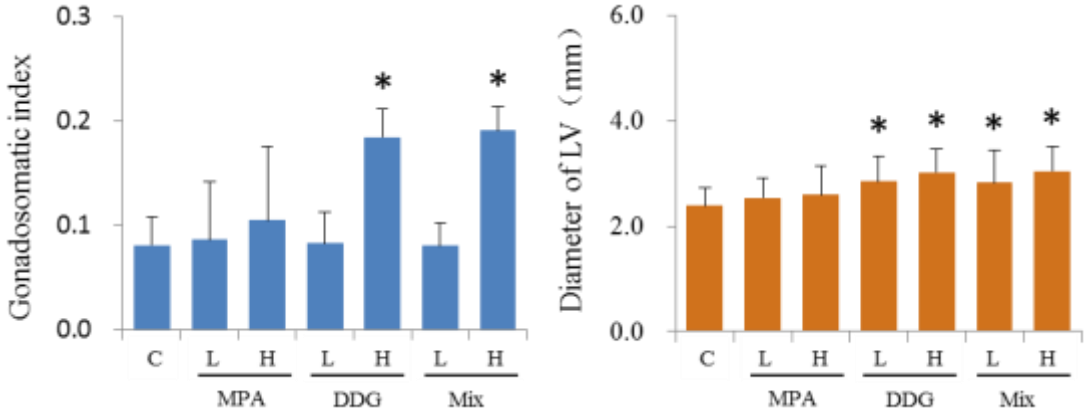


Figure S5. Explicative transverse sections of zebrafish ovaries after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO) (A), and different concentrations of MPA (B), DDG (C–D) and their mixtures (E-F). Examples of oocyte maturation stages include po: perinucleolar oocyte, co: cortical alveolar oocyte, er: early vitellogenic oocyte, lv: mid-late vitellogenic oocyte. The observed alterations included post-ovulatory follicles (pof) and atretic follicles (af). (40x magnification).

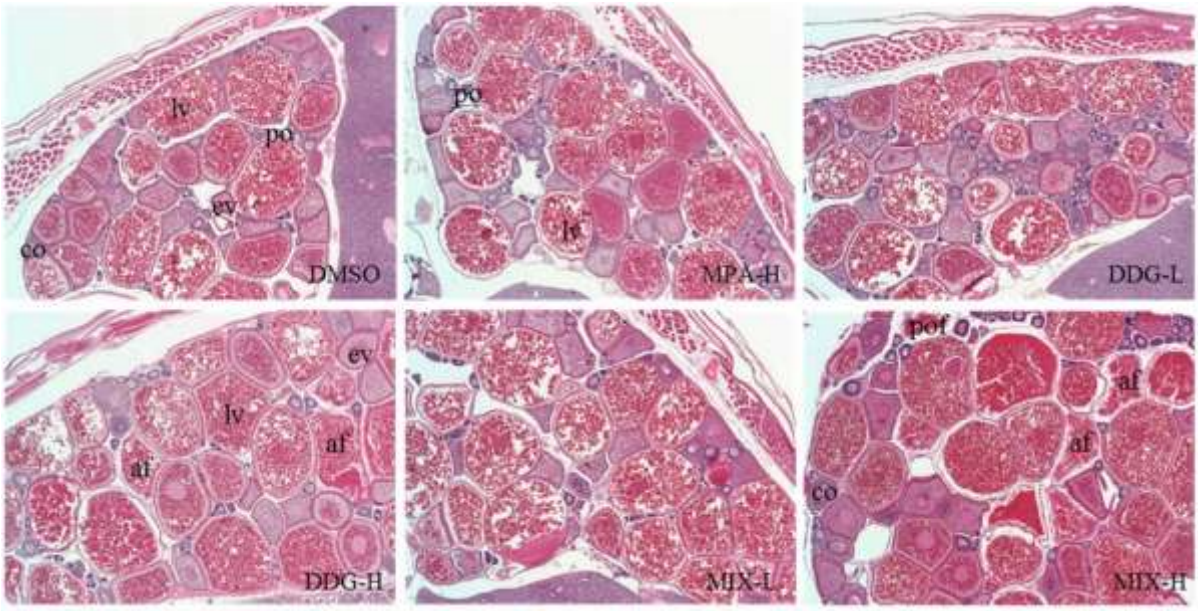


Figure S6. Explicative transverse sections of zebrafish testis after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO) (A), and different concentrations of MPA (B), DDG (C–D) and their mixtures (E-F). Individual testes were evaluated by calculating the relative percentage of mature (spermatids and spermatozoa) and immature (spermatogonia and spermatocytes) spermatocytes. (100x magnification).

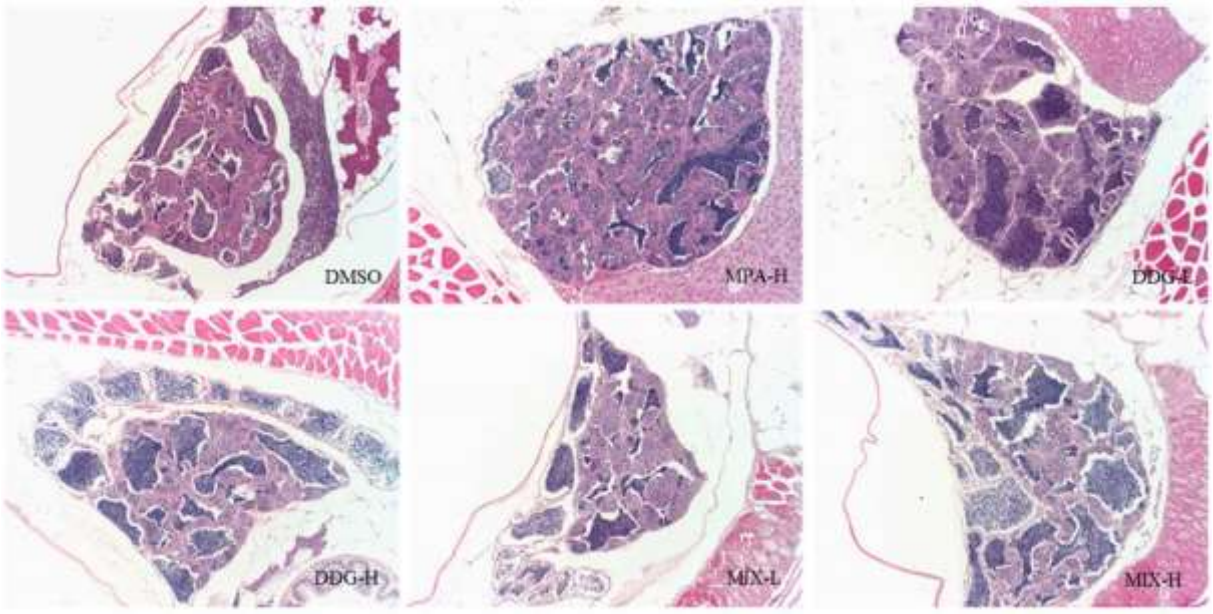


Figure S7. Concentrations of 17β-estradiol (E2) and 11-ketotestosterone (11-KT) measured in blood plasma of adult zebrafish females and males, respectively, after two weeks pre-exposure followed by three weeks exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. Concentrations are given as mean values±S.D. (n = 4 samples per treatment).

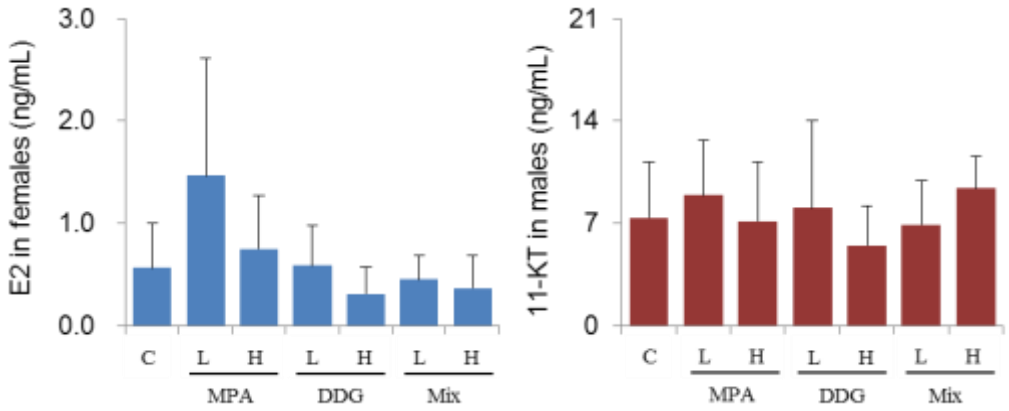
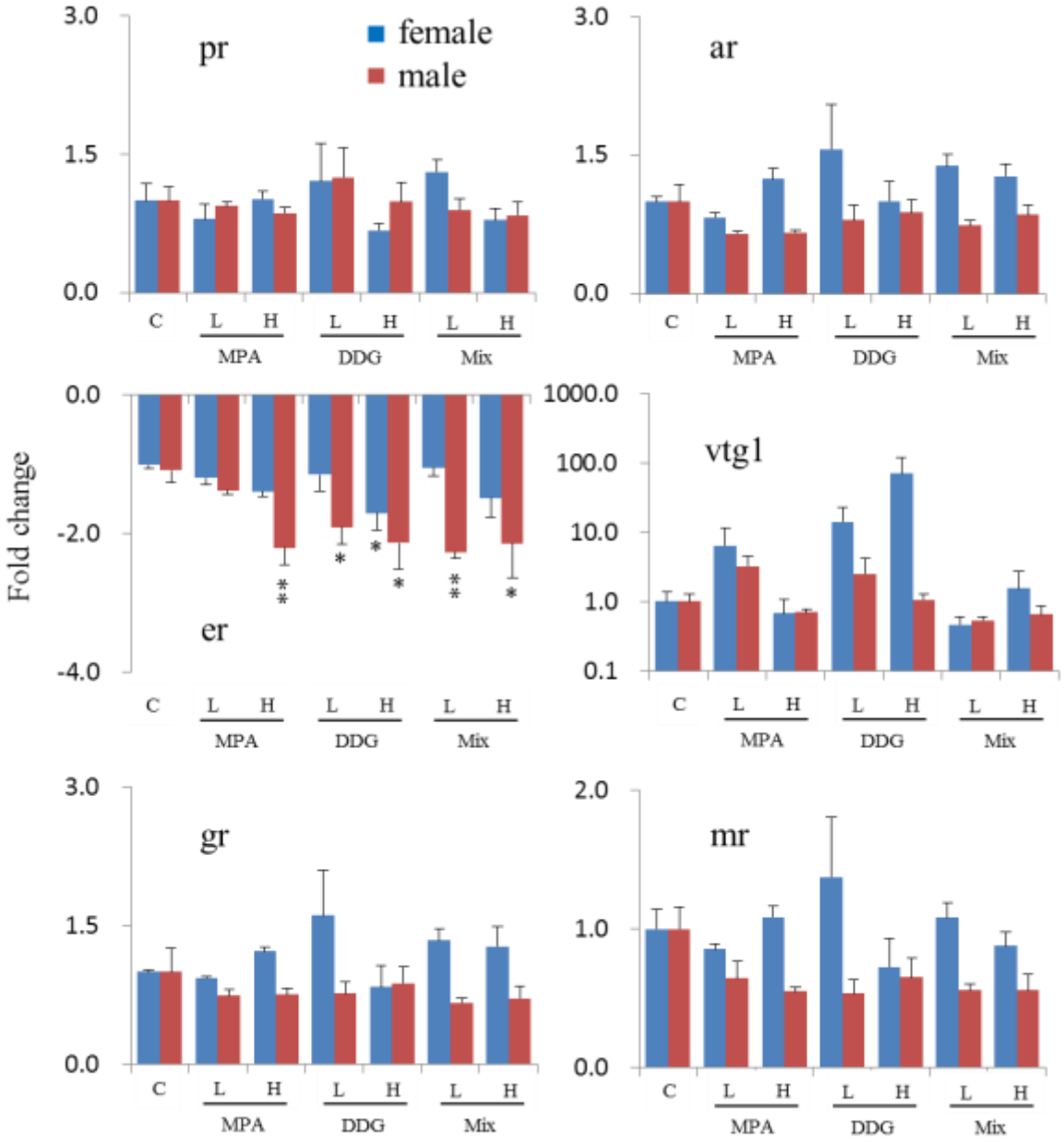
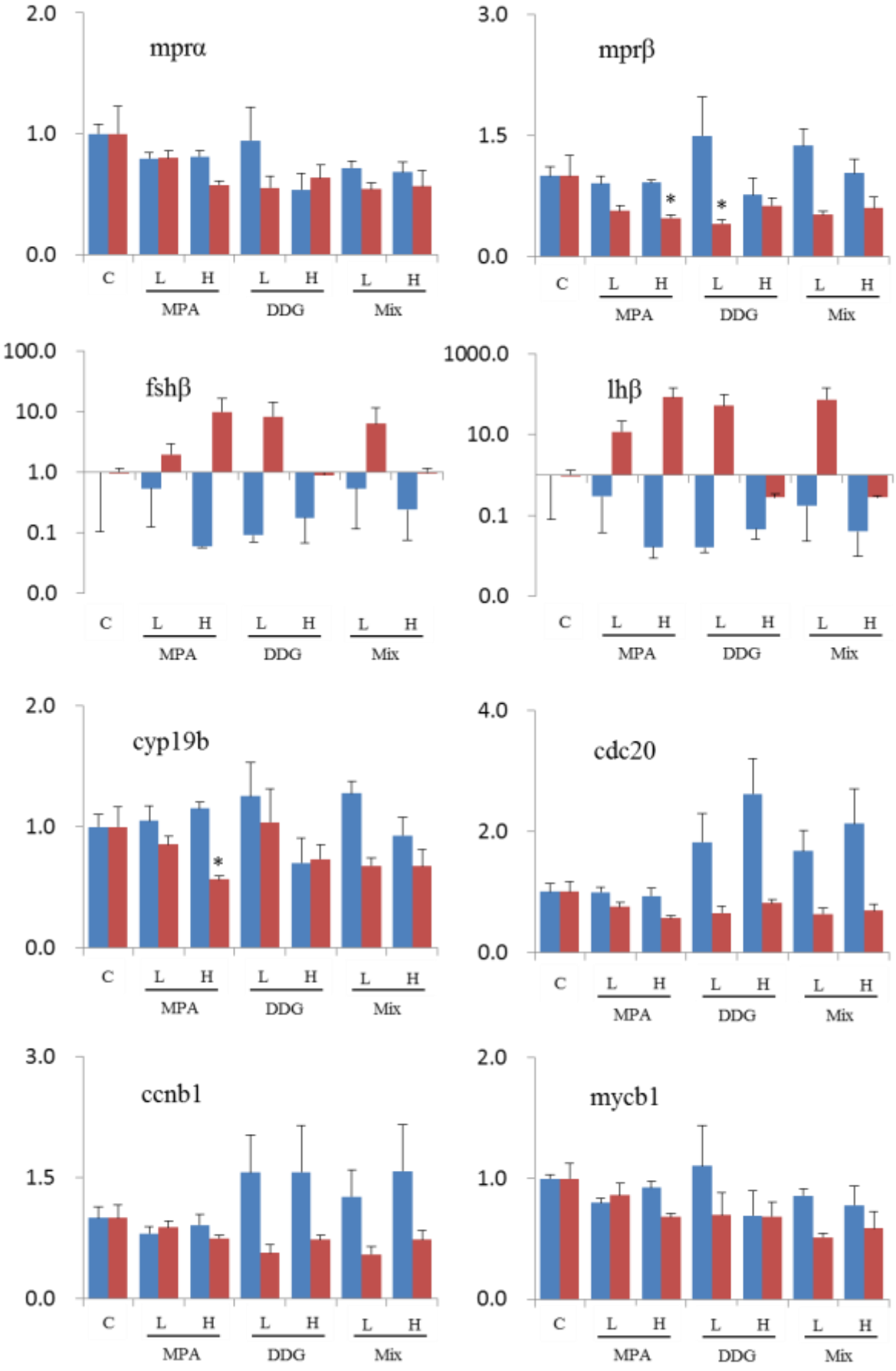


Figure S8. Heat map depicts the gene expression alterations in adult zebrafish brain of all the 20 investigated transcripts in a color scheme: the dark green color represents significant down-regulation with ≥2 fold change, light green represents significant down-regulation with 1-2 fold change, and yellow represents no significant alterations.

Figure S9. Relative gene expressions of all 20 investigated transcripts in adult zebrafish brain after exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. Relative transcript abundance was quantified by real-time reverse transcription PCR. Results are given as the mean value ±S.D. (n = 4 replicates). Asterisks indicate significantly higher expression than control (*p < 0.05), (**p < 0.01), and (***p < 0.001).




mprα
mprβ
fshβ
lhβ
cyp19b
cdc20
ccnb1
mycb1
C
L
H
MPA
DDG
Mix

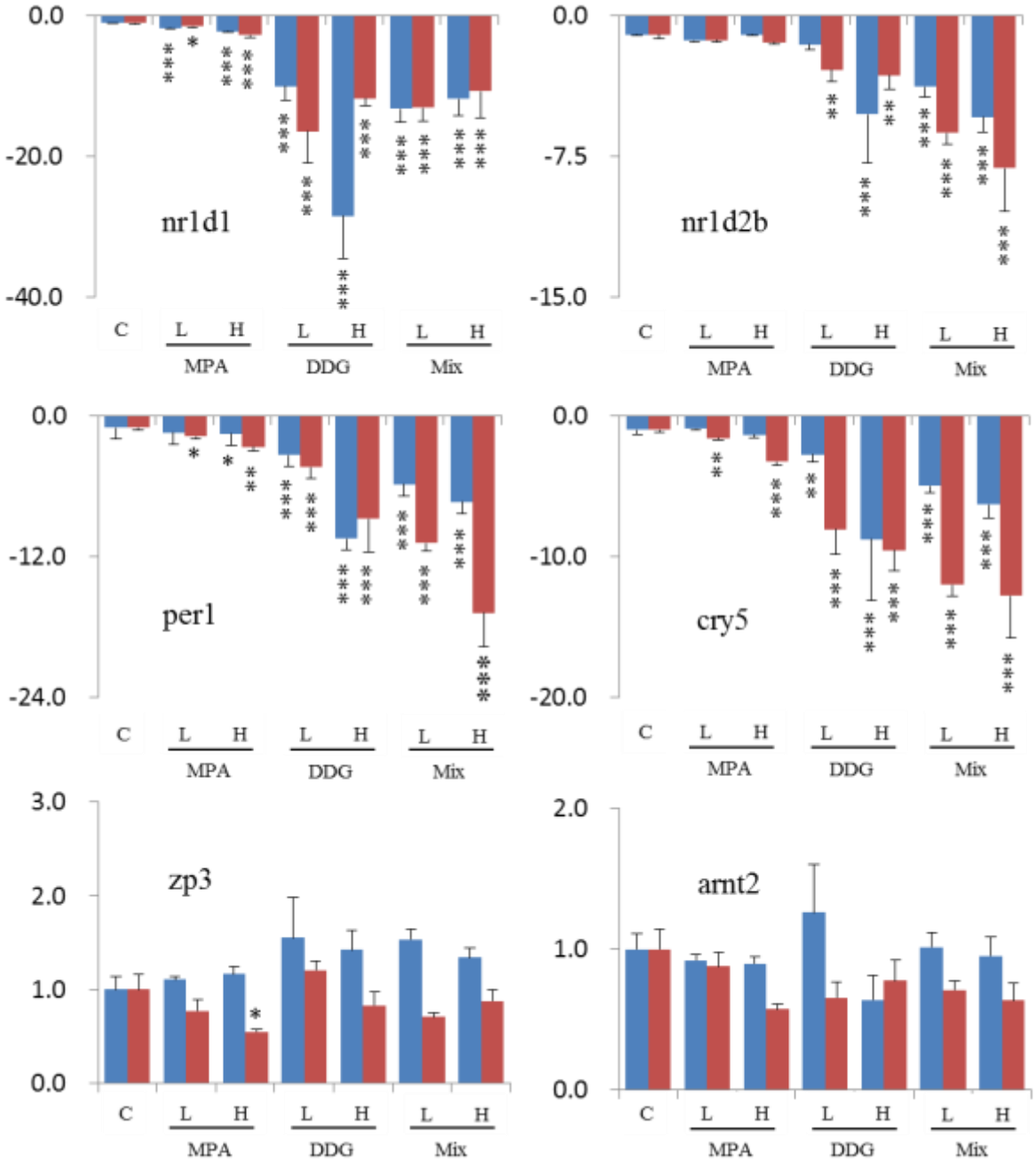
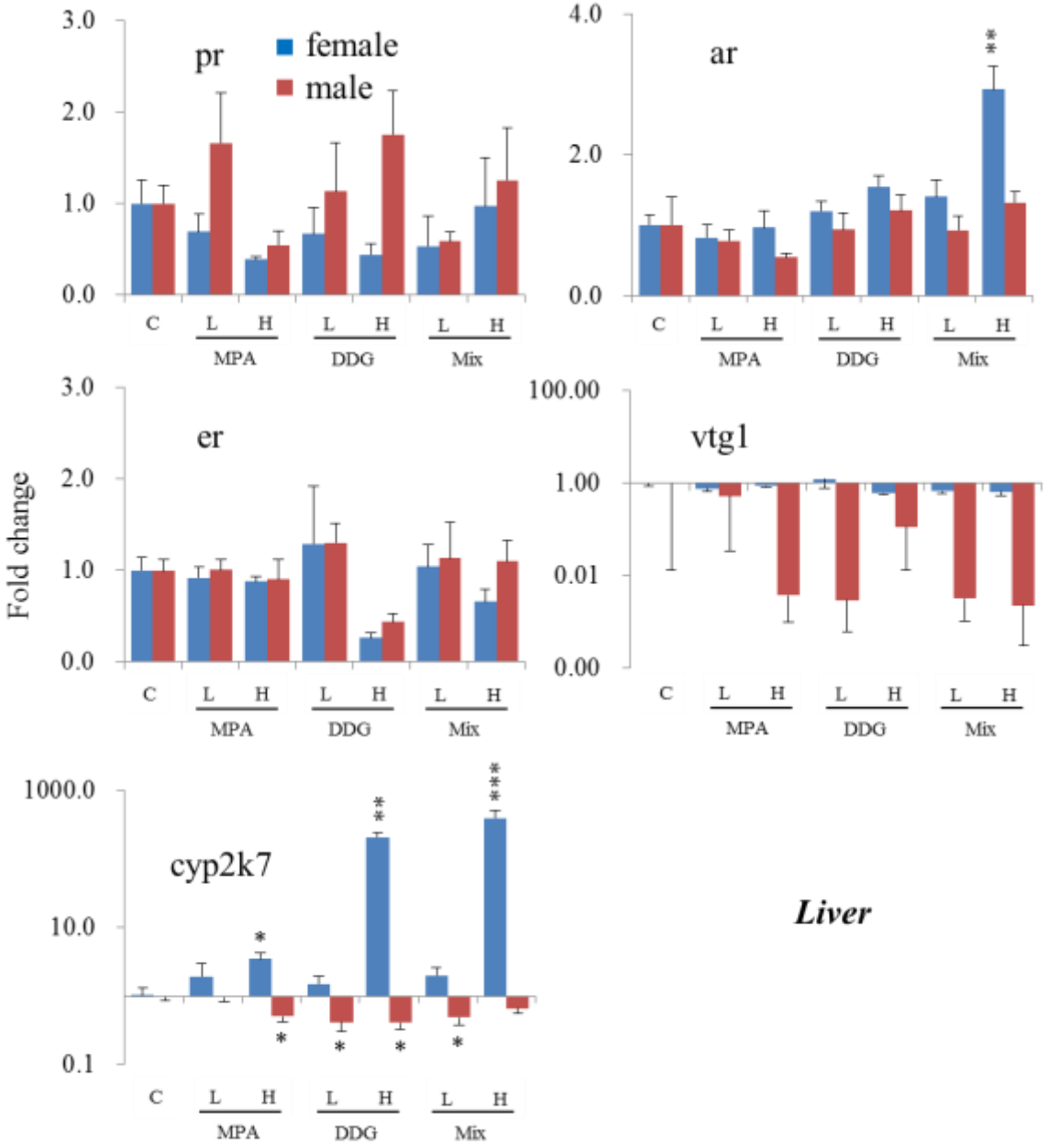
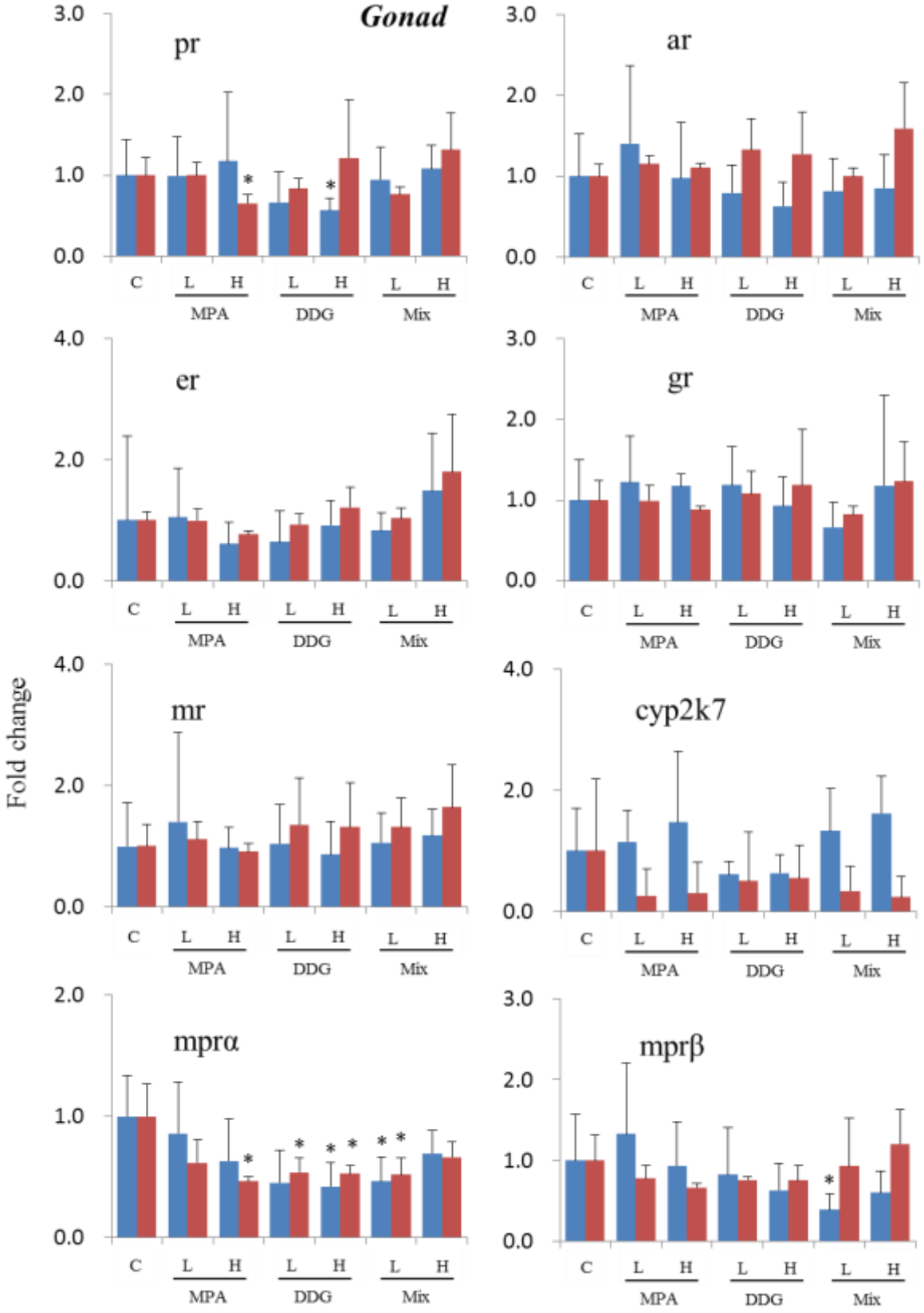

nr1d1
nr1d2b
per1
cry5
zp3
arnt2
C
L
H
MPA
DDG
Mix

Figure S10. Heat map depicts the gene expression alterations in adult zebrafish liver and gonad of the investigated transcripts in a color scheme: the dark red color represents significant up-regulation with ≥2 fold change, light red represents significant up-regulation with 1-2 fold change, the dark green color represents significant down-regulation with ≥2 fold change, light green represents significant down-regulation with 1-2 fold change, and yellow represents no significant alterations.

Figure S11. Relative gene expressions of the investigated 20 transcripts in adult female and male zebrafish liver and gonads after exposure to solvent control (0.01% DMSO), and different concentrations of MPA, DDG and their mixtures. Relative transcript abundance was quantified by quantitative real-time reverse transcription PCR. Results are given as the mean value ±S.D. (n = 4 replicates for liver and testis samples and n = 8 replicates for ovary samples). Asterisks indicate significantly higher expression than control (*p < 0.05), (**p < 0.01), and (***p < 0.001).




Gonad
pr
ar
er
gr
mr
cyp2k7
mprα
mprβ
Fold change
C
L
H
MPA
DDG
Mix

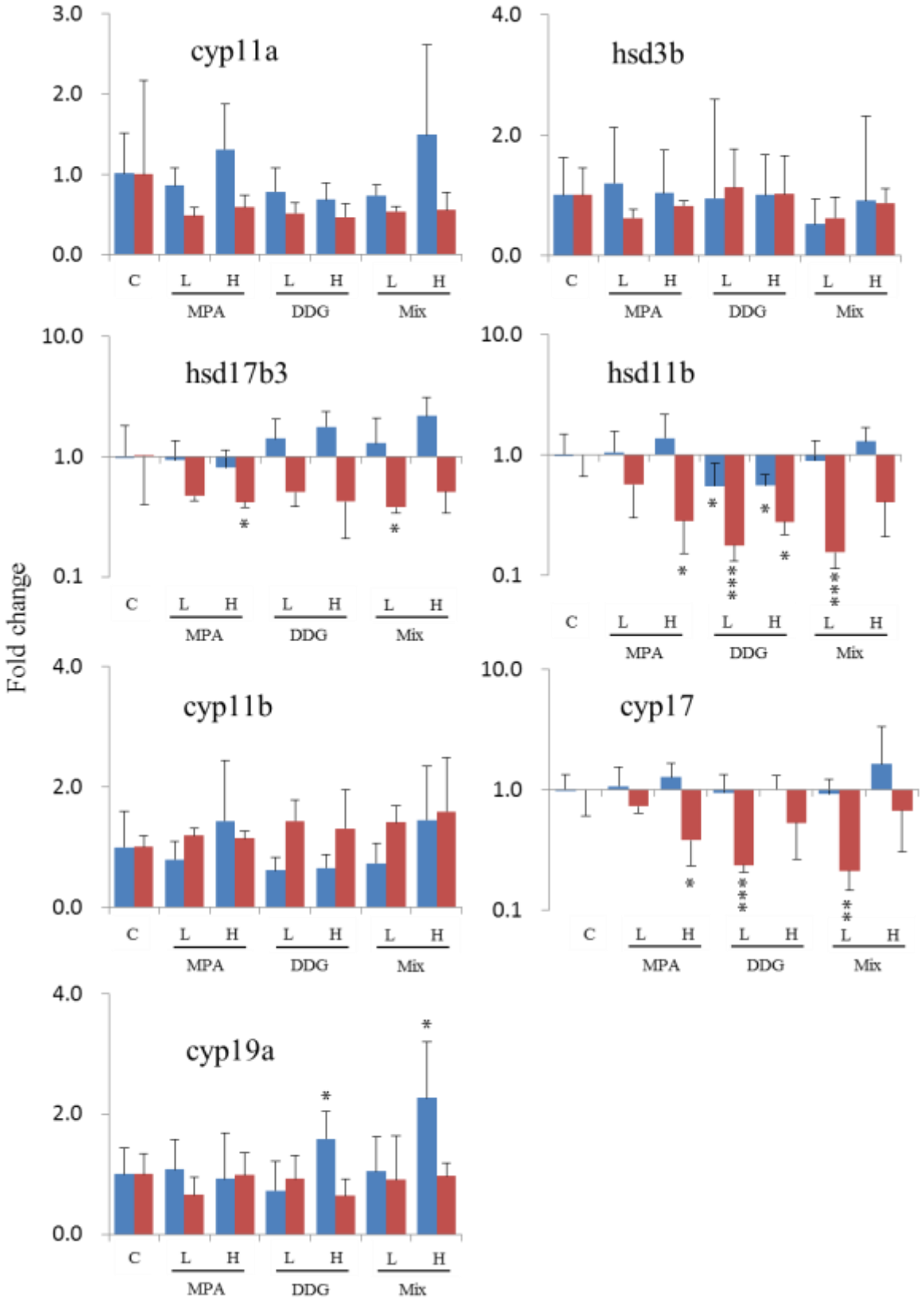

Fold change
cyp11a
hsd3b
hsd17b3
hsd11b
cyp11b
cyp17
cyp19a
C
L
H
MPA
DDG
Mix

Figure S12. Heat map depicts the gene expression alterations in zebrafish embryos of the 28 investigated transcripts in a color scheme. The dark red color represents significant up-regulation with ≥2 fold change, light red represents significant up-regulation with 1-2 fold change, the dark green color represents significant down-regulation with ≥2 fold change, light green represents significant down-regulation with 1-2 fold change, and yellow represents no significant alterations.

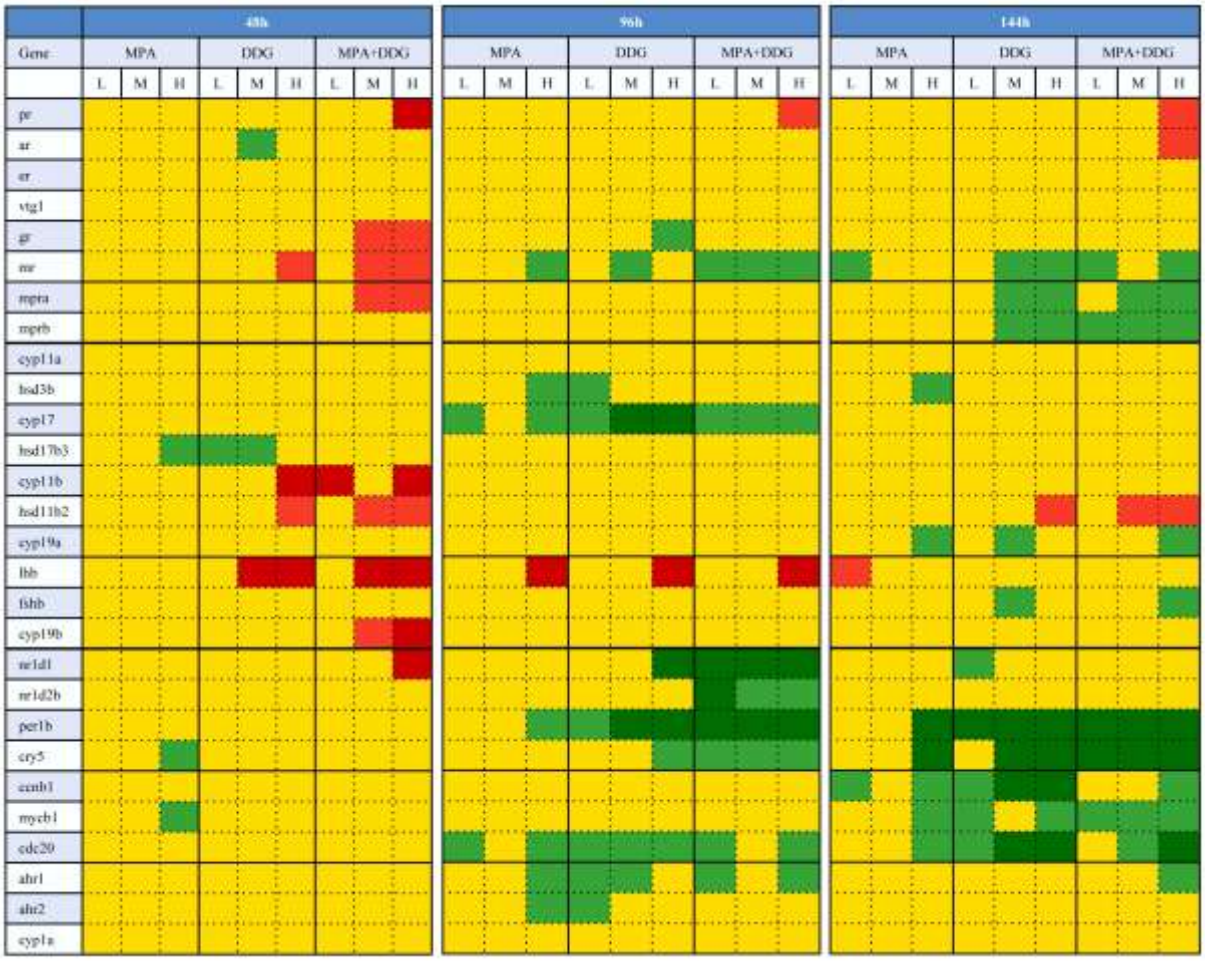
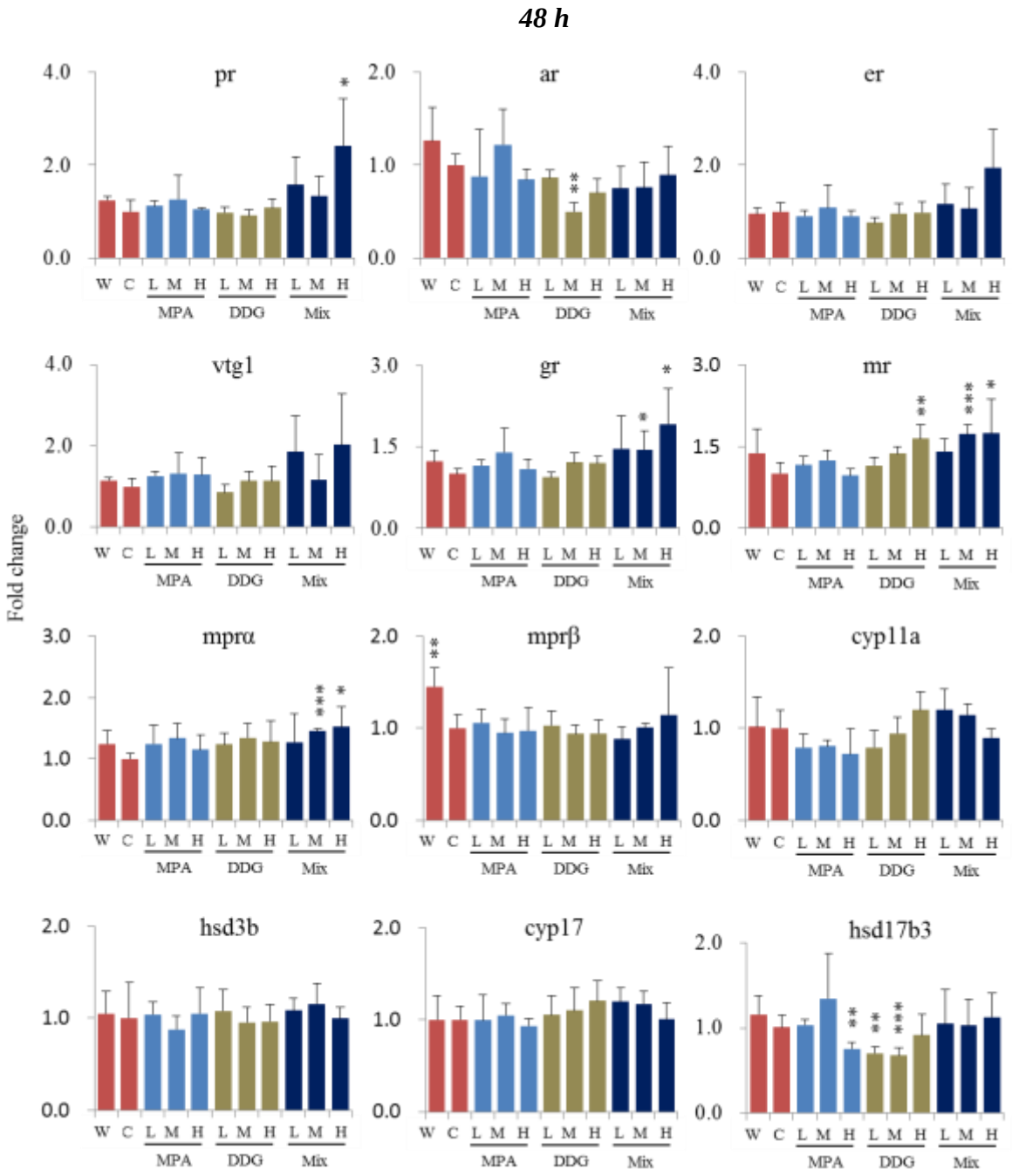
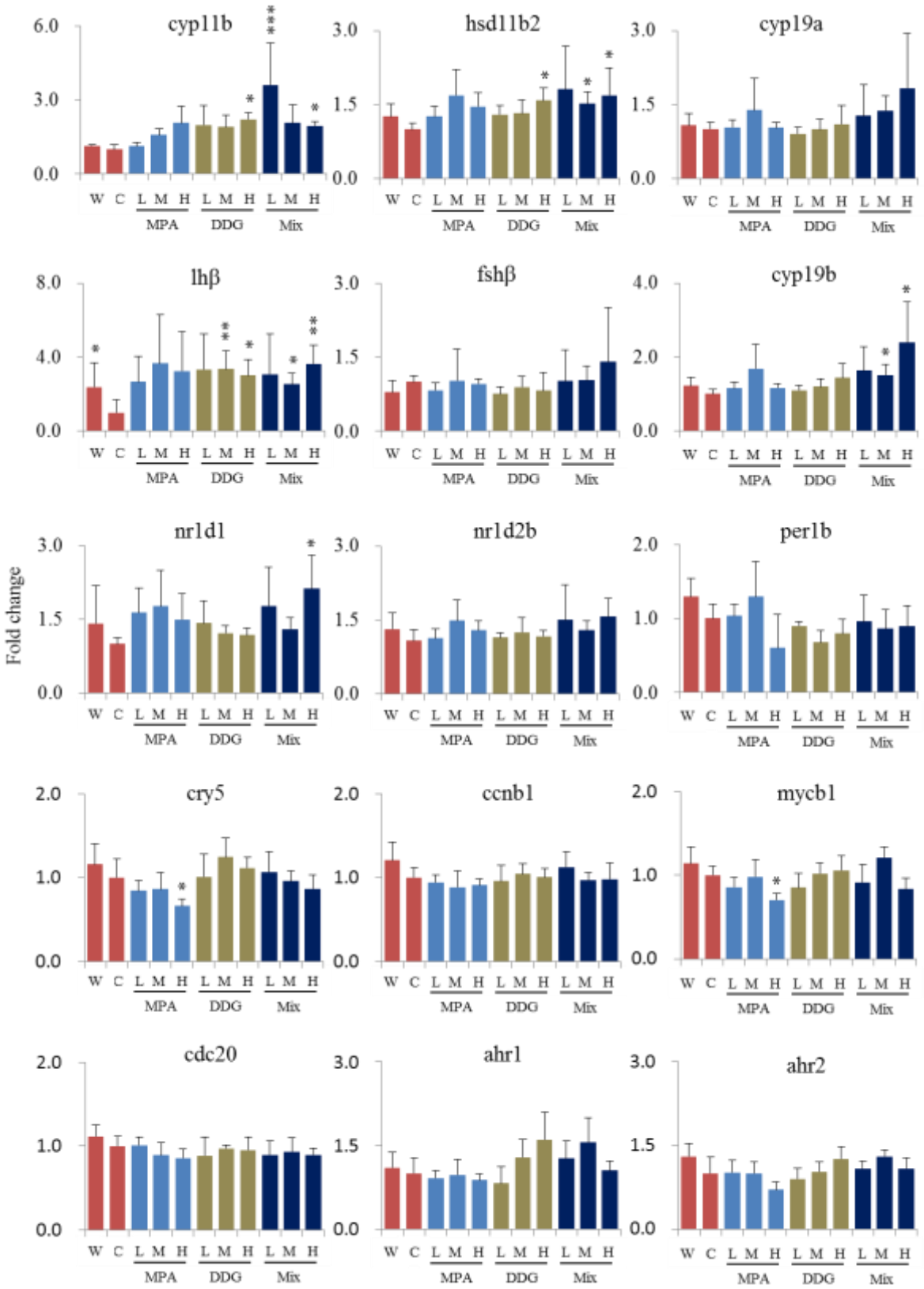
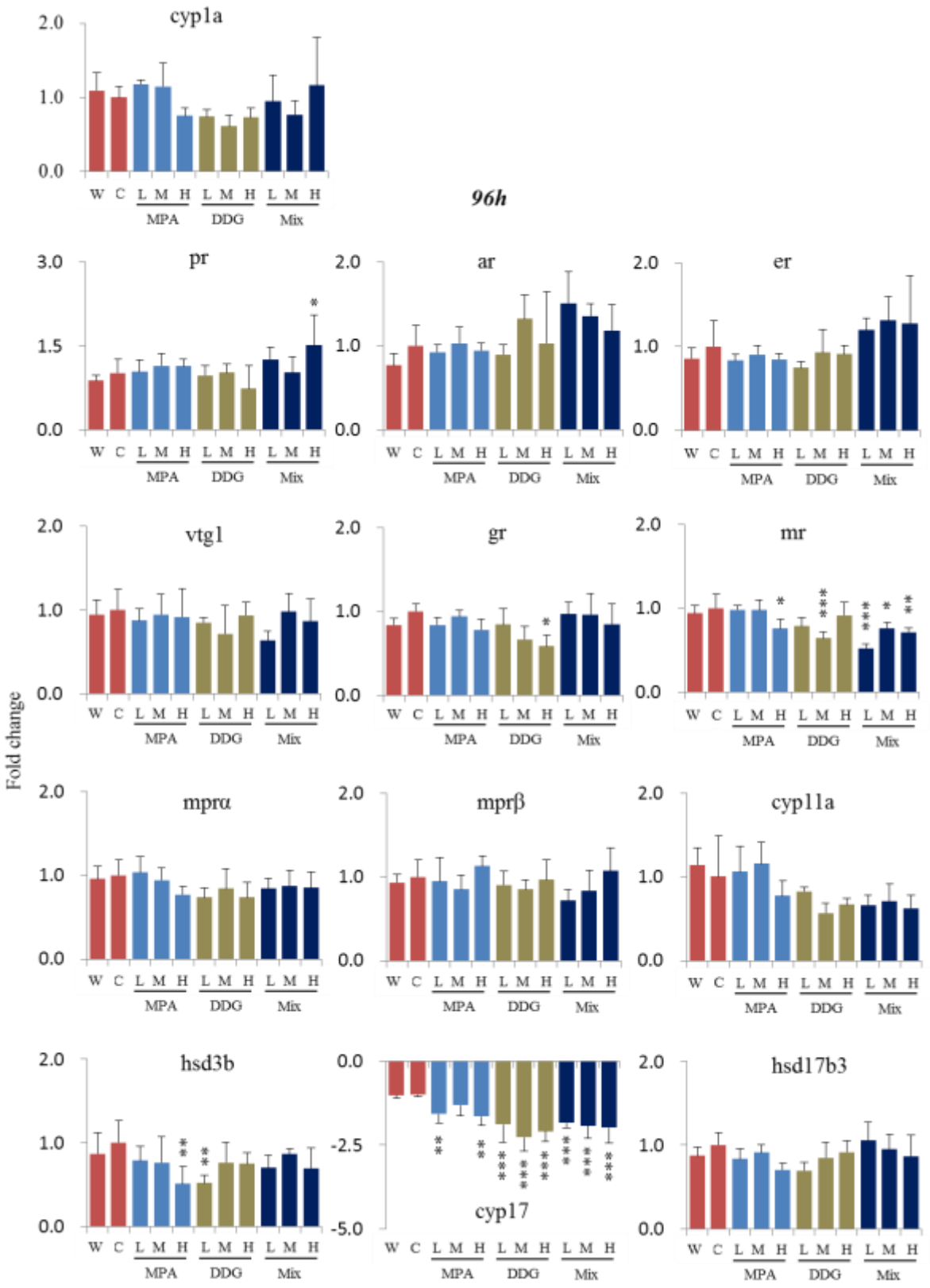
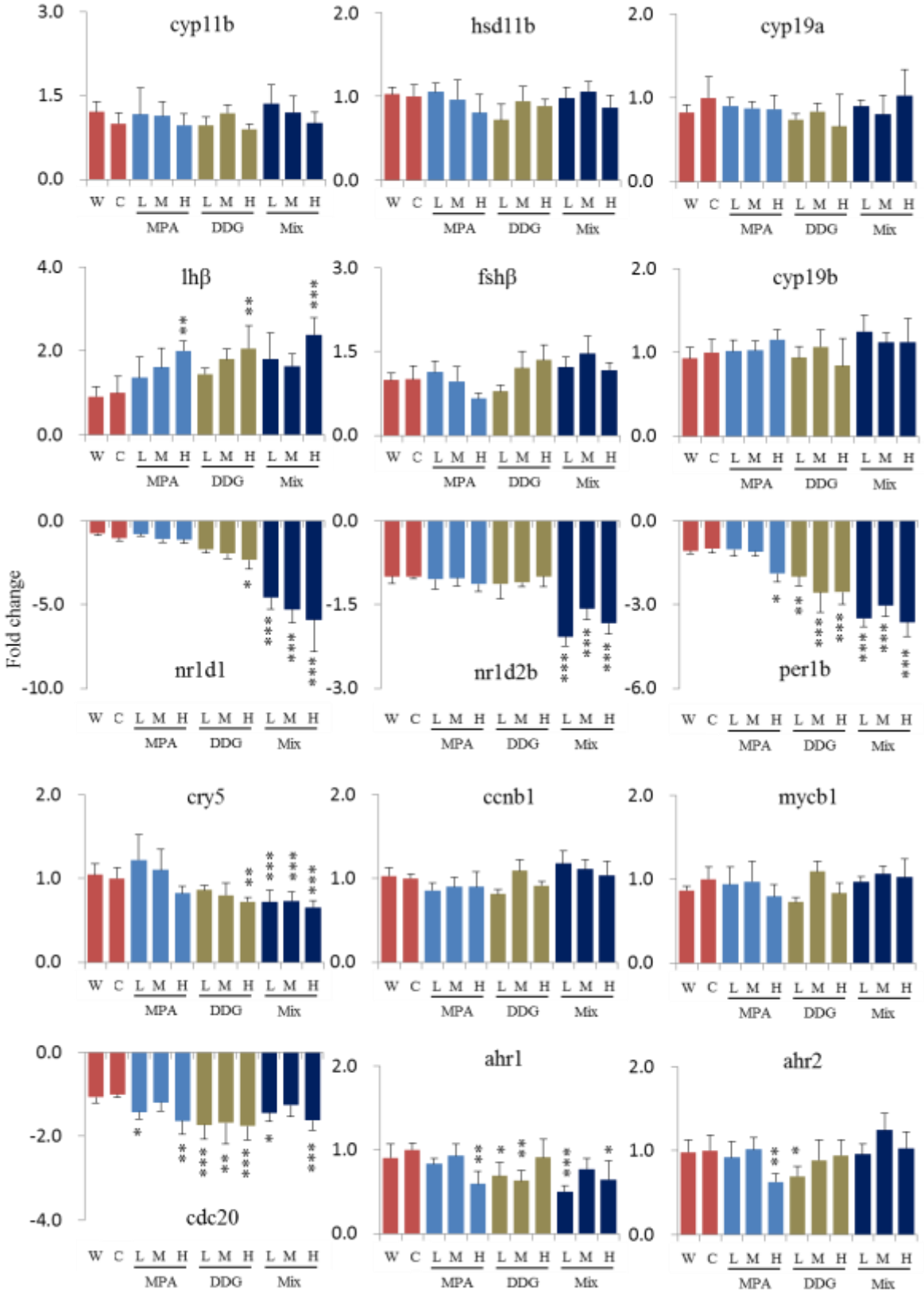


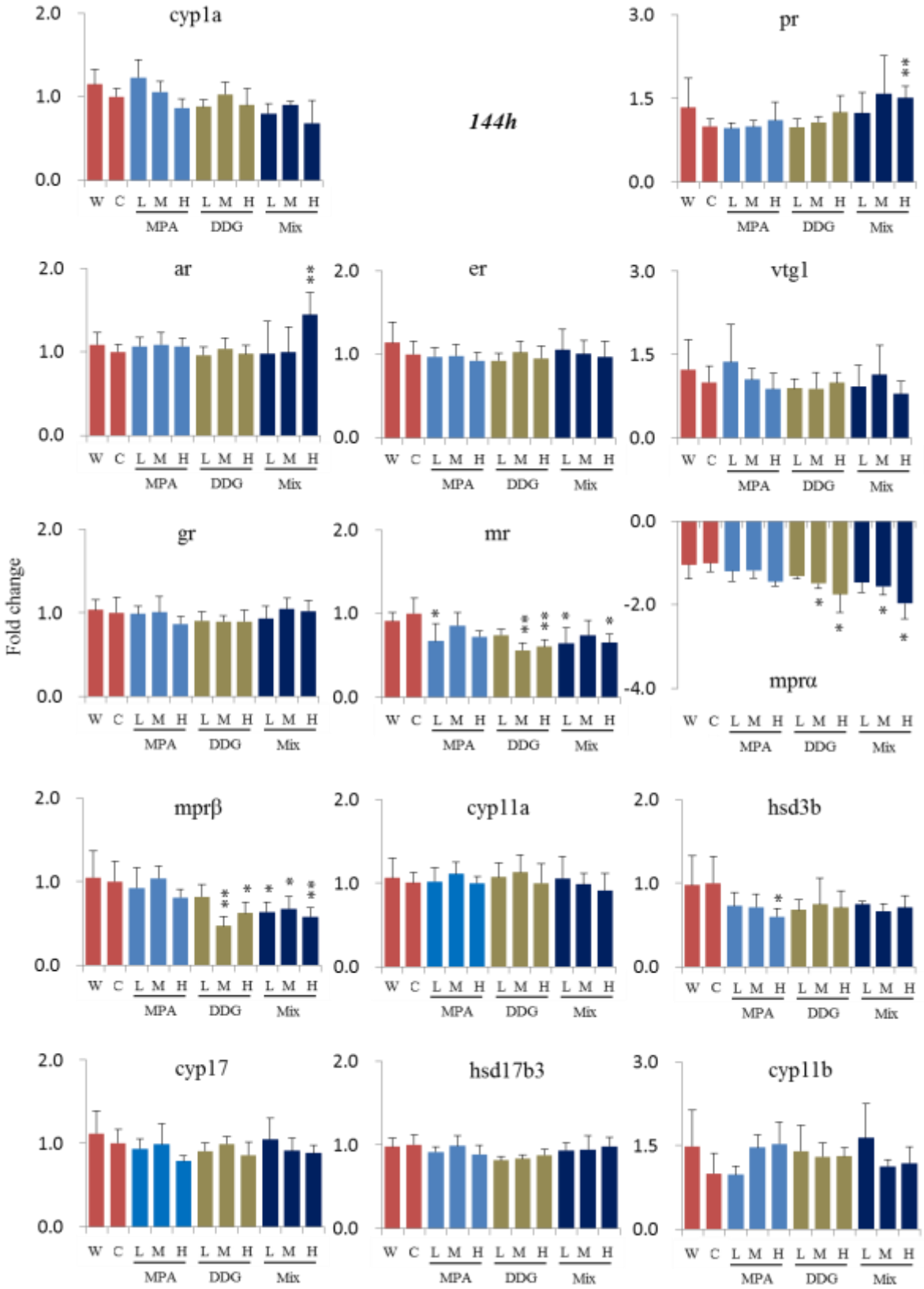
Figure S13. Relative gene expressions of the 28 investigated transcripts in zebrafish embryos after exposure to water control (W), solvent control (C) and three different concentrations of MPA, DDG and their mixtures. Relative transcript abundance was quantified by quantitative real-time reverse transcription PCR. Results are given as the mean value ±S.D. (n = 5). Asterisks indicate significantly different expression than the solvent control (*p < 0.05), (**p < 0.01), and (***p < 0.001).

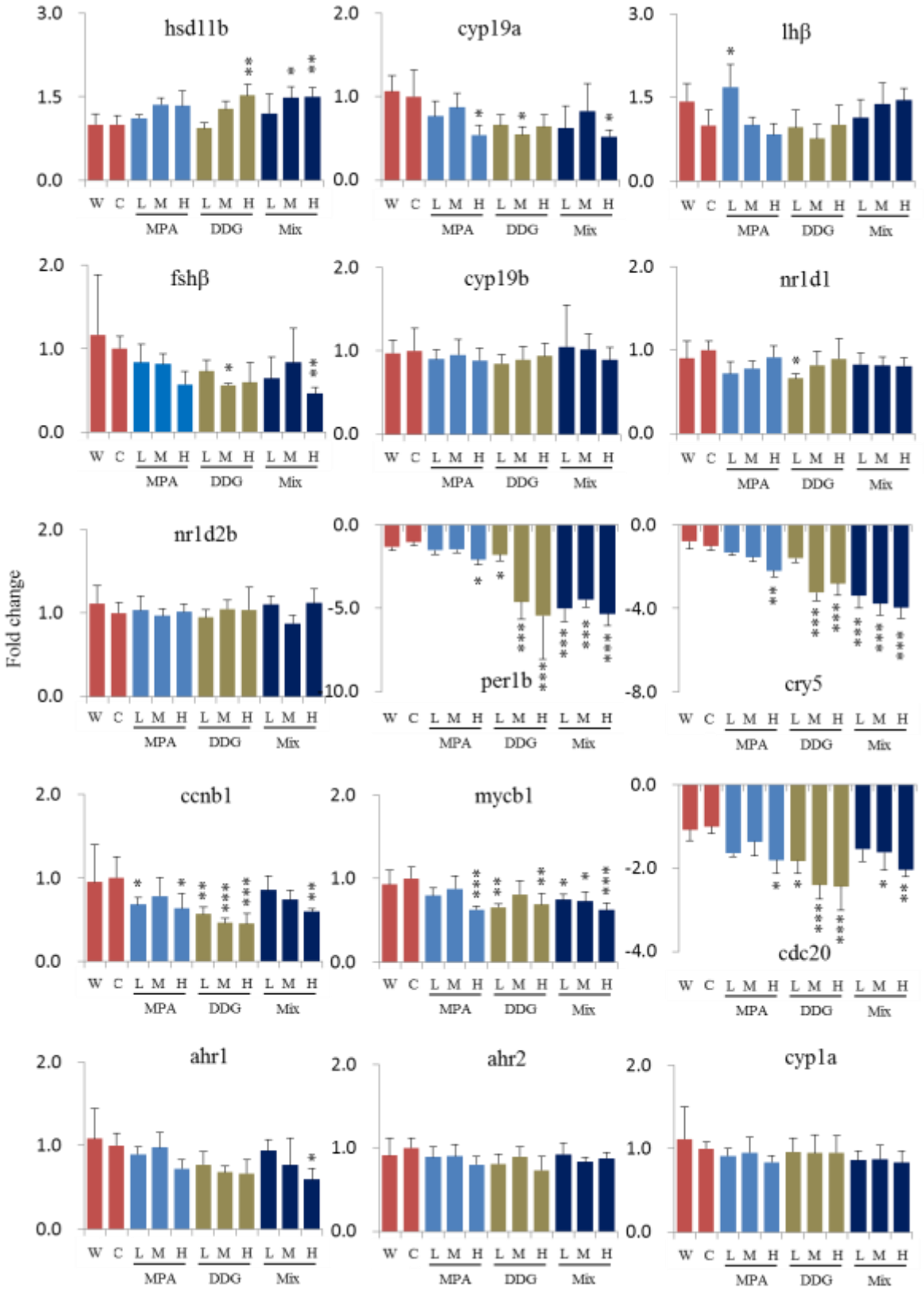



cyp11b
hsd11b2
cyp19a
lhβ
fshβ
cyp19b
nr1d1
nr1d2b
per1b
cry5
ccnb1
mycb1
cdc20
ahr1
ahr2
Fold change
W C L M H L M H L M H
MPA DDG Mix


96h
Fold change
cyp1a
pr
ar
er
vtg1
gr
mr
mprα
mprβ
cyp11a
hsd3b
cyp17
hsd17b3
W C L M H L M H L M H
MPA DDG Mix


Fold change
cyp11b
hsd11b
cyp19a
lhβ
fshβ
cyp19b
nr1d1
nr1d2b
per1b
cry5
ccnb1
mycb1
cdc20
ahr1
ahr2
W C L M H L M H L M H
MPA DDG Mix


144h
Fold change
cyp1a
pr
ar
er
vtg1
gr
mr
mprα
mprβ
cyp11a
hsd3b
cyp17
hsd17b3
cyp11b
W C L M H L M H L M H
MPA DDG Mix

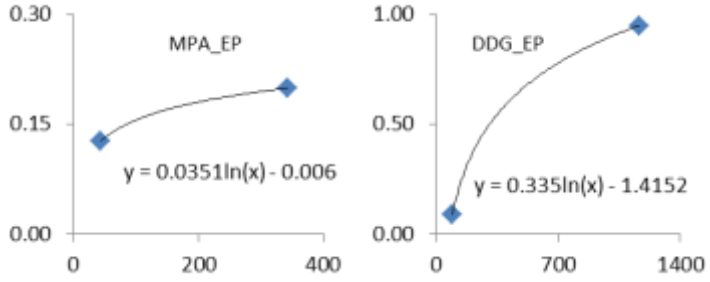

Fold change
hsd11b
cyp19a
lhβ
fshβ
cyp19b
nr1d1
nr1d2b
per1b
cry5
ccnb1
mycb1
cdc20
ahr1
ahr2
cyp1a
W C L M H L M H L M H
MPA DDG Mix

Mixture estimations are shown in the following Tables S7 and S8 followed by the graphics showing the basis of the calculations. The potential additive effects in the mixture for egg production and transcriptional alterations were investigated. Data analyses were performed according to the method described previously for P4 and DRG mixtures.[2] In brief, the response was plotted against concentration for each endpoint for individual exposures. A logarithmic curve was fitted to the data and the response for the concentrations used in individual exposures were predicted based on the equation. The difference between the observed and predicted responses for individual compounds was quantified and was used as margin of error for the equations. The expected response was predicted for the concentrations used in the mixture studies ± error rate. The expected additive effect (A+B) was calculated based on the predicted response and compared to the observed response.

Table S7. The measured concentrations and observed reproductive changes for zebrafish after exposure to MPA and DDG. Egg production (EP) of 14-21 days was used for this estimation, since the significant change occurred during this period at low concentration mixture group. Graphs are based on the measured concentrations (shown as Conc. in the table below=x axis) and percentage of changes normalized to solvent control (shown as average change (AC) in the table below=Y axis). Based on these graphs, the expected response for the investigated concentrations were re-calculated to estimate the accuracy of the formula. L, low exposure concentrations, H, high exposure concentration.

Group	14-21d Egg production							Percentage of change						
DMSO	33.7	34.4	18.8	30.3	19.9	34.5	28.3	-0.18	-0.20	0.34	-0.06	0.30	-0.21	0.01
MPA-L	20.6	24.4	33.7	18.6	25.3	31.9	20.2	0.28	0.15	-0.18	0.35	0.11	-0.12	0.29
MPA-H	44.9	21.7	22.7	9.7	22.2	24.4	14.5	-0.57	0.24	0.20	0.66	0.22	0.15	0.49
DDG-L	34.0	36.9	17.8	26.2	15.8	32.8	18.7	-0.19	-0.29	0.38	0.08	0.45	-0.15	0.35
DDG-H	1.2	2.3	0.9	2.2	1.1	1.6	0.9	0.96	0.92	0.97	0.92	0.96	0.94	0.97
Mix-L	15.9	30.8	13.0	15.5	20.4	23.3	10.6	0.44	-0.08	0.54	0.46	0.29	0.18	0.63
Mix-H	3.6	0.0	1.1	0.0	0.0	1.0	0.0	0.87	1.00	0.96	1.00	1.00	0.96	1.00

	MPA	Conc. (ng/L)	AC (100%)	SD	SEM	Predicted based on formula	DDG	Conc. (ng/L)	AC (100%)	SD	SEM	Predicted based on formula
EP	L	42.9	0.13	0.21	0.08	0.13	L	89.0	0.09	0.30	0.12	0.09
	H	342.3	0.20	0.39	0.15	0.20	H	1162.0	0.95	0.02	0.01	0.95



Calculation the difference between observed changes and the predicated changes for MPA and DDG treatments. These values were used for predicting additive effects. The reproductive response at high mixture concentration was not determined as the effect of DDG was the driving factor in the mixture.

	MPA	Conc. (ng/L)	AC	Predicted based on formula	Difference	DDG	Conc. (ng/L)	AC	Predicted based on formula	Difference	Predicted MPA_Mix	Predicted DDG_Mix	A+B (Predicted)
	L	42.9	0.28	0.13	-0.15	L	89.0	-0.19	0.09	0.28	-0.03	0.53	0.50
	L	42.9	0.15	0.13	-0.02	L	89.0	-0.29	0.09	0.38	0.10	0.63	0.74
	L	42.9	-0.18	0.13	0.31	L	89.0	0.38	0.09	-0.29	0.43	-0.04	0.39
	L	42.9	0.35	0.13	-0.22	L	89.0	0.08	0.09	0.01	-0.10	0.26	0.16
	L	42.9	0.11	0.13	0.01	L	89.0	0.45	0.09	-0.36	0.13	-0.10	0.03
	L	42.9	-0.12	0.13	0.24	L	89.0	-0.15	0.09	0.24	0.37	0.49	0.86
EP	L	42.9	0.29	0.13	-0.17	L	89.0	0.35	0.09	-0.26	-0.04	0.00	-0.05
	H	342.3	-0.57	0.20	0.77	H	1162.0	0.96	0.95	-0.01	0.98	1.06	2.04
	H	342.3	0.24	0.20	-0.04	H	1162.0	0.92	0.95	0.03	0.17	1.10	1.27
	H	342.3	0.20	0.20	0.00	H	1162.0	0.97	0.95	-0.02	0.20	1.05	1.25
	H	342.3	0.66	0.20	-0.46	H	1162.0	0.92	0.95	0.03	-0.25	1.09	0.84
	H	342.3	0.22	0.20	-0.02	H	1162.0	0.96	0.95	-0.01	0.18	1.06	1.24
	H	342.3	0.15	0.20	0.05	H	1162.0	0.94	0.95	0.00	0.26	1.07	1.33
	H	342.3	0.49	0.20	-0.29	H	1162.0	0.97	0.95	-0.02	-0.09	1.05	0.96

Mixture		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	AC	AC_ Predicted		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	AC	AC_ Predicted
	L	39.3	145.3	0.44	0.50	H	431.8	1662.5	0.87	2.04
	L	39.3	145.3	-0.08	0.74	H	431.8	1662.5	1.00	1.27
	L	39.3	145.3	0.54	0.39	H	431.8	1662.5	0.96	1.25
EP	L	39.3	145.3	0.46	0.16	H	431.8	1662.5	1.00	0.84
	L	39.3	145.3	0.29	0.03	H	431.8	1662.5	1.00	1.24
	L	39.3	145.3	0.18	0.86	H	431.8	1662.5	0.96	1.33
	L	39.3	1662.5	0.63	-0.05	H	431.8	1662.5	1.00	0.96

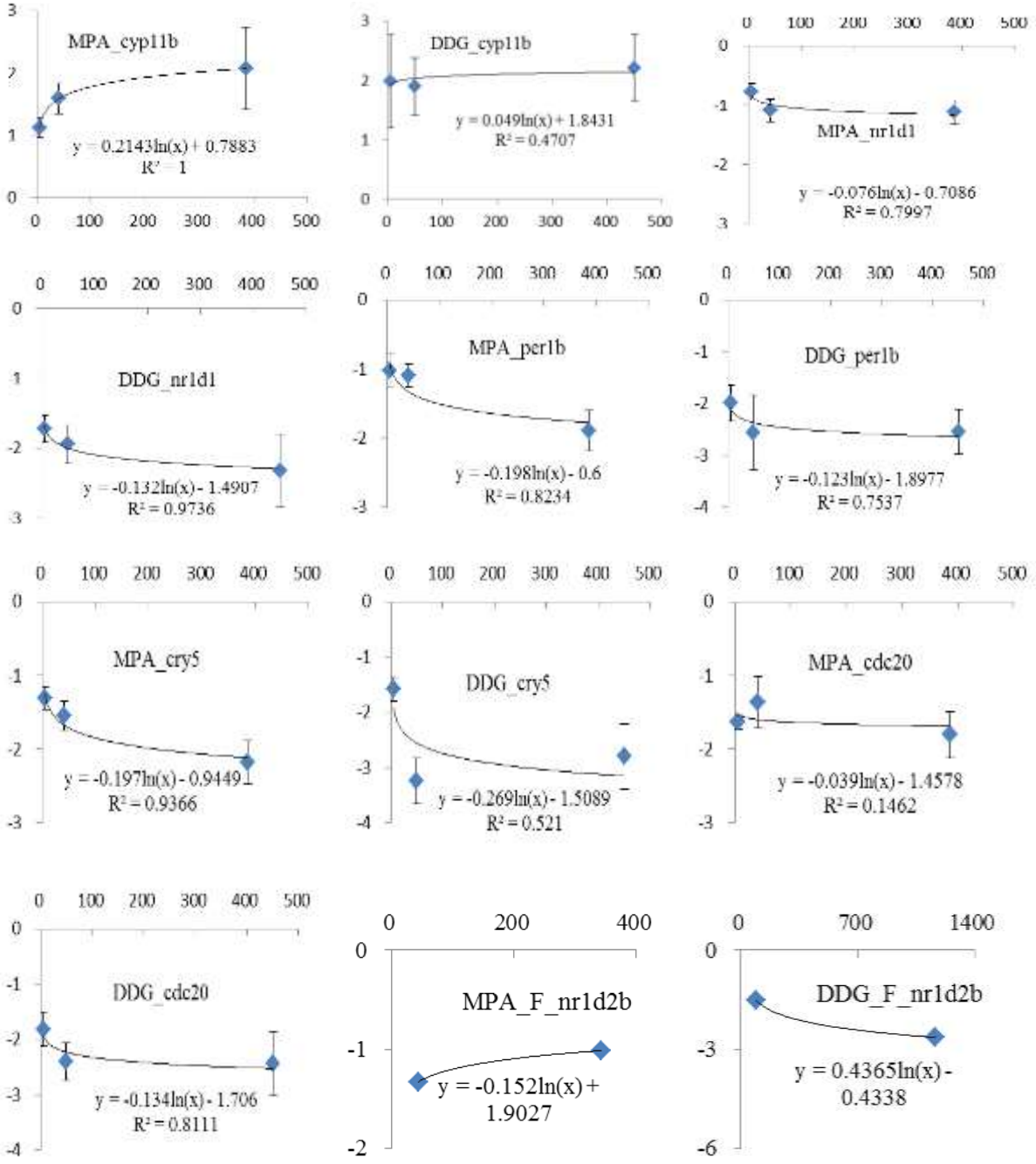
Mixture		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	Observed_ Ave FC	SD	SEM	Predicted_ Ave FC	SD	SEM	*P Value*
PO	L	39.3	145.3	0.35	0.24	0.09	0.38	0.35	0.13	0.799
	H	431.8	1662.5	0.97	0.05	0.02				

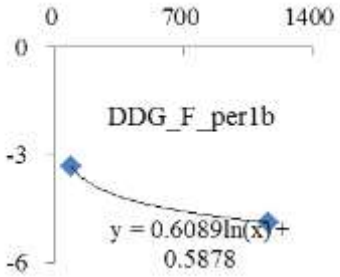
Table S8. Investigation into potential additive effect in mixtures for transcriptional responses. In total, 9 genes (5 genes in embryo exposures and 4 genes in adult zebrafish exposures) were identified as ideal candidates for investigating the additive effect due to their statistically different expression levels compared to the control and different magnitude of responses in the mixture compared to single MPA and DDG. The same approach described in Table S7 was employed.

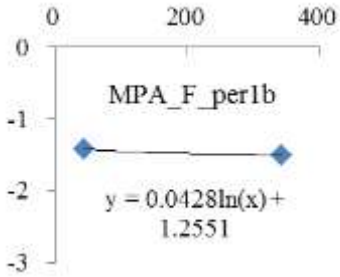
Table S8.1. The measured concentrations and the observed transcriptional responses for these 9 genes in zebrafish (*cyp11b*, *nr1d1*, *per1b*, *cry5* and *cdc20* in eleuthero-embryos and *nr1d2b*, *per1b*, *hsd11b* and *cyp17* in adult zebrafish) after exposure to MPA and DDG. Graphs are based on the measured concentrations (shown as Conc. in the table below=x axis) and observed fold change (shown as average fold change (Ave FC) in the table below=Y axis). Based on these graphs the expected response for all the investigated concentrations were re-calculated to estimate the accuracy of the formula. L, low exposure concentrations, M, medium exposure concentration, H, high exposure concentration.

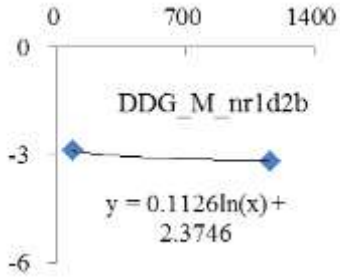
Transcript	MPA	Conc. (ng/L)	Ave FC	SD	SEM	Predicted based on formula	DDG	Conc. (ng/L)	Ave FC	SD	SEM	Predicted based on formula
	L	4.7	1.12	0.16	0.07	1.12	L	4.6	1.98	0.78	0.35	1.92
cyp11b	M	41.1	1.58	0.24	0.11	1.58	M	49.1	1.90	0.48	0.21	2.03
	H	385.5	2.06	0.66	0.30	2.06	H	450.5	2.21	0.55	0.25	2.14
	L	4.7	-0.78	0.14	0.06	-0.83	L	4.6	-1.72	0.19	0.09	-1.69
nr1d1	M	41.1	-1.09	0.19	0.08	-0.99	M	49.1	-1.95	0.28	0.13	-2.00
	H	385.5	-1.12	0.20	0.09	-1.16	H	450.5	-2.33	0.52	0.25	-2.30
	L	4.7	-1.02	0.23	0.10	-0.91	L	4.6	-1.99	0.35	0.15	-2.08
per1b	M	41.1	-1.10	0.17	0.07	-1.34	M	49.1	-2.56	0.72	0.32	-2.38
	H	385.5	-1.89	0.30	0.13	-1.78	H	450.5	-2.55	0.43	0.19	-2.65
	L	4.7	-1.32	0.15	0.07	-1.25	L	4.6	-1.59	0.22	0.10	-1.92
cry5	M	41.1	-1.55	0.20	0.09	-1.68	M	49.1	-3.24	0.41	0.18	-2.56
	H	385.5	-2.18	0.29	0.13	-2.12	H	450.5	-2.80	0.59	0.26	-3.15
	L	4.7	-1.64	0.09	0.04	-1.52	L	4.6	-1.83	0.30	0.13	-1.91
cdc20	M	41.1	-1.36	0.35	0.16	-1.60	M	49.1	-2.40	0.34	0.15	-2.23
	H	385.5	-1.81	0.32	0.14	-1.69	H	450.5	-2.44	0.58	0.26	-2.53
nr1d2b	L	42.9	-1.33	0.09	0.05	-1.33	L	89.0	-1.53	0.54	0.27	-1.53
(female)	H	342.3	-1.02	0.12	0.06	-1.02	H	1162.0	-2.65	0.44	0.22	-2.65
nr1d2b	L	42.9	-1.30	0.24	0.12	-1.30	L	89.0	-2.88	1.23	0.61	-2.88
(male)	H	342.3	-1.41	0.28	0.14	-1.41	H	1162.0	-3.17	1.49	0.75	-3.17
per1b	L	42.9	-1.42	0.27	0.13	-1.42	L	89.0	-3.32	1.49	0.74	-3.32
(female)	H	342.3	-1.50	0.30	0.15	-1.50	H	1162.0	-4.89	0.68	0.34	-4.89
per1b	L	42.9	-1.75	0.42	0.21	-1.75	L	89.0	-4.38	1.85	0.93	-4.38
(male)	H	342.3	-2.61	0.84	0.42	-2.61	H	1162.0	-8.78	5.64	2.82	-8.78
hsd11b	L	42.9	1.05	0.53	0.19	1.05	L	89.0	0.55	0.28	0.10	0.55
(female)	H	342.3	1.38	0.78	0.28	1.38	H	1162.0	0.55	0.13	0.04	0.55

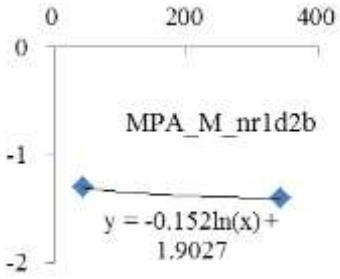
hsd11b	L	42.9	-1.94	0.97	0.49	-1.94	L	89.0	-5.45	1.44	0.72	-5.45
(male)	H	342.3	-3.75	1.51	0.76	-3.75	H	1162.0	-3.42	0.80	0.40	-3.43
cyp17	L	42.9	1.07	0.48	0.17	1.07	L	89.0	0.94	0.37	0.13	0.94
(female)	H	342.3	1.27	0.40	0.14	1.27	H	1162.0	1.00	0.31	0.11	1.00
cyp17	L	42.9	-1.21	0.14	0.07	-1.21	L	89.0	-3.75	0.47	0.23	-3.74
(male)	H	342.3	-2.53	0.85	0.42	-2.53	H	1162.0	-1.92	0.78	0.39	-1.91

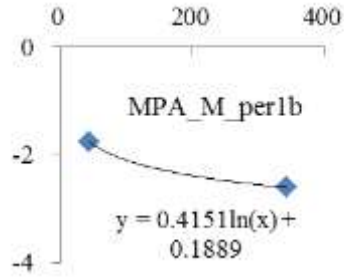


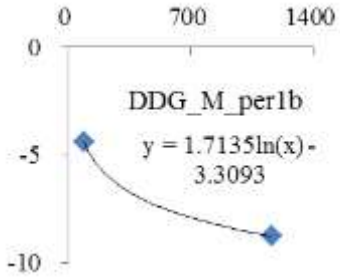

DDG_F_per1b
y = 0.6089ln(x) + 0.5878

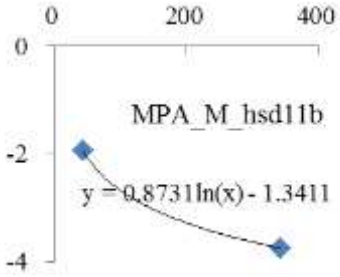

MPA_F_per1b
y = 0.0428ln(x) + 1.2551

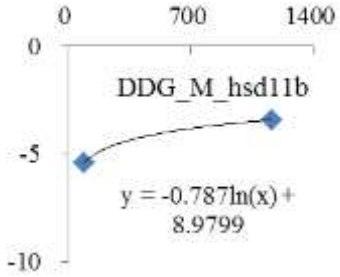

DDG_M_nr1d2b
y = 0.1126ln(x) + 2.3746

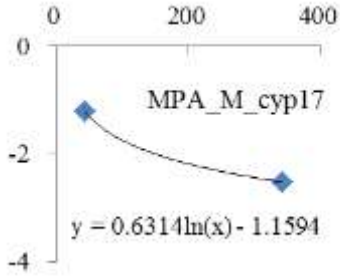

MPA_M_nr1d2b
y = -0.152ln(x) + 1.9027

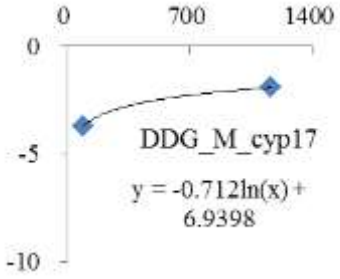

MPA_M_per1b
y = 0.4151ln(x) + 0.1889

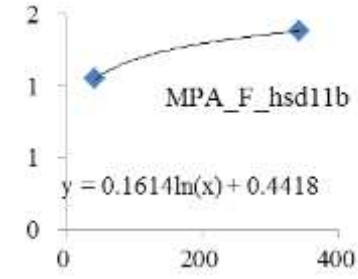

DDG_M_per1b
y = 1.7135ln(x) - 3.3093

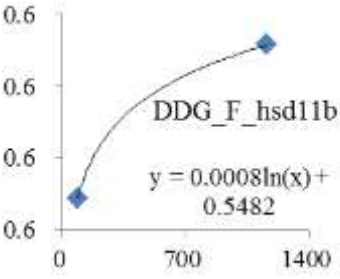

MPA_M_hsd11b
y = 0.8731ln(x) - 1.3411

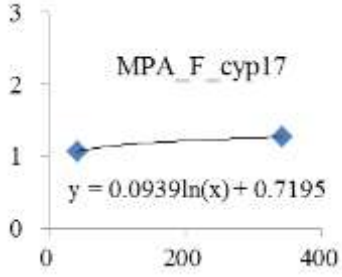

DDG_M_hsd11b
y = -0.787ln(x) + 8.9799


MPA_M_cyp17
y = 0.6314ln(x) - 1.1594


DDG_M_cyp17
y = -0.712ln(x) + 6.9398


MPA_F_hsd11b
y = 0.1614ln(x) + 0.4418


DDG_F_hsd11b
y = 0.0008ln(x) + 0.5482


MPA_F_cyp17
y = 0.0939ln(x) + 0.7195

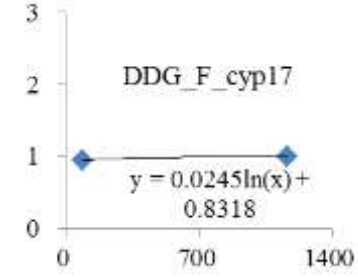

DDG_F_cyp17
y = 0.0245ln(x) + 0.8318

Table S8.2. Calculating the difference between the observed fold change (FC) and the predicated FC for MPA and DDG treatments. These values were used for predicting additive effects.

	MPA	Conc. (ng/L)	FC	Predicted based on formula	Difference	DDG	Conc. (ng/L)	FC	Predicted based on formula	Difference	Predicted MPA_Mix	Predicted DDG_Mix	A+B (Predicted)
	L	4.7	0.99	1.12	0.13	L	4.6	1.78	1.92	0.14	1.25	2.05	3.30
	L	4.7	1.05	1.12	0.07	L	4.6	0.96	1.92	0.96	1.18	2.87	4.06
	L	4.7	1.02	1.12	0.10	L	4.6	1.63	1.92	0.28	1.21	2.20	3.41
	L	4.7	1.39	1.12	-0.27	L	4.6	2.76	1.92	-0.85	0.85	1.07	1.92
	L	4.7	1.15	1.12	-0.03	L	4.6	2.78	1.92	-0.86	1.09	1.06	2.14
	M	41.1	1.55	1.58	0.04	M	49.1	1.51	2.03	0.53	1.63	2.55	4.19
	M	41.1	1.81	1.58	-0.23	M	49.1	1.32	2.03	0.71	1.37	2.74	4.11
cyp11b	M	41.1	1.19	1.58	0.40	M	49.1	2.44	2.03	-0.41	2.00	1.62	3.62
	M	41.1	1.67	1.58	-0.08	M	49.1	1.95	2.03	0.09	1.51	2.12	3.63
	M	41.1	1.71	1.58	-0.13	M	49.1	2.27	2.03	-0.23	1.47	1.80	3.27
	H	385.5	1.46	2.06	0.60	H	450.5	1.46	2.14	0.68	2.68	2.82	5.50
	H	385.5	1.45	2.06	0.61	H	450.5	2.57	2.14	-0.43	2.69	1.71	4.39
	H	385.5	2.94	2.06	-0.87	H	450.5	2.44	2.14	-0.30	1.20	1.84	3.04
	H	385.5	2.54	2.06	-0.47	H	450.5	1.83	2.14	0.32	1.60	2.45	4.06
	H	385.5	1.92	2.06	0.14	H	450.5	2.77	2.14	-0.63	2.22	1.51	3.72
	L	4.7	-0.60	-0.83	-0.22	L	4.6	-1.41	-1.69	-0.28	-1.05	-1.97	-3.02
	L	4.7	-0.76	-0.83	-0.06	L	4.6	-1.90	-1.69	0.21	-0.89	-1.48	-2.37
	L	4.7	-0.87	-0.83	0.04	L	4.6	-1.66	-1.69	-0.03	-0.78	-1.72	-2.50
nr1d1	L	4.7	-0.96	-0.83	0.13	L	4.6	-1.79	-1.69	0.10	-0.70	-1.59	-2.28
	L	4.7	-0.69	-0.83	-0.13	L	4.6	-1.83	-1.69	0.14	-0.96	-1.54	-2.50
	M	41.1	-1.38	-0.99	0.39	M	49.1	-1.69	-2.00	-0.31	-0.61	-2.31	-2.91
	M	41.1	-1.13	-0.99	0.14	M	49.1	-2.36	-2.00	0.36	-0.86	-1.64	-2.49

	M	41.1	-1.10	-0.99	0.1	M	49.1	-1.85	-2.00	-0.15	-0.89	-2.14	-3.03
	M	41.1	-0.93	-0.99	-0.06	M	49.1	-1.73	-2.00	-0.27	-1.06	-2.26	-3.32
	M	41.1	-0.91	-0.99	-0.09	M	49.1	-2.09	-2.00	0.09	-1.08	-1.91	-2.99
	H	385.5	-1.36	-1.16	0.2	H	450.5	-1.86	-2.30	-0.44	-0.97	-2.73	-3.70
	H	385.5	-1.11	-1.16	-0.05	H	450.5	-2.25	-2.30	-0.04	-1.22	-2.33	-3.55
	H	385.5	-1.00	-1.16	-0.17	H	450.5	-2.00	-2.30	-0.30	-1.34	-2.58	-3.92
	H	385.5	-0.85	-1.16	-0.31	H	450.5	-3.19	-2.30	0.90	-1.48	-1.39	-2.87
	H	385.5	-1.26	-1.16	0.1	H	450.5	-2.33	-2.30	0.03	-1.07	-2.25	-3.33
	L	4.7	-0.87	-0.91	-0.03	L	4.6	-1.58	-2.08	-0.50	-0.94	-2.58	-3.52
per1b	L	4.7	-0.97	-0.91	0.07	L	4.6	-2.10	-2.08	0.02	-0.84	-2.06	-2.90
	L	4.7	-1.43	-0.91	0.52	L	4.6	-1.84	-2.08	-0.24	-0.38	-2.32	-2.70
	L	4.7	-0.95	-0.91	0.05	L	4.6	-1.93	-2.08	-0.16	-0.86	-2.24	-3.09
	L	4.7	-0.90	-0.91	-0.01	L	4.6	-2.51	-2.08	0.43	-0.91	-1.65	-2.56
	M	41.1	-1.23	-1.34	-0.10	M	49.1	-3.24	-2.38	0.86	-1.45	-1.50	-2.96
	M	41.1	-1.13	-1.34	-0.21	M	49.1	-3.43	-2.38	1.06	-1.56	-1.31	-2.86
	M	41.1	-1.01	-1.34	-0.33	M	49.1	-2.22	-2.38	-0.15	-1.67	-2.52	-4.19
	M	41.1	-1.27	-1.34	-0.06	M	49.1	-1.93	-2.38	-0.44	-1.41	-2.81	-4.22
	M	41.1	-0.87	-1.34	-0.47	M	49.1	-1.98	-2.38	-0.39	-1.81	-2.76	-4.57
	H	385.5	-2.33	-1.78	0.55	H	450.5	-1.98	-2.65	-0.67	-1.24	-3.31	-4.55
	H	385.5	-1.65	-1.78	-0.13	H	450.5	-2.25	-2.65	-0.39	-1.92	-3.03	-4.96
	H	385.5	-1.77	-1.78	-0.01	H	450.5	-2.92	-2.65	0.27	-1.80	-2.37	-4.17
	H	385.5	-2.07	-1.78	0.29	H	450.5	-2.97	-2.65	0.33	-1.50	-2.31	-3.81
	H	385.5	-1.66	-1.78	-0.12	H	450.5	-2.63	-2.65	-0.02	-1.91	-2.65	-4.56
	L	4.7	-1.24	-1.25	-0.01	L	4.6	-1.62	-1.92	-0.29	-1.25	-2.20	-3.46
	L	4.7	-1.37	-1.25	0.12	L	4.6	-1.67	-1.92	-0.25	-1.13	-2.15	-3.28
cry5	L	4.7	-1.33	-1.25	0.08	L	4.6	-1.70	-1.92	-0.21	-1.17	-2.12	-3.29
	L	4.7	-1.11	-1.25	-0.14	L	4.6	-1.20	-1.92	-0.71	-1.38	-2.62	-4.01
	L	4.7	-1.53	-1.25	0.28	L	4.6	-1.74	-1.92	-0.18	-0.97	-2.09	-3.06

	M	41.1	-1.54	-1.68	-0.14	M	49.1	-3.10	-2.56	0.55	-1.82	-1.99	-3.81
	M	41.1	-1.20	-1.68	-0.47	M	49.1	-2.98	-2.56	0.42	-2.16	-2.11	-4.27
	M	41.1	-1.64	-1.68	-0.04	M	49.1	-2.78	-2.56	0.23	-1.73	-2.31	-4.03
	M	41.1	-1.65	-1.68	-0.03	M	49.1	-3.67	-2.56	1.11	-1.71	-1.42	-3.13
	M	41.1	-1.70	-1.68	0.02	M	49.1	-3.67	-2.56	1.11	-1.67	-1.42	-3.09
	H	385.5	-2.27	-2.12	0.16	H	450.5	-3.72	-3.15	0.57	-1.97	-2.56	-4.54
	H	385.5	-2.00	-2.12	-0.11	H	450.5	-2.09	-3.15	-1.06	-2.24	-4.19	-6.43
	H	385.5	-2.63	-2.12	0.52	H	450.5	-2.64	-3.15	-0.52	-1.61	-3.65	-5.26
	H	385.5	-1.87	-2.12	-0.24	H	450.5	-2.82	-3.15	-0.34	-2.37	-3.47	-5.84
	H	385.5	-2.12	-2.12	0.00	H	450.5	-2.73	-3.15	-0.42	-2.12	-3.55	-5.68
	L	4.70	-1.74	-1.52	0.22	L	4.55	-1.67	-1.91	-0.24	-1.29	-2.14	-3.44
	L	4.70	-1.70	-1.52	0.18	L	4.55	-1.80	-1.91	-0.11	-1.33	-2.02	-3.35
	L	4.70	-1.55	-1.52	0.03	L	4.55	-1.68	-1.91	-0.23	-1.49	-2.14	-3.63
	L	4.70	-1.67	-1.52	0.15	L	4.55	-2.35	-1.91	0.44	-1.37	-1.46	-2.83
	L	4.70	-1.54	-1.52	0.02	L	4.55	-1.64	-1.91	-0.27	-1.49	-2.18	-3.67
	M	41.10	-0.87	-1.60	-0.73	M	49.10	-2.61	-2.23	0.38	-2.34	-1.84	-4.18
	M	41.10	-1.72	-1.60	0.11	M	49.10	-2.88	-2.23	0.65	-1.49	-1.57	-3.06
cdc20	M	41.10	-1.44	-1.60	-0.16	M	49.10	-2.32	-2.23	0.09	-1.76	-2.13	-3.89
	M	41.10	-1.16	-1.60	-0.44	M	49.10	-2.13	-2.23	-0.10	-2.04	-2.32	-4.36
	M	41.10	-1.63	-1.60	0.02	M	49.10	-2.08	-2.23	-0.15	-1.58	-2.37	-3.95
	H	385.50	-1.60	-1.69	-0.09	H	450.50	-3.18	-2.53	0.65	-1.78	-1.87	-3.65
	H	385.50	-1.90	-1.69	0.21	H	450.50	-1.76	-2.53	-0.77	-1.48	-3.29	-4.77
	H	385.50	-1.36	-1.69	-0.33	H	450.50	-2.72	-2.53	0.19	-2.02	-2.32	-4.34
	H	385.50	-2.14	-1.69	0.45	H	450.50	-1.96	-2.53	-0.56	-1.24	-3.08	-4.32
	H	385.50	-2.04	-1.69	0.35	H	450.50	-2.57	-2.53	0.04	-1.34	-2.47	-3.82
	L	42.9	-1.35	-1.33	0.02	L	89.0	-1.71	-1.53	0.19	-1.33	-1.55	-2.88
nr1d2b	L	42.9	-1.40	-1.33	0.07	L	89.0	-0.72	-1.53	-0.81	-1.28	-2.55	-3.83
(female)	L	42.9	-1.20	-1.33	-0.13	L	89.0	-1.80	-1.53	0.28	-1.48	-1.46	-2.94

	L	42.9	-1.38	-1.33	0.05	L	89.0	-1.87	-1.53	0.34	-1.30	-1.40	-2.69
	H	342.3	-0.92	-1.02	-0.10	H	1162.0	-2.23	-2.65	-0.42	-1.08	-3.22	-4.30
	H	342.3	-0.92	-1.02	-0.10	H	1162.0	-2.65	-2.65	0.00	-1.08	-2.80	-3.88
	H	342.3	-1.05	-1.02	0.03	H	1162.0	-2.45	-2.65	-0.19	-0.95	-3.00	-3.95
	H	342.3	-1.18	-1.02	0.16	H	1162.0	-3.26	-2.65	0.61	-0.82	-2.20	-3.01
nr1d2b (male)	L	42.9	-1.26	-1.30	-0.04	L	89.0	-1.53	-2.88	-1.35	-1.34	-4.28	-5.62
	L	42.9	-1.55	-1.30	0.25	L	89.0	-3.06	-2.88	0.18	-1.05	-2.75	-3.80
	L	42.9	-1.40	-1.30	0.10	L	89.0	-2.47	-2.88	-0.41	-1.19	-3.35	-4.54
	L	42.9	-0.99	-1.30	-0.31	L	89.0	-4.46	-2.88	1.58	-1.61	-1.35	-2.96
	H	342.3	-1.19	-1.41	-0.22	H	1162.0	-1.56	-3.17	-1.60	-1.64	-4.81	-6.45
	H	342.3	-1.15	-1.41	-0.26	H	1162.0	-3.25	-3.17	0.08	-1.68	-3.13	-4.81
	H	342.3	-1.67	-1.41	0.27	H	1162.0	-2.72	-3.17	-0.45	-1.15	-3.66	-4.81
	H	342.3	-1.62	-1.41	0.21	H	1162.0	-5.14	-3.17	1.97	-1.21	-1.24	-2.44
per1b (female)	L	42.9	-1.40	-1.42	-0.01	L	89.0	-3.08	-3.32	-0.24	-1.43	-3.86	-5.28
	L	42.9	-1.35	-1.42	-0.06	L	89.0	-1.42	-3.32	-1.90	-1.48	-5.52	-7.00
	L	42.9	-1.13	-1.42	-0.28	L	89.0	-4.97	-3.32	1.65	-1.69	-1.97	-3.66
	L	42.9	-1.77	-1.42	0.36	L	89.0	-3.82	-3.32	0.49	-1.05	-3.13	-4.18
	H	342.3	-1.26	-1.50	-0.25	H	1162.0	-4.23	-4.89	-0.65	-1.76	-5.76	-7.52
	H	342.3	-1.24	-1.50	-0.27	H	1162.0	-4.89	-4.89	0.00	-1.78	-5.10	-6.89
	H	342.3	-1.73	-1.50	0.22	H	1162.0	-4.60	-4.89	-0.28	-1.29	-5.39	-6.68
	H	342.3	-1.80	-1.50	0.29	H	1162.0	-5.82	-4.89	0.94	-1.22	-4.17	-5.39
per1b (male)	L	42.9	-1.55	-1.75	-0.19	L	89.0	-1.83	-4.38	-2.55	-1.91	-7.77	-9.68
	L	42.9	-2.36	-1.75	0.61	L	89.0	-5.93	-4.38	1.54	-1.10	-3.68	-4.78
	L	42.9	-1.66	-1.75	-0.09	L	89.0	-4.23	-4.38	-0.15	-1.80	-5.38	-7.17
	L	42.9	-1.42	-1.75	-0.33	L	89.0	-5.54	-4.38	1.16	-2.04	-4.06	-6.11
	H	342.3	-2.07	-2.61	-0.54	H	1162.0	-4.21	-8.78	-4.57	-3.25	-13.97	-17.22
	H	342.3	-1.76	-2.61	-0.85	H	1162.0	-16.41	-8.78	7.62	-3.56	-1.78	-5.33
	H	342.3	-3.56	-2.61	0.95	H	1162.0	-4.82	-8.78	-3.96	-1.76	-13.36	-15.12

	H	342.3	-3.06	-2.61	0.45	H	1162.0	-9.69	-8.78	0.91	-2.26	-8.49	-10.75
hsd11b (female)	L	42.9	0.67	1.05	0.38	L	89.0	0.37	0.55	0.18	1.41	0.74	2.15
	L	42.9	1.20	1.05	-0.15	L	89.0	0.80	0.55	-0.25	0.88	0.30	1.18
	L	42.9	1.96	1.05	-0.91	L	89.0	0.55	0.55	0.00	0.13	0.55	0.68
	L	42.9	0.34	1.05	0.71	L	89.0	0.95	0.55	-0.40	1.75	0.15	1.90
	L	42.9	1.46	1.05	-0.41	L	89.0	0.32	0.55	0.23	0.62	0.78	1.41
	L	42.9	1.30	1.05	-0.25	L	89.0	0.32	0.55	0.24	0.78	0.79	1.57
	L	42.9	0.88	1.05	0.17	L	89.0	0.27	0.55	0.28	1.20	0.84	2.04
	L	42.9	0.58	1.05	0.47	L	89.0	0.84	0.55	-0.29	1.51	0.26	1.77
	H	342.3	0.53	1.38	0.85	H	1162.0	0.54	0.55	0.02	2.28	0.57	2.84
	H	342.3	0.85	1.38	0.54	H	1162.0	0.60	0.55	-0.04	1.96	0.51	2.47
	H	342.3	1.31	1.38	0.07	H	1162.0	0.47	0.55	0.09	1.49	0.64	2.13
	H	342.3	1.92	1.38	-0.54	H	1162.0	0.77	0.55	-0.22	0.89	0.34	1.22
	H	342.3	0.70	1.38	0.69	H	1162.0	0.44	0.55	0.12	2.11	0.67	2.78
	H	342.3	2.44	1.38	-1.06	H	1162.0	0.73	0.55	-0.18	0.36	0.38	0.74
	H	342.3	2.44	1.38	-1.06	H	1162.0	0.47	0.55	0.09	0.37	0.64	1.01
	H	342.3	0.88	1.38	0.50	H	1162.0	0.42	0.55	0.13	1.92	0.68	2.61
hsd11b (male)	L	42.9	-1.03	-1.94	-0.91	L	89.0	-4.07	-5.45	-1.37	-2.78	-6.44	-9.21
	L	42.9	-2.06	-1.94	0.12	L	89.0	-5.93	-5.45	0.48	-1.74	-4.58	-6.32
	L	42.9	-1.42	-1.94	-0.52	L	89.0	-4.52	-5.45	-0.92	-2.38	-5.99	-8.37
	L	42.9	-3.25	-1.94	1.31	L	89.0	-7.25	-5.45	1.81	-0.56	-3.25	-3.81
	H	342.3	-1.96	-3.75	-1.79	H	1162.0	-2.62	-3.43	-0.81	-5.75	-3.95	-9.70
	H	342.3	-4.27	-3.75	0.51	H	1162.0	-4.51	-3.43	1.09	-3.45	-2.06	-5.50
	H	342.3	-3.26	-3.75	-0.50	H	1162.0	-3.46	-3.43	0.03	-4.45	-3.11	-7.57
	H	342.3	-5.53	-3.75	1.78	H	1162.0	-3.11	-3.43	-0.32	-2.18	-3.46	-5.64
cyp17 (female)	L	42.9	0.70	1.07	0.37	L	89.0	0.97	0.94	-0.03	1.43	0.92	2.36
	L	42.9	1.03	1.07	0.04	L	89.0	0.94	0.94	0.01	1.11	0.96	2.07
	L	42.9	2.17	1.07	-1.10	L	89.0	0.94	0.94	0.00	-0.03	0.95	0.92

	L	42.9	0.82	1.07	0.25	L	89.0	1.68	0.94	-0.74	1.32	0.21	1.53
	L	42.9	0.91	1.07	0.17	L	89.0	0.64	0.94	0.31	1.23	1.26	2.49
	L	42.9	1.32	1.07	-0.24	L	89.0	0.73	0.94	0.21	0.82	1.17	1.99
	L	42.9	0.85	1.07	0.22	L	89.0	0.48	0.94	0.46	1.29	1.42	2.71
	L	42.9	0.79	1.07	0.28	L	89.0	1.16	0.94	-0.22	1.35	0.74	2.09
	H	342.3	1.55	1.27	-0.28	H	1162.0	1.31	1.00	-0.31	1.01	0.71	1.72
	H	342.3	1.54	1.27	-0.28	H	1162.0	1.06	1.00	-0.06	1.01	0.95	1.97
	H	342.3	1.96	1.27	-0.69	H	1162.0	0.77	1.00	0.24	0.60	1.25	1.85
	H	342.3	1.19	1.27	0.08	H	1162.0	1.61	1.00	-0.60	1.37	0.41	1.78
	H	342.3	0.91	1.27	0.35	H	1162.0	0.91	1.00	0.09	1.64	1.11	2.75
	H	342.3	1.30	1.27	-0.03	H	1162.0	0.69	1.00	0.32	1.26	1.33	2.59
	H	342.3	0.89	1.27	0.38	H	1162.0	0.92	1.00	0.08	1.66	1.10	2.76
	H	342.3	0.79	1.27	0.47	H	1162.0	0.77	1.00	0.23	1.76	1.25	3.01
cyp17 (male)	L	42.9	-1.21	-1.21	-0.01	L	89.0	-3.35	-3.74	-0.39	-1.16	-3.79	-4.95
	L	42.9	-1.36	-1.21	0.15	L	89.0	-4.16	-3.74	0.42	-1.01	-2.98	-3.99
	L	42.9	-1.26	-1.21	0.04	L	89.0	-3.33	-3.74	-0.41	-1.11	-3.81	-4.92
	L	42.9	-1.03	-1.21	-0.19	L	89.0	-4.14	-3.74	0.39	-1.34	-3.00	-4.34
	H	342.3	-1.47	-2.53	-1.06	H	1162.0	-0.95	-1.91	-0.97	-3.73	-2.63	-6.35
	H	342.3	-2.53	-2.53	0.01	H	1162.0	-2.85	-1.91	0.94	-2.66	-0.72	-3.38
	H	342.3	-2.55	-2.53	0.02	H	1162.0	-1.94	-1.91	0.02	-2.65	-1.64	-4.29
	H	342.3	-3.55	-2.53	1.03	H	1162.0	-1.93	-1.91	0.02	-1.65	-1.64	-3.29

4

Table S8.3. Calculating the difference between the observed FC and the predicated FC for mixture treatments. These values were used for predicting additive effects.

Mixture *Transcript*		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	FC	FC_ Predicted	Mixture		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	FC	FC_ Predicted
	L	4.6	4.4	5.44	3.30		L	4.6	4.4	-3.66	-3.02
	L	4.6	4.4	1.57	4.06		L	4.6	4.4	-4.48	-2.37
	L	4.6	4.4	2.08	3.41		L	4.6	4.4	-4.37	-2.50
	L	4.6	4.4	4.85	1.92		L	4.6	4.4	-5.44	-2.28
	L	4.6	4.4	4.09	2.14		L	4.6	4.4	-4.98	-2.50
	M	43.5	45.1	1.92	4.19		M	43.5	45.1	-4.91	-2.91
	M	43.5	45.1	2.30	4.11		M	43.5	45.1	-4.78	-2.49
cyp11b	M	43.5	45.1	1.88	3.62	*nr1d1*	M	43.5	45.1	-4.47	-3.03
	M	43.5	45.1	2.11	3.63		M	43.5	45.1	-6.01	-3.32
	M	43.5	45.1	2.08	3.27		M	43.5	45.1	-6.27	-2.99
	H	407.5	414.0	2.94	5.50		H	407.5	414.0	-3.30	-3.70
	H	407.5	414.0	1.60	4.39		H	407.5	414.0	-5.11	-3.55
	H	407.5	414.0	1.06	3.04		H	407.5	414.0	-6.19	-3.92
	H	407.5	414.0	1.75	4.06		H	407.5	414.0	-8.48	-2.87
	H	407.5	414.0	2.44	3.72		H	407.5	414.0	-6.47	-3.33
	L	4.6	4.4	-3.66	-3.52		L	4.6	4.4	-2.64	-3.46
	L	4.6	4.4	-4.48	-2.90		L	4.6	4.4	-3.98	-3.28
	L	4.6	4.4	-4.37	-2.70		L	4.6	4.4	-3.32	-3.29
	L	4.6	4.4	-5.44	-3.09		L	4.6	4.4	-2.91	-4.01
	L	4.6	4.4	-4.98	-2.56		L	4.6	4.4	-4.02	-3.06
	M	43.5	45.1	-4.91	-2.96		M	43.5	45.1	-2.78	-3.81
	M	43.5	45.1	-4.78	-2.86		M	43.5	45.1	-3.94	-4.27
per1b	M	43.5	45.1	-4.47	-4.19	*cry5*	M	43.5	45.1	-3.93	-4.03
	M	43.5	45.1	-6.01	-4.22		M	43.5	45.1	-4.16	-3.13
	M	43.5	45.1	-6.27	-4.57		M	43.5	45.1	-3.97	-3.09
	H	407.5	414.0	-3.30	-4.55		H	407.5	414.0	-3.80	-4.54
	H	407.5	414.0	-5.11	-4.96		H	407.5	414.0	-3.95	-6.43
	H	407.5	414.0	-6.19	-4.17		H	407.5	414.0	-4.71	-5.26
	H	407.5	414.0	-8.48	-3.81		H	407.5	414.0	-3.19	-5.84
	H	407.5	414.0	-6.47	-4.56		H	407.5	414.0	-4.10	-5.68
	L	4.6	4.4	-1.43	-3.44		L	39.3	145.3	-2.85	-2.88
	L	4.6	4.4	-1.33	-3.35		L	39.3	145.3	-3.02	-3.83
	L	4.6	4.4	-1.39	-3.63		L	39.3	145.3	-4.89	-2.94
cdc20	L	4.6	4.4	-1.50	-2.83	*nr1d2b*	L	39.3	145.3	-4.48	-2.69
	L	4.6	4.4	-2.07	-3.67	female	H	431.8	1662.5	-4.67	-4.30
	M	43.5	45.1	-1.18	-4.18		H	431.8	1662.5	-6.67	-3.88
	M	43.5	45.1	-1.36	-3.06		H	431.8	1662.5	-3.50	-3.95
	M	43.5	45.1	-2.10	-3.89		H	431.8	1662.5	-6.87	-3.01

	M	43.5	45.1	-1.41	-4.36		L	39.3	145.3	-5.73	-5.62
	M	43.5	45.1	-2.08	-3.95		L	39.3	145.3	-7.94	-3.80
	H	407.5	414.0	-2.05	-3.65		L	39.3	145.3	-6.32	-4.54
	H	407.5	414.0	-2.27	-4.77	*nr1d2b*	L	39.3	145.3	-5.13	-2.96
	H	407.5	414.0	-1.99	-4.34	male	H	431.8	1662.5	-4.46	-6.45
	H	407.5	414.0	-1.81	-4.32		H	431.8	1662.5	-14.91	-4.81
	H	407.5	414.0	-2.05	-3.82		H	431.8	1662.5	-7.28	-4.81
				0.00	0.00		H	431.8	1662.5	-5.95	-2.44
	L	39.3	145.3	-4.79	-5.28		L	39.3	145.3	-9.22	-9.68
	L	39.3	145.3	-6.01	-7.00		L	39.3	145.3	-10.70	-4.78
	L	39.3	145.3	-7.49	-3.66		L	39.3	145.3	-12.73	-7.17
per1b	L	39.3	145.3	-4.85	-4.18	*per1b*	L	39.3	145.3	-10.66	-6.11
female	H	431.8	1662.5	-7.51	-7.52	male	H	431.8	1662.5	-9.83	-17.22
	H	431.8	1662.5	-10.60	-6.89		H	431.8	1662.5	-23.88	-5.33
	H	431.8	1662.5	-6.45	-6.68		H	431.8	1662.5	-17.24	-15.12
	H	431.8	1662.5	-8.62	-5.39		H	431.8	1662.5	-16.19	-10.75
	L	39.3	145.3	1.31	2.15		L	39.3	145.3	1.35	2.36
	L	39.3	145.3	0.33	1.18		L	39.3	145.3	0.80	2.07
	L	39.3	145.3	0.55	0.68		L	39.3	145.3	0.56	0.92
	L	39.3	145.3	1.57	1.90		L	39.3	145.3	0.91	1.53
	L	39.3	145.3	1.11	1.41		L	39.3	145.3	1.01	2.49
	L	39.3	145.3	0.76	1.57		L	39.3	145.3	0.58	1.99
	L	39.3	145.3	0.99	2.04		L	39.3	145.3	0.79	2.71
hsd11b	L	39.3	145.3	0.51	1.77	*cyp17*	L	39.3	145.3	1.32	2.09
female	H	431.8	1662.5	2.01	2.84	female	H	431.8	1662.5	1.36	1.72
	H	431.8	1662.5	1.15	2.47		H	431.8	1662.5	0.96	1.97
	H	431.8	1662.5	0.92	2.13		H	431.8	1662.5	1.05	1.85
	H	431.8	1662.5	0.87	1.22		H	431.8	1662.5	1.03	1.78
	H	431.8	1662.5	1.29	2.78		H	431.8	1662.5	1.05	2.75
	H	431.8	1662.5	1.38	0.74		H	431.8	1662.5	0.85	2.59
	H	431.8	1662.5	1.14	1.01		H	431.8	1662.5	0.91	2.76
	H	431.8	1662.5	1.55	2.61		H	431.8	1662.5	1.17	3.01
	L	39.3	145.3	-4.41	-9.21		L	39.3	145.3	-2.91	-4.95
	L	39.3	145.3	-5.66	-6.32		L	39.3	145.3	-4.55	-3.99
	L	39.3	145.3	-8.41	-8.37		L	39.3	145.3	-6.14	-4.92
hsd11b	L	39.3	145.3	-6.51	-3.81	*cyp17*	L	39.3	145.3	-4.15	-4.34
male	H	431.8	1662.5	-1.31	-9.70	male	H	431.8	1662.5	-0.72	-6.35
	H	431.8	1662.5	-2.99	-5.50		H	431.8	1662.5	-1.73	-3.38
	H	431.8	1662.5	-2.91	-7.57		H	431.8	1662.5	-1.51	-4.29
	H	431.8	1662.5	-2.99	-5.64		H	431.8	1662.5	-2.22	-3.29

8
9

10

Mixture *Transcript*		MPA_Conc. (ng/L)	DDG_Conc. (ng/L)	Observed_ Ave FC	SD	SEM	Predicted_ Ave FC	SD	SEM
	L	4.6	4.4	3.60	1.71	0.76	2.97	0.91	0.41
cyp11b	M	43.5	45.1	2.06	0.17	0.07	3.76	0.38	0.17
	H	407.5	414.0	1.96	0.74	0.33	4.14	0.91	0.41
	L	4.6	4.4	-4.59	0.67	0.30	-2.53	0.28	0.13
nr1d1	M	43.5	45.1	-5.29	0.80	0.36	-2.95	0.30	0.13
	H	407.5	414.0	-5.91	1.90	0.85	-3.47	0.40	0.18
	L	4.6	4.4	-3.50	0.28	0.13	-2.95	0.37	0.17
per1b	M	43.5	45.1	-3.04	0.38	0.17	-3.76	0.79	0.35
	H	407.5	414.0	-3.63	0.52	0.23	-4.41	0.43	0.20
	L	4.6	4.4	-3.38	0.62	0.28	-3.42	0.36	0.16
cry5	M	43.5	45.1	-3.76	0.55	0.25	-3.67	0.53	0.24
	H	407.5	414.0	-3.95	0.55	0.24	-5.55	0.71	0.31
	L	4.6	4.4	-1.54	0.30	0.13	-3.38	0.34	0.15
cdc20	M	43.5	45.1	-1.63	0.43	0.19	-3.89	0.50	0.22
	H	407.5	414.0	-2.03	0.16	0.07	-4.18	0.45	0.20
nr1d2b	L	39.3	145.3	-3.81	1.03	0.51	-3.08	0.51	0.25
female	H	431.8	1662.5	-5.43	1.62	0.81	-3.78	0.55	0.27
nr1d2b	L	39.3	145.3	-6.28	1.21	0.60	-4.23	1.13	0.56
male	H	431.8	1662.5	-8.15	4.65	2.32	-4.63	1.65	0.83
per1b	L	39.3	145.3	-5.79	1.27	0.63	-5.03	1.48	0.74
female	H	431.8	1662.5	-8.29	1.77	0.89	-6.62	0.89	0.45
per1b	L	39.3	145.3	-10.83	1.45	0.72	-6.93	2.07	1.04
male	H	431.8	1662.5	-16.78	5.75	2.88	-12.11	5.26	2.63
hsd11b	L	39.3	145.3	0.89	0.43	0.15	1.59	0.49	0.17
female	H	431.8	1662.5	1.29	0.37	0.13	1.98	0.85	0.30
hsd11b	L	39.3	145.3	-6.25	1.68	0.84	-6.93	2.41	1.20
male	H	431.8	1662.5	-2.55	0.83	0.41	-7.10	1.97	0.99
cyp17	L	39.3	145.3	0.91	0.30	0.11	2.02	0.57	0.20
female	H	431.8	1662.5	1.05	0.16	0.06	2.30	0.53	0.19
cyp17	L	39.3	145.3	-4.44	1.33	0.67	-4.55	0.47	0.23
male	H	431.8	1662.5	-1.55	0.62	0.31	-4.33	1.42	0.71

11

12

References

(1) Blüthgen, N.; Castiglioni, S.; Sumpter, J. P.; Fent, K. Effects of low concentrations of the antiprogestin mifepristone (RU486) in adults and embryos of zebrafish (Danio rerio): 1. Reproductive and early developmental effects. *Aquat. Toxicol.* **2013**, *15* (144−145), 83−95.

(2) Zucchi, S.; Mirbahai, L.; Castiglioni, S.; Fent, K. Transcriptional and physiological responses induced by binary mixtures of drospirenone and progesterone in zebrafish (Danio rerio) at environmental concentrations. *Environ. Sci. Technol.* **2014**, *48* (6), 3523–3531.

(3) Routledge, E. J.; Sumpter, J. P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* **1996**, *15* (3), 241-248.

(4) Sohoni, P.; Sumpter, J. P. Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **1998**, *158* (3), 327-339.

(5) Liu, Z.-H.; Ogejo, J. A.; Pruden, A.; Knowlton, K. F., Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment, a review. *Sci. Total Environ.* **2011**, *409* (24), 5149–5161.

(6) Oggier, D. M.; Weisbrod, C. J.; Stoller, A. M.; Zenker, A. K.; Fent, K. Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish Danio rerio. *Environ. Sci. Technol.* **2010**, *44* (19), 7685-7691.

(7) Chen, S. X.; Bogerd, J.; Garcia-López, A.; de Jonge, H.; de Wall, P. P.; Hong, W. S.; Shulz, R. W. Molecular cloning and functional characterization of a zebrafish nuclear progesterone receptor. *Biol. Reprod.* **2010**, *82* (1), 171−181.

(8) Hossain, M. S.; Larsson, A.; Scherbak, N.; Olsson, P. E.; Orban, L. Zebrafish androgen receptor: Isolation, molecular, and biochemical characterization. *Biol. Reprod.* **2008**, *78* (2), 361−369.

(9) Martyniuk, C. J.; Gerrie, E. R.; Popesku, J. T.; Ekker, M.; Trudeau, V. L. Microarray analysis in the zebrafish (Danio rerio) liver and telencephalon after exposure to low concentration of 17alpha-ethinylestradiol. *Aquat. Toxicol.* **2007**, *84* (1), 38−49.

(10) Alsop, D.; Vijayan, M. M. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am. *J. Physiol. Regul. Integr. Comp. Physiol.* **2008**, *294* (3), 711−719.

(11) Hoffmann, J. L.; Torontali, S. P.; Thomason, R. G.; Lee, D. M.; Brill, J. L.; Price, B. B.; Carr, G. J.; Versteeg, D. J. Hepatic gene expression profiling using genechips in zebrafish exposed to 17alpha-ethynylestradiol. *Aquat. Toxicol.* **2006**, *79* (3), 233−246.

(12) Hoffmann, J. L.; Thomason, R. G.; Lee, D. M.; Brill, J. L.; Price, B. B.; Carr, G. J.; Versteeg, D. J. Hepatic gene expression profiling using genechips in zebrafish exposed to 17alpha- methyldihydrotestosterone. *Aquat. Toxicol.* **2008**, *87* (2), 69−80.

(13) Hanna, R. N.; Zhu, Y. Controls of meiotic signaling by membrane or nuclear progestin receptor in zebrafish follicle-enclosed oocytes. *Mol. Cell Endocrinol.* **2011**, *337* (1-2), 80–88.

(14) Liang, Y. Q.; Huang, G. Y.; Ying, G. G.; Liu, S. S.; Jiang, Y. X.; Liu, S.; Peng, F. J. A time-course transcriptional kinetics of the hypothalamic-pituitary-gonadal and

hypothalamic-pituitary-adrenal axes in zebrafish eleuthero-embryos after exposure to norgestrel. *Environ. Toxicol. Chem.* **2015**, *34* (1), 112-119.
(15) Arukwe, A.; Nordtug, T.; Kortner, T. M.; Mortensen, A. S.; Brakstad, O. G. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (Danio rerio) exposed to water-soluble fraction of crude oil. *Environ. Res.* **2008**, *107* (3), 362–70.
(16) Amaral, I. P.; Johnston, I. A. Circadian expression of clock and putative clock-controlled genes in skeletal muscle of the zebrafish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2012**, *302* (1), R193-206.
(17) Idda, M. L.; Kage, E.; Lopez-Olmeda, J. F.; Mracek, P.; Foulkes, N. S.; Vallone, D. Circadian Timing of Injury-Induced Cell Proliferation in Zebrafish. *PLoS ONE*. **2012**, *7* (3), e34203.
(18) Luo, D.; Wei, H.; Chen, S.; Xiao, Y.; Sun. Y.; Zhu, Z. Identification of Differentially Expressed Genes Between Cloned and Zygote-Developing Zebrafish (*Danio rerio*) Embryos at the Dome Stage Using Suppression Subtractive Hybridization. *Biol. Reprod*. **2009**, *80* (4), 674–684.
(19) Andreasen, E. A.; Spitsbergen, J. M.; Tanguay, R. L.; Stegeman, J. J.; Heideman, W.; Peterson, R. E. Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxinexposure. *Toxicol. Sci.* **2002**, *68* (2), 403–419.
(20) Behrendt, L.; Jonsson, M. E.; Goldston, J. V.; Stegeman, J. J. Induction of cytochrome P450 1genes and stress response genes in developing zebrafish exposed to ultraviolet radiation. *Aquat. Toxicol*. **2010**, *98* (1), 74–82.
(21) Zucchi, S.; Castiglioni, S.; Fent, K. Progesterone alters global transcription profiles at environmental concentrations in brain and ovary of female zebrafish (Danio rerio). *Environ. Sci. Technol.* **2013**, *47* (21), 12548−12556.