Environmental Science & Technology

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Transcriptional Responses of the Brain–Gonad–Liver Axis of Fathead Minnows Exposed to Untreated and Ozone-Treated Oil Sands Process-Affected Water

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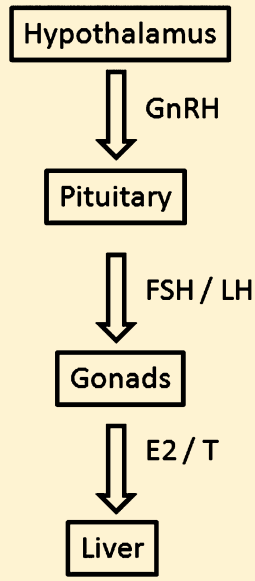
ABSTRACT: Oil sands process-affected water (OSPW) produced by the surface mining oil sands industry in Alberta, Canada, is toxic to aquatic organisms. Ozonation of OSPW attenuates this toxicity. Altered concentrations of sex steroid hormones, impaired reproductive performance, and less prominent secondary sexual characteristics have been reported for fish exposed to OSPW. However, the mechanism(s) by which these effects occur and whether ozonation can attenuate these effects in fish was unknown. The objective of this in vivo study was to investigate the endocrine-disrupting effects of OSPW and ozone-treated OSPW on the abundances of transcripts of genes in the brain–gonad–liver (BGL) axis in male and female fathead minnows (*Pimephales promelas*). Abundances of transcripts of genes important for synthesis of gonadotropins were greater in brains from both male and female fish exposed to untreated OSPW compared to that of control fish. In gonads from male fish exposed to untreated OSPW the abundances of transcripts of gonadotropin receptors and several enzymes of sex hormone steroidogenesis were greater than in control fish. The abundances of transcripts of estrogen-responsive genes were greater in livers from male fish exposed to untreated OSPW than in control fish. In female fish exposed to untreated OSPW there was less abundance of transcripts of gonadotropin receptors in gonads, as well as less abundance of transcripts of estrogen-responsive genes in livers. Many effects were either fully or partially attenuated in fish exposed to ozone-treated OSPW. The results indicate that (1) OSPW has endocrine-disrupting effects at all levels of BGL axis, (2) OSPW has different effects in male and female fish, (3) ozonation attenuates the effects of OSPW on abundances of transcripts of some genes, and the attenuation is more prominent in males than in females, but effects of ozonation on endocrine-disrupting effects of OSPW were less clear than in previous in vitro studies. The results provide a mechanistic basis for the endocrine-disrupting effects of OSPW from other studies.



INTRODUCTION

Oil sands process-affected water (OSPW) is a byproduct of the extraction of bitumen from the oil sands in Alberta, Canada. OSPW is a mixture of water, residual bitumen, silts, clays, and other inorganic and organic compounds. The water-soluble organic fraction of OSPW has been shown to be responsible for the majority of the toxicity of OSPW.[1−5] Naphthenic acids (NAs) are one of the primary persistent organic constituents of OSPW. These are a group of carboxylic acids with the general formula $C_{n}H_{2n+z}O_2$, where n indicates the number of carbons and z relates to the number of rings or double bonds.[6−10]

OSPW has endocrine-disrupting effects. Exposure to OSPW decreases concentrations of testosterone (T) and estradiol (E2) in plasma of yellow perch (*Perca flavescens*)[11] and goldfish (*Carassius auratus*)[14] and causes less synthesis of T and E2 by explants of ovarian and testicular tissue from goldfish exposed to OSPW.[12] Exposure to OSPW impairs reproductive capacity of fathead minnows (*Pimephales promelas*) as exemplified by decreased fecundity, altered synthesis of sex steroid hormones, and less pronounced secondary sex characteristics of male and female minnows.[13] In vitro, OSPW reduces synthesis of T and

Received: May 15, 2012
Revised: July 30, 2012
Accepted: August 2, 2012
Published: August 2, 2012

   dx.doi.org/10.1021/es3019258 | Environ. Sci. Technol. 2012, 46, 9701−9708

increases synthesis of E2 in the H295R cell line[14] and stimulates estrogenic and antiandrogenic effects in T47D-kbluc and MDA-kb2 cell lines, respectively.[15] Although the components of OSPW that are responsible for these effects are unknown, some NAs are structurally similar to sex steroid hormones.[10]

In accordance with a policy of zero-discharge, OSPW is stored on-site in active settling basins, otherwise known as tailings ponds. Currently, there are greater than 1 billion m^3 of OSPW stored on-site in tailings ponds of several companies operating in this region.[16] Eventually this OSPW needs to be remediated and reclaimed either as viable aquatic habitats or released to the receiving environment. In order for OSPW to be reclaimed it is essential that toxicity of the water-soluble organic fraction be reduced. Currently, this is attempted by aging OSPW in the tailing ponds, or experimental ponds, to decrease the concentrations of NAs through natural in situ biodegradation.[9,16,17] However, some NAs in OSPW are resistant to biodegradation.[17,18] Consequently, aging is only moderately effective for removing the toxicity of OSPW. Therefore, to more rapidly remediate OSPW, a treatment approach that targets NAs, and other persistent dissolved organics, is required. Ozonation has been identified as a potentially effective treatment method.[19−21] Ozone preferentially degrades NAs with more rings which are most resistant to biodegradation, thereby accelerating subsequent microbial remediation.[22] Ozonation reduces the acute toxicity of OSPW toward *Vibrio fischeri* as measured by the Microtox assay[19,20] and attenuates some of the endocrine-disrupting effects on eukaryotic cells in vitro.[11,12]

The mechanism(s) of endocrine disruption caused by exposure to OSPW are unknown. To develop effective treatment, monitoring, and remediation programs, as well as to be able to conduct risk assessments and set regulatory standards, knowledge of the mechanism of toxic action would be beneficial. Therefore, in the current study, a PCR array was utilized to investigate the mechanistic basis of the endocrine-disrupting effects of OSPW by examining transcriptional responses of key genes along the brain−gonad−liver (BGL) axis of male and female fathead minnows. In addition, to further investigate the usefulness of ozonation for reducing the toxicity of OSPW the effects of ozone-treated OSPW on transcriptional responses of these genes were determined.

MATERIALS AND METHODS

OSPW Collection and Ozonation. OSPW was collected in February 2010, from the West-In-Pit (WIP), an active settling basin on the site of Syncrude Canada Ltd. (Fort McMurray, AB, Canada). The WIP-OSPW is untreated process water from the main bitumen extraction plant as described previously.[17] The total concentration of NAs in WIP-OSPW, as determined by ultra pressure liquid chromatography high resolution mass spectrometry (UPLC-HRMS), was 19.7 mg/L.[23] Ozonation of WIP-OSPW was conducted at the University of Alberta (Edmonton, AB, Canada) using a semibatch ozonation system and following a standard protocol described elsewhere.[20,23] Ozonation was continued until approximately 90% degradation of parent NAs was achieved, as determined by the remaining sum response of all UPLC-HRMS peak area corresponding to NAs. The total concentration of NAs in the ozone-treated WIP-OSPW (O3-OSPW) was 1.9 mg/L. A more detailed description of the effects of ozonation on the distribution of the NAs in this sample is given by Wang.[23]

Experimental Protocol. This study was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use. The experiment was conducted in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan's Toxicology Centre. Adult male and female fathead minnows of approximately 6 months of age were randomly selected from a culture that is maintained within the ATRF. Minnows were maintained in 200-L tanks supplied with running water at approximately 20 °C, maintained under a 12 L:12D photoperiod and fed approximately 2% body weight of frozen bloodworms once daily. Thirty-six fathead minnows (18 male and 18 female) were randomly assigned to one of six 25-L aquaria containing 20 L of either dechlorinated city of Saskatoon tap water as control, untreated OSPW at full-strength, or O3-OSPW at full-strength. All exposures were performed in duplicate aquaria, with six males and six females per treatment. The aquaria were supplied with constant aeration, the water temperature was maintained at 22 °C, and a 12 L:12D photoperiod was used. Approximately 50% of the water volume of each aquarium was replaced daily, and the exposure was maintained for 7 days. Minnows were fed approximately 2% body weight of frozen bloodworms once daily. There were no mortalities in either of the exposure groups. At the end of exposure period, minnows were netted and immediately anesthetized with 150 mg/L MS-222. Brain (including the pituitary gland), liver, and gonad were collected from each fish and frozen at −80 °C for analysis of the abundances of transcripts of the genes of interest.

Quantification of Transcript Abundance by Real-Time PCR. Total RNA was extracted from livers by use of the Qiagen RNeasy Plus Mini Kit according to the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). Total RNA was extracted from brains and gonads by use of a Qiazol RNeasy lipid tissue mini kit acording to the manufacturer's protocol (Qiagen). Purified RNA was quantified by use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and samples were stored at −80 °C until analysis. First-strand cDNA was synthesized from 1 μg of total RNA by use of an iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions. The cDNA samples were stored at −80 °C until further analysis.

Real-time PCR (qPCR) was performed on an ABI 7300 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, Foster City, CA). A PCR reaction mixture for one reaction contained 10 μL of SYBR Green master mix (Applied Biosystems), 2 μL of sense/antisense gene-specific primers (Invitrogen, Carlsbad, CA), and 8 μL of cDNA that was diluted in RNase-free water (Qiagen). The PCR reaction mix was denatured at 95 °C for 10 min before the first PCR cycle. The thermal cycle profile was denaturizing for 15 s at 95 °C and annealing and extension for 1 min at 60 °C for a total of 40 PCR cycles. After amplification reactions were completed, dissociation curves were generated to ensure amplification of a single product. Efficiency, uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves by use of serially diluted cDNA standards. Changes in abundances of transcripts of target genes were quantified by normalizing to 18s rRNA, according to the method of Simon.[24] Amplification of genes of interest and reference genes was performed in separate reactions.

Table 1. Nucleotide Sequence and Efficiencies of Primer Pairs Used in qPCR

abbreviation	target gene	category	sense (5′−3′)	antisense (5′−3′)	size (bp)	efficiency	GenBank
18S	ribosomal RNA 18S	reference gene	GCCCTGTAATTGGAATGAGC	TCCCGAGATCCAACTACGAG	147	1.99	AY855349
AR	androgen receptor	steroid receptor	CAACGCGTCTAAATCCCATT	TGTTCGAACTGACACGAAGC	147	2.04	AY727529
ERα	estrogen receptor alpha	steroid receptor	CGGTGTGCAGTGACTATGCT	CTCTTCCTGCGGTTTCTGTC	151	2.06	AY775183
ERβ	estrogen receptor beta	steroid receptor	CGTTTTGGCATAACCATGTG	TGCTGTCAGACTTCCGAATG	152	1.99	AY566178
KISS1R	kisspeptin 1 receptor	peptide receptor	GATGAGTGGAGACCGTTGCT	CCTCAAGCCTCTGGTACAGG	149	2.02	EF672266
GnRH2	gonadotropin releasing hormone 2	hormone	TCACTCGAGGGAAAGCAGAC	AACTGGGCACTTAAACACAGC	155	2.03	EF672264
GnRH3	gonadotropin releasing hormone 3	hormone	TCTATTCCTGCGGACACTCC	CTCCAAGGGTTCAACATCGT	152	2.04	EF672265
GnRHR	gonadotropin releasing hormone receptor	peptide receptor	TGCAAAGCCAGTGAAAATTG	TTGTCAAACTGGGACGTGAG	150	2.05	*a*
FSHβ	follicle-stimulating hormone beta subunit	hormone	AGCTGCATCACAATCGACAC	AGGGCAGCCTTTAAACTCGT	147	1.99	DQ242616
FSHR	follicle-stimulating hormone receptor	peptide receptor	CACGTACTGCTGTCCAGACG	GTGGCTGGGGTATGTCAGAT	146	2.01	EF219401
LHβ	luteinizing hormone beta subunit	hormone	GTCGTTGCTCAAAGCTCCTT	TGGAGAACGGGCTCTTGTAT	157	1.85	DQ242617
LHR	luteinizing hormone receptor	peptide receptor	CTTTCAACCACCTTCCCAAG	AGCATTTGGTGGGACTGAAC	152	1.92	DT281016
StAR	steroidogenic acute regulatory protein	steroidogenesis	ATGCCCGAGAAGAAAGGATT	CCCGGTTGATGACTGTTTTT	151	1.98	DQ360497
CYP11A	cholesterol side-chain cleavage enzyme	steroidogenesis	CACACTGATGTGGACGCTCT	AGGGCTCCTTTAAGCAGAGG	144	1.95	DQ360498
CYP17	17α-hydroxylase	steroidogenesis	CTGCCCATCATTGGAAGTCT	GCATGATGGTGGTTGTTCAC	146	1.98	AJ277867
CYP19a	aromatase α	steroidogenesis	GCTGCACAAGAAGCACAAG	CGTGGCTCTGAGCGAATATC	146	1.93	AF288755
CYP19b	aromatase β	steroidogenesis	AGGGTGTATCCTGGCAACTG	ATCTGCACCCGTTTCATTTC	156	1.97	AJ277866
3βHSD	3β-hydroxysteroid delta dehydrogenase	steroidogenesis	TAACTGGAGGATGCGGTTTT	TGCACCACTACCACCTTCAC	151	1.99	DT361291
17βHSD	17β-hydroxysteroid dehydrogenase	steroidogenesis	ATCCAGAGTGTGCTGCCTTT	AGGGAAATAGCCGTTGGTCT	144	1.98	DT161033
VTG	vitellogenin	yolk precursor	TTGCTCTCCAGACCTTTGCT	GCAGAGCCTCCACCTTGTAG	150	1.97	AF130354
CHG-L	choriogenin L	yolk precursor	CAAGCACAATCGCAGAGAAC	GTCCCTGTTGGGTTTGTGAG	133	2.08	*a*
CHG-H	choriogenin H minor	yolk precursor	GCAGCATCAATTGCGTTTAC	TCTTCTGGGGATCAAACCAT	142	2.06	*a*

[a]Sequence information obtained by Illumina RNA sequencing (unpublished data).

Gene Selection and Model Development. Twenty-three genes representing key signaling pathways and functional process of the BGL axis in fathead minnows were selected based on principles of a previous study using zebrafish.[25] All primers were designed based on the sequences available in the NCBI GeneBank database or from sequences obtained by Illumina RNA sequencing (unpublished data). Sequences of nucleotide primers are given in Table 1.

Statistical Analysis. Fathead minnows were exposed to the treatment waters in duplicate tanks. The authors are aware that this is technically pseudoreplication; however, there were no differences in the responses to the same treatment between fish in different tanks. Therefore, individual male and female fish were considered the experimental unit. Statistical analyses were conducted by use of SPSS16.0 (SPSS, Chicago, IL). All data are expressed as mean ± SEM. Normality of each data set was assessed by use of Kolomogrov−Smirnov one-sample test, and homogeneity of variance was determined by use of Levene's test. When necessary to meet assumptions of parametric tests, data were log-transformed to ensure homogeneity of variance. Nontransformed data are presented in figures. Where data met the required assumptions, statistical differences were evaluated by one-way ANOVA followed by posthoc Tukey test. A Kruskal−Wallis test was used when neither the untransformed nor transformed data met the assumptions of parametric statistics. Differences were considered statistically different at $p < 0.05$.

RESULTS

Effects on Males. Exposure to untreated OSPW affected the abundances of transcripts of target genes expressed in brains from male fathead minnows. The abundances of transcripts of *erα*, *kiss1r*, *fshβ*, *lhβ*, and *cyp19b* in brains from male fish exposed to untreated OSPW were greater by 5.14 ± 3.22, 6.11 ± 1.51, 3.96 ± 0.96, 3.04 ± 1.80, and 3.44 ± 1.20-fold, respectively, than in brains from male fish exposed to freshwater. Ozonation of OSPW attenuated these effects. Abundances of transcripts of *erα*, *kiss1r*, *fshβ*, *lhβ*, and *cyp19b* in brains from male fish exposed to O3-OSPW were not different from abundances of these transcripts in brains from male fish exposed to freshwater. The abundance of transcripts of *gnrhr* was less in brains from males exposed to untreated OSPW or O3-OSPW by 0.13 ± 0.05 and 0.11 ± 0.06-fold, respectively, compared to the abundance of transcripts of *gnrhr* in brains from male fish exposed to freshwater. Abundances of transcripts of *erB*, *ar*, *gnrh2*, and *gnrh3* were not different among brains from male fish exposed to any of the treatments (Figure 1a).

Exposure to untreated OSPW significantly affected abundances of transcripts of target genes expressed in gonads from male fathead minnows. Abundances of transcripts of *fshr* and *lhr* were greater in gonads from male fish exposed to OSPW by 3.7 ± 0.43 and 2.5 ± 0.59-fold, respectively, compared to abundances in gonads from male fish exposed to freshwater. Ozonation of OSPW partially attenuated effects on abundances of transcripts of *fshr* and *lhr*. The abundance of transcripts of *lhr*

dx.doi.org/10.1021/es3019258 | Environ. Sci. Technol. 2012, 46, 9701−9708

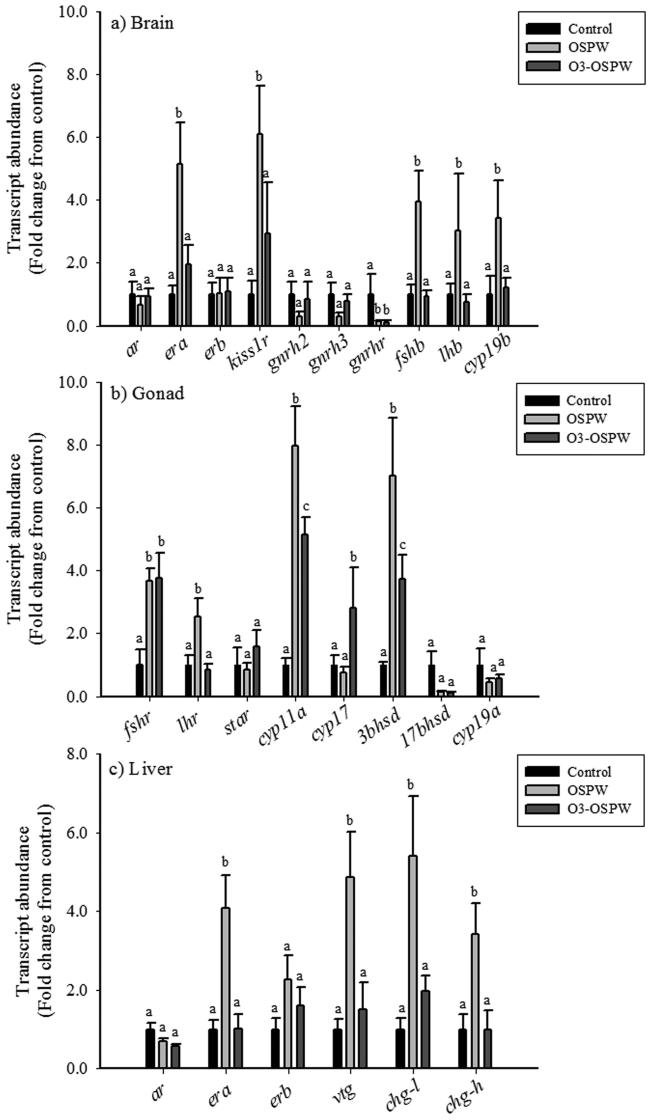


Figure 1. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in male fathead minnows exposed to freshwater, OSPW, or O3-OSPW. (a) Brain, (b) gonad, (c) liver. Different letters indicate significant differences in the abundance of transcripts between treatments ($p < 0.05$).

was not different in gonads from male fish exposed to O3-OSPW compared to abundance in gonads from male fish exposed to freshwater and was less (0.86 ± 0.19-fold relative to freshwater) than in gonads from male fish exposed to freshwater. In contrast, the abundance of transcripts of *fshr* was not different in gonads from male fish exposed to O3-OSPW compared to that for untreated OSPW but was greater, by 3.8 ± 0.77-fold, than that in gonads from male fish exposed to untreated OSPW. Abundances of transcripts of *cyp11a* and *3βhsd* were greater in gonads from male fish exposed to OSPW by 8.0 ± 1.3 and 7.0 ± 1.8-fold, respectively, compared to abundances in gonads from fish exposed to freshwater. Abundances of transcripts *cyp11a* and *3βhsd* in gonads from male fish exposed to O3-OSPW (5.16 ± 0.56 and 3.72 ± 0.78-fold relative to freshwater, respectively) were less than abundances in gonads from male fish exposed to untreated OSPW but were greater than abundances in gonads from male fish exposed to freshwater. The abundance of transcripts of *cyp17* was greater in gonads from male fish exposed to O3-OSPW compared to abundance in gonads from male fish exposed to freshwater and untreated OSPW. Abundances of transcripts of other genes including *star*, *17βhsd*, and *cyp19a* were not affected by any of the treatments (Figure 1b).

Exposure to untreated OSPW affected abundances of transcripts of target genes in livers from male fish. Abundances of transcripts of *erα*, *vtg*, *chg-l*, and *chg-h* in livers from male fish exposed to untreated OSPW were greater by 4.1 ± 0.85, 4.9 ± 1.2, 5.4 ± 1.5, and 3.4 ± 0.78-fold, respectively, compared to abundances in livers from male fish exposed to freshwater. Exposure to O3-OSPW attenuated these effects, and abundances of transcripts of *erα*, *vtg*, *chgl*, and *chgh* in livers from male fish exposed to O3-OSPW were not different from abundances of transcripts in male fish exposed to freshwater. The abundance of transcripts of *ar* and *erβ* were not different in livers from male fish exposed to either untreated OSPW or O3-OSPW compared to the abundance in livers from male fish exposed to freshwater (Figure 1c).

Effects on Females. Exposure to untreated OSPW significantly affected abundances of transcripts of several target genes expressed in brains from female fathead minnows. The abundance of transcripts of *lhβ* was greater by 5.3 ± 2.2-fold in brains from female fish exposed to OSPW compared to the abundance in brains from female fish exposed to freshwater. However, in brains from female fish exposed to O3-OSPW, the abundance of transcripts of *lhβ* was less than the abundance in brains from female fish exposed to untreated OSPW and not different from the abundance in brains from female fish exposed to freshwater. The abundance of transcripts of *fshβ* in brains from female fish exposed to untreated OSPW was not different from that of female fish exposed to freshwater. However, the abundance of transcripts of *fshβ* in brains from female fish exposed to O3-OSPW was greater by 2.0 ± 0.25-fold compared to the abundance in brains from female fish exposed to freshwater but not different from the abundance in brains from female fish exposed to untreated OSPW. Abundances of transcripts of *gnrh2* or *gnrh3* in brains from female fish exposed to OSPW were not affected. However, the abundance of transcripts of *gnrh2*, but not *gnrh3*, was significantly greater in brains of female fish exposed to O3-OSPW, by 1.6 ± 0.36-fold, compared to the abundance in brains from female fish exposed to freshwater. Abundances of transcripts of *kiss1r*, *cyp19b*, *erα*, *erβ*, or *ar* were not different between brains from female fish exposed to either of the waters (Figure 2a).

Exposure to untreated OSPW affected abundances of transcripts of several target genes expressed in gonads from female fathead minnows. Abundances of transcripts of *fshr*, *lhr*, and *cyp19a* were less by 0.02 ± 0.004, 0.33 ± 0.056, and 0.28 ± 0.10-fold in gonads from female fish exposed to untreated OSPW compared to abundance in gonads from female fish exposed to freshwater. Ozonation did not attenuate these effects, and abundances of these transcripts were not different between fish exposed to untreated OSPW and O3-OSPW. However, abundances of transcripts of *fshr*, *lhr*, and *cyp19a* were less by 0.01 ± 0.002, 0.14 ± 0.045, and 0.07 ± 0.013-fold in gonads from female fish exposed to O3-OSPW compared to abundances in gonads from female fish exposed to freshwater. Abundances of transcripts of *cyp11a* and *cyp17* were not different between gonads from fish exposed to freshwater or those exposed to untreated OSPW. However, abundances of transcripts of *cyp11a* and *cyp17* were less by 0.044 ± 0.017 and 0.28 ± 0.078-fold in gonads from fish exposed to O3-OSPW compared to gonads from fish exposed to freshwater.

dx.doi.org/10.1021/es3019258 | Environ. Sci. Technol. 2012, 46, 9701−9708

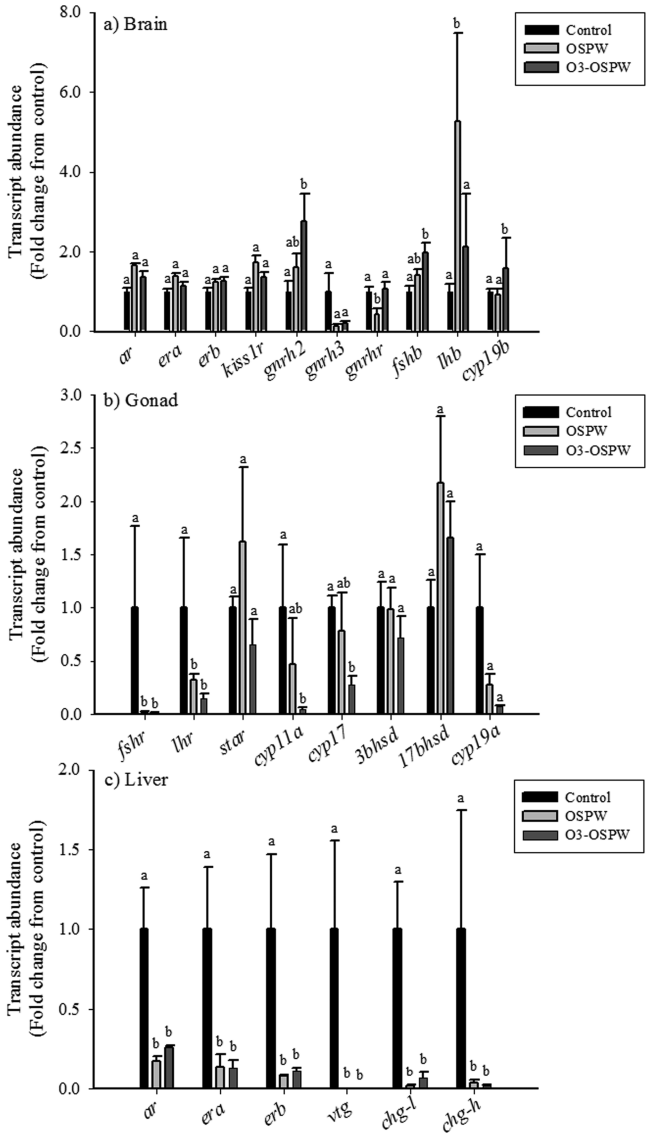


Figure 2. Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in female fathead minnows exposed to freshwater, OSPW, or O3-OSPW. (A) Brain, (B) gonad, (C) liver. Different letters indicate significant differences in the abundance of transcripts between treatments ($p < 0.05$).

Abundances of transcripts of *star*, *3βhsd*, and *17βhsd* were not significntly different in gonads from female fish exposed to either untreated OSPW or O3-OSPW compared to the abundance in gonads from female fish exposed to freshwater (Figure 2b).

Exposure to untreated OSPW significantly affected abundances of transcripts of target genes in livers from female fish. In livers from female fish exposed to OSPW, abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* were less by 0.18 ± 0.025, 0.14 ± 0.075, 0.080 ± 0.011, 0.002 ± 0.0011, 0.022 ± 0.007, and 0.036 ± 0.024-fold compared to abundances in livers from female fish exposed to freshwater. Ozonation did not attenuate these effects. Abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* were not different between brains from female fish exposed to untreated OSPW and those exposed to O3-OSPW. Abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* were less by 0.26 ± 0.015, 0.13 ± 0.050, 0.11 ± 0.016, 0.0015 ± 0.0004, 0.072 ± 0.033, and 0.021 ± 0.008-fold in livers from female fish exposed to O3-OSPW compared to abundance of transcripts in livers from female fish exposed to freshwater (Figure 2c).

DISCUSSION

In teleost fishes, regulation of sexual reproduction is dependent upon a complex network of signaling pathways between brain (the hypothalamus and pituitary), gonads, and liver. GnRHs act via a G-protein-coupled receptor, termed the GnRHR, to regulate synthesis and release of FSH and LH from the pituitary.[26] Gonadotropins consist of a noncovalently linked glycoprotein−hormone α-subunit (GTHα) and a specific β-subunit (FSHβ and LHβ).[27] The gonadotropins stimulate gonadal development by regulating synthesis of the sex steroid hormones T and E2.[28] In teleost fishes, FSH is involved in early gamete maturation, and LH is mainly involved in the final stage of gamete maturation which results in ovulation and/or spermiation.[29] E2 and T synthesized in response to LH and FSH also feedback to the hypothalamus and the pituitary, thereby regulating the synthesis and release of FSH and LH.[28] Results of recent studies indicate a important role of Kiss peptides and Kiss peptide receptors, in particular the Kiss1 system, in HPG axis regulation via its action on GnRH1 neurons, thereby regulating synthesis and release of LH and FSH in fishes.[30,31]

Genes involved in synthesis and regulation of gonadotropins, which are expressed in the hypothalamus and pituitary, including Kiss1R, FSH, LH, GnRHs, and GnRHR, are critical for control and regulation of sexual maturation and reproduction of male and female fathead minnows. The greater abundance of transcripts of *kiss1r* in male fathead minnows exposed to OSPW and the attenuation of this effect by ozonation of OSPW suggest that OSPW might stimulate secretion of FSH and LH via the Kiss1 system. Although the abundances of transcripts of *gnrh2* and *gnrh3* were not different in fathead minnows exposed to OSPW, the abundance of transcripts of *gnrhr* was less in males and females exposed to untreated OSPW. The reason for down-regulation of expression of the GnRHR might be due to compensation feedback via activation of GnRH or direct inhibition by dissolved organic compounds in OSPW. Exposure to OSPW caused greater abundances of transcripts of *fshβ* and *lhβ* in brains from male fathead minnows, but in brains from female fish only the abundance of transcripts of *lhβ* was up-regulated. Greater abundances of transcripts of the gonadotropin hormones, in particular in brains from male minnows, might be due to stimulation of ERα signaling. Effects of estrogens on the pituitary are exerted primarily through the ERα isoform,[32] and exposure to OSPW caused greater abundance of transcripts of *erα*, but not *erβ*, in brains from male fathead minnows exposed to untreated OSPW. These effects on transcription were all attenuated by ozonation. The results suggest that exposure to OSPW might have a greater effect on hypothalamic and pituitary control of sexual maturation in male fathead minnows than in maturing female fathead minnows.

After FSH and LH are released from the pituitary, they are transported in blood to the gonads where they bind to FSHR and LHR and regulate sex hormone steroidogenesis.[29,33,34] In addition to acting via their respective receptors, there is some cross-activation of FSHR by LH.[29] The greater abundances of transcripts of *fshr* and *lhr* in gonads from male fish exposed to OSPW compared to gonads from fish exposed to freshwater might be a response to greater FSHβ and LHβ released from

dx.doi.org/10.1021/es3019258 | Environ. Sci. Technol. 2012, 46, 9701−9708

the pituitary in response to OSPW as suggested by the greater abundances of transcripts of these gonadotropins. Several studies have demonstrated FSHR- and LHR-activating properties by FSH and LH, respectively.[35−37] In contrast, lesser abundances of transcripts of *fshr* and *lhr* in gonads from female fish exposed to OSPW compared to that for freshwater might be a mechanism of regulating steroidogenesis in response to a greater concentration of LH. Another possible mechanism for less *fshr* and *lhr* in gonads from female fish is that E2-like compounds in OSPW directly interact with the ER and, in turn, feedback to inhibit steroidogenesis and E2 synthesis. The combination of these two effects might explain the conflicting effects between male and female, thus feminization in males and less fecundity in females that was reported by Kavanagh and colleagues.[15] The reason zonation only attenuated effects on *lhr* in male gonad is unknown at this time and requires further study.

If concentrations of FSHR and LHR are greater in gonads from male fish exposed to OSPW, it might increase sensitivity of the gonads to circulating FSH and LH, which would cause greater activity of steroidogenic enzymes.[38,39] Greater abundances of transcripts of *cyp11a*, *3βhsd*, and *11βhsd* observed in male fathead minnows exposed to OSPW suggest that the capacity for steroidogenesis is greater in these fish. Ozonation partially attenuated effects of OSPW on this signaling pathway. In female fathead minnows exposed to untreated OSPW and O3-OSPW, abundances of transcripts of *11βhsd*, *cyp11a*, and *cyp17*, respectively, was less than in gonads from minnows exposed to freshwater. This suggests a down-regulation of steroidogenesis and is consistent with the down-regulation of *fshr* and *lhr*. These results suggest that OSPW, possibly due to the "steroid-like" NAs,[10] might decrease steroidogenic activity in female fathead minnows, by directly inhibiting transcription of genes in the gonads.

Exposure to untreated OSPW resulted in sex-specific effects on expression of target genes in livers of male and female fathead minnows. Up-regulation of ER*α* and estrogen-response genes such as VTG, CHG-L, and CHG-H in livers of fish exposed to estrogens has been previously demonstrated in Japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), and fathead minnow.[40−42] The fact that the sex steroid receptor ER*α*, as well as the estrogen-responsive genes VTG, CHG-L, and CHG-H, were up-regulated in livers from male fathead minnows exposed to OSPW suggests that exposure to OSPW resulted in an estrogenic effect. This is consistent with results of a previous in vitro study that demonstrated the stimulation of ER signaling by OSPW and identification of estrogen-like compounds in OSPW.[10,12] In addition, activation of ER signaling might have been due to greater synthesis of E2 caused by stimulation of steroidogenesis by FSH and LH released from the pituitary. Either way, activation of ER signaling suggests that male fathead minnows exposed to untreated OSPW are exposed to an estrogenic internal environment, and this might explain the less pronounced secondary sex characteristics in male fathead minnows exposed to OSPW.[15] Ozonation of OSPW attenuated these effects on male fathead minnows. The presence of estrogen-like compounds in OSPW[10,12] might explain lesser abundances of transcripts of *ar*, *erα*, *erβ*, *vtg*, *chg-l*, and *chg-h* in livers of female fathead minnows exposed to OSPW compared to that in females exposed to freshwater. These results suggest that OSPW might impair HPG signaling and E2 synthesis. Lesser abundances of transcripts of *fshr*, *lhr*, *cyp11a*, and *cyp17* support this mechanism of action of OSPW. Regardless of the mechanism of action, any impairment of ER signaling and subsequent inhibition of synthesis of egg envelope proteins might explain the lesser fecundity of female fathead minnows exposed to OSPW.[15]

In summary, exposure to OSPW resulted in changes in abundances of transcripts at all levels of the BGL axis in male and female fathead minnows. This is perhaps not surprising for a mixture with the complexity of OSPW. Estrogen-like NAs in OSPW might have caused some of the effects in the liver and might have influenced negative feedback pathways that regulate synthesis and release of gonadotropins and sex steroid hormones. It is also possible that organic compounds in OSPW, including NAs, might have directly affected actions of gonadotropin releasing hormones in the hypothalamus, gonadotropins in the pituitary, and sex steroid hormones in the gonads. The results of this study provide a mechanistic basis for the impaired reproductive performance and less pronounced secondary sexual characteristics of fathead minnows exposed to OSPW. Effects of ozonation on endocrine-disrupting effects of OSPW were less clear than previous in vitro studies where ozonation of OSPW either attenuated or had no effect on the endocrine-disruptive effects of OSPW. Ozonation attenuated the effects of OSPW on some endocrine end points, and the effects were more prominent in males than in females.

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Notes

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ACKNOWLEDGMENTS

This research was supported by a research grant from the Alberta Water Research Institute to J. P. Giesy, J. W. Martin, and M. Gamal El-Din (project no. C4288), a Discovery Grant from the Natural Science and Engineering Research Council of Canada (project no. 326415-07), and grants from Western Economic Diversification Canada (project nos. 6578 and 6807) to J. P. Giesy. The research was also supported by research grants from the Helmholtz Alberta Initiative (HAI) to M. Gamal El-Din and J. W. Martin. The authors acknowledge the support of an instrumentation grant from the Canada Foundation for Innovation. J. P. Giesy was supported by the Canada Research Chair program. J. P. Giesy was further supported by an at large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution at City University of Hong Kong and the Einstein Professor Program of the Chinese Academy of Sciences. We acknowledge the support of the Aquatic Toxicology Research Facility (ARTF) at the Toxicology Centre, University of Saskatchewan, in providing space for the exposure study. The authors thank Warren Zubot of Syncrude Canada Ltd. for supplying the OSPW.

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