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Exposure of fathead minnows to municipal wastewater effluent affects intracellular signaling pathways in the liver

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ABSTRACT

Municipal wastewater effluent can impact its receiving environment. In the St. Lawrence River, male fish living downstream from Montreal exhibit increased hepatic vitellogenin, intersex, delayed spermatogenesis and altered immune function. Few studies have examined genome-wide effects associated with municipal effluent exposure in fish to decipher the mechanisms of toxicity. The present objective was to identify hepatic cellular signaling pathways in fathead minnows following exposure to municipal wastewater effluent. Immature minnows were exposed for 21 days to either 0% (Control) or 20% municipal effluent, the highest concentration in the St. Lawrence River. Hepatic RNA was extracted and used to hybridize a fathead minnow oligonucleotide microarray containing approximately 15 k gene sequences. A total of 1300 genes were differentially expressed, of which 309 genes had more than 2-fold change in expression level between control and MWWE-exposed fish. Of those, 118 were up-regulated and 191 were down-regulated. Altered genes grouped according to function, indicated effects on various signaling pathways, apoptosis, immune responses, and cellular metabolism. Pathway analysis software predicted at least 5 signaling pathways that were altered by treatment: cell adhesion, inflammation, various kinases, estrogen receptor signaling and WNT signaling. Various components of the canonical Wnt pathway were dramatically down-regulated, while several other genes involved in the non-canonical Wnt pathway, such as Wnt4, LRP6, and PPP2R5E, which are known to inhibit the canonical Wnt pathway, were increased. These results indicate that municipal wastewater effluent from Montreal can target and inhibit various signaling including those implicated in hepatic Wnt signaling pathway in fathead minnows.

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1. Introduction

There is well-established evidence of the deleterious impact of municipal wastewater effluent on fish living in the receiving aquatic environment (Jobling et al., 1998, 2002; Gregory et al., 2008; Thorpe et al., 2009; Vidal-Dorsch et al., 2012; Cyr et al., 2014). Sewage is collected from all municipalities on the island of Montreal, representing approximately 1.8 million people and approximately 8000 commercial establishments and industrial plants and is treated at the Montreal wastewater treatment plant (MWWTP) (Pham and Proulx, 1997; Sabik et al., 2003; Marcogliese et al., 2009). The MWWTP is among the largest primary physico-chemical treatment plant in North America (Sabik et al., 2003). This facility is located on the eastern tip of the island of Montreal and treats approximately 1.3 million m³ of raw wastewater daily (Pham and Proulx, 1997; Sabik et al., 2003; Marcogliese et al., 2009). During

periods of heavy rain or thaw, the volume of water can reach up to 7.6 million m³ (Pham and Proulx, 1997; Sabik et al., 2003; Marcogliese et al., 2009). Over 90% of Montreal wastewater effluent (MWWE) currently undergoes some form of wastewater treatment before reaching the St. Lawrence River (Chambers et al., 1997). This process includes mechanical screening, grit removal, a coagulant, usually ferric chloride, and an anionic polymer that are added to increase the settling of suspended particles (Chambers et al., 1997; Pham and Proulx, 1997; Gagné et al., 2002). Large particles are sieved and removed (Gagné et al., 2002) and the resulting effluent is released directly into the St. Lawrence River (Pham and Proulx, 1997; Sabik et al., 2003; Marcogliese et al., 2009).

Previous studies have shown that spottail shiners (*Notropis hudsonius*) caught in the St. Lawrence River downstream from the Montreal wastewater effluent discharge point displayed induced hepatic VTG (vitellogenin) mRNA levels, delayed spermatogenesis, produced fewer sperm that were less motile, and had a higher incidence of intersex (Aravindakshan et al., 2004). These fish also displayed suppressed immune function (Ménard et al., 2010) and altered parasitic abundance (Marcogliese et al., 2006). Recently, northern pike (*Esox lucius*) sampled downstream of the Montreal wastewater effluent

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outfall also had increased levels of hepatic VTG mRNA levels, as well as blood VTG levels as compared to fish sampled upstream (Houde et al., 2013). The fish also displayed increased gill metallothionein, superoxide dismutase, glutathion-S-transferase, and cytochrome P4501A1 (Cyp4501A1) levels, suggesting that metals or organic compounds from the effluent may result in the production of free radicals (Houde et al., 2013).

Short-term exposure of rainbow trout (*Oncorhynchus mykiss*) to this municipal effluent under laboratory conditions altered immune function and decreased thyroid function (Salo et al., 2007; Escarné et al., 2008). Long-term laboratory-based exposure studies, in which brook trout (*Salvelinus fontinalis*) were exposed to 1, 10 and 20% MWWE concentrations for 4 and 12 weeks, indicated that hepatic VTG mRNA levels were unaltered after 4 weeks of treatment. However, VTG mRNA levels increased significantly at the two highest exposure concentrations at 12 weeks, suggesting that estrogenic compounds in the wastewater effluent likely accumulated over time (de Montgolfier et al., 2008). The expression of gap junction proteins, connexins, in the testes of these fish was also altered at both 4 and 12 weeks, which may, at least in part, explain the delayed spermatogenesis observed in wild-caught shiners in the St. Lawrence River downstream from Montreal (de Montgolfier et al., 2008). Rainbow trout hepatocyte primary cultures exposed in vitro to water samples collected 8 km downstream of the wastewater outfall displayed increased levels of metallothionein mRNA, Cyp1A1 enzyme activity, and increased cytotoxicity (Gagné and Blaise, 1995).

Together these studies clearly indicate that fish living downstream of the City of Montreal are negatively impacted by MWWE, and that these effects are directly related to contaminants present in the effluent. Still, there is limited information on the cellular and molecular mechanisms by which these contaminants affect the physiology of varying fish species in the St. Lawrence River. This information would provide a better understanding of the toxicity of the effluent and may help direct critical research avenues for understanding the impact of these contaminants on these fish. While other studies have examined the effects of MWWE on fish, differences in the composition of wastewater effluents due to differences in the industrial make-up of municipalities or treatment of effluents may alter different mechanisms by which physiological effects occur. The objectives of this study were to examine the effects of MWWE on gene expression in the liver of fathead minnow (*Pimephales promelas*), a species present in the St. Lawrence River, in order to identify key regulatory pathways implicated in the mechanism toxicity of MWWE. We elected to examine the liver, since it represents a site of action for many classes of contaminants can serve as a baseline for subsequent studies on other organ systems.

2. Materials & methods

2.1. Experimental protocol

Fathead minnows were raised at the Quebec Aquarium (Québec, QC, Canada) and transferred to the Montreal wastewater treatment plant (Montreal, QC, Canada). Fish were maintained in 15 L glass aquariums with flowing water at a constant temperature ($25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) under 16 h of light and 8 h of dark. Fish were fed 4 mL of brine shrimp (Ocean Nutrition, Dartmouth, NS, Canada) twice per day. Minnows ($n = 6$ per group) were exposed for 21 days to either 0 (control) or 20% v/v wastewater effluent in a flow-through system. Each treatment condition was carried out in duplicate. Effluent concentrations corresponded to concentrations previously determined to be equivalent to those measured 0.3 km downstream from the discharge pipe in the St. Lawrence River (de Montgolfier et al., 2008). After 21 days, juvenile minnows were anesthetized in 3-aminobenzoic-ethyl-ester-acid (MS222; 0.05 g/L). Fish were euthanized, weighed, measured and livers were removed, weighed, frozen and stored at $-80\text{ }^{\circ}\text{C}$. The sexual status, immature versus mature, of the fish was confirmed using a dissecting microscope. The condition

factor of the fish was calculated according to Williams (2000). Only immature animals were used for subsequent analyses in order to minimize estrogen-induced changes in gene expression that would be apparent in adult fish. All animal procedures were approved by Environment Canada's Animal Care Committee according to the guidelines of the Canadian Council on Animal Care.

2.2. RNA extraction

Frozen liver samples were ground under liquid nitrogen using a mortar and pestle and total RNA was extracted using the Illustra RNAspin Mini kit (GE Healthcare, Baie D'Urfe, QC, Canada) according to the manufacturer's protocol. The quality of the RNA was verified using an RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, USA) and Bioanalyzer model 2100 (Agilent Technologies). The isolated RNA was treated with DNase (1 U/ μg of RNA, deoxyribonuclease I, amplification grade; GE Healthcare) to remove any contaminating genomic DNA. The RNA was then reverse transcribed using oligo d(T) 12–18 (R&D Systems, Minneapolis, MS, USA) and M-MLV reverse transcriptase (Sigma Aldrich, Mississauga, ON, Canada), according to the suppliers' instructions.

2.3. Microarray processing

Gene expression profiling was carried out using a fathead minnow oligonucleotide array containing 15,208 oligonucleotides designed by EcoArray (Fat Head Minnow Microarray 016349, Alachua, FL, USA) and purchased from Agilent (Agilent Technologies). An aliquot of 500 ng of total RNA was prepared for amplification and labeling with Cy3 using the Low RNA Input Linear Amplification kit and Quick Amp Labeling Kit, according to the manufacturer's instructions (Agilent Technologies). A 40 μL -aliquot of labeled cRNA was hybridized to each microarray according to the manufacturer's instructions using the In Situ Hybridization Kit Plus (Agilent Technologies). Following hybridization, the microarray slides were scanned using a ScanArray Express laser scanner (Perkin Elmer, Woodbridge, ON, Canada) and imported into Gene Pix Pro 7 (Molecular Devices Inc., Sunnyvale, CA, USA). Two slides, each containing 8 one-color microarrays, were used to hybridize RNA from control ($n = 4$) and MWWE-exposed ($n = 4$) fish.

2.4. Microarray analysis

Fluorescent images were processed using the GenePix Pro 7 software (Molecular Devices Inc.). Data were imported into GeneSpring GX11 software (Agilent Technologies) for further analysis. The intensity of each spot was characterized by the median pixel intensity and between-array scale normalization on median intensities was carried out using GeneSpring GX11 (Zahurak et al., 2007). Genes with a greater than 2-fold change in at least seven of the eight samples were included for further analysis. Analyses were done according to MIAME standards.

Network analysis and functional analysis (biological functions and biological/toxicological processes) of the differentially expressed genes with a greater than 4-fold change was carried out using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA). For these analyses, mammalian signaling pathways were considered to be similar to those in fish. Statistical analyses were carried out using a one-way ANOVA (significance level set at $p < 0.05$) using SigmaStat (SPSS Inc., Chicago, IL, USA).

2.5. Western blot analyses

Nuclear and cytosolic proteins were extracted from liver of control ($n = 5$) and MWWE-exposed ($n = 4$; 20% v/v) fish using the Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). Frozen tissue was homogenized with 3 mL of 1X Hypotonic Buffer (containing 3 μL of 1 M DTT and 3 μL of detergent/g of tissue) and incubated on ice for 15 min.

Samples were centrifuged for 10 min at 850 g at 4 °C and the supernatant transferred to a chilled microcentrifuge tube. The pellet was resuspended in 500 µL of 1X Hypotonic Buffer and incubated on ice for 15 min. After incubation, 25 µL of detergent was added to each sample and the tubes were vortexed for 10 s. Samples were subsequently centrifuged for 45 s at 14 000 g in a pre-cooled microcentrifuge (4 °C). The supernatant was used as the cytosolic fraction. The nuclear pellet was resuspended in 50 µL of complete lysis buffer (10 mM DTT, lysis buffer AM1 and protease inhibitor cocktail) and vortexed for 10 s. The suspension was then incubated for 30 min on a rocking platform at 4 °C. Samples were then vortexed for 10 s and centrifuged at 4 °C for 10 min at 14 000 g. The supernatant, containing the nuclear fraction, was then transferred to pre-cooled microcentrifuge tubes. Protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada). The purity of the nuclear fraction was determined using alpha-tubulin (α -tubulin) as a marker for the cytoplasmic fraction and histone protein3 (H3) as a marker for the nuclear fraction. Overexposed films showed <7% cross-contamination between cytoplasmic and nuclear extracts. Aliquots of control and treated samples containing 25 µg of nuclear proteins and 12.5 µg of cytosolic protein were loaded onto a 4–20% gradient gel (Bio-Rad Laboratories) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were then transferred onto a nitrocellulose membrane at 100 V for 30 min and then at 80 V for 1 h at 4 °C. The resulting membranes were stained with Ponceau red S to evaluate the transfer efficiency.

Membranes containing transferred proteins were blocked with phosphate-buffered saline (PBS) containing 5% powdered milk and 0.05% Tween and then hybridized overnight at 4 °C with the appropriate primary antibody (rabbit anti-H3, 6 ng/mL, Cell Signalling Technology, Danvers, MA, USA; rabbit anti- β -catenin, 48 ng/mL, Cell Signalling Technology; rabbit anti-alpha-tubulin, 60–120 ng/mL, Abcam, Toronto, ON, Canada). Following the hybridization, the membranes were washed in PBS containing 0.05% Tween and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (0.08 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein signal was revealed by chemiluminescence using a commercial kit (Lumilight, Roche Diagnostic, Laval, QC, Canada).

Protein loading was verified using Ponceau red (5% Ponceau red in 5% acetic acid) coloration and quantified using a Fluor-S MultiImager (Bio-Rad Laboratories). A comparison of cytosolic and nuclear protein levels of CTNBN1 for control and treated samples was carried out. Cytoplasmic protein loading was standardized by measuring α -tubulin levels and nuclear protein loading was standardized according to H3 levels. Since levels for these two proteins were not affected by treatment, they were considered appropriate loading controls.

3. Results

Fathead minnows were exposed to either dechlorinated tap water alone (control) or 20% wastewater effluent for 21 days. At the end of the exposure period there were no significant differences in either body weight, length, condition K-factor, or HSI between control and exposed fish (Table 1).

The fathead minnow oligonucleotide microarray used in this study contained 15 208 genes. A total of 1300 genes were differentially expressed of which 309 genes were differentially expressed with a greater than 2-fold change in steady-state mRNA levels as compared

Table 1

Fish mass, length, condition K-factor and HSI in fish exposed to 20% municipal effluent for 21 days. Control fish were exposed to dechlorinated tap water alone. There were no significant differences in between groups.

Group	Mass (g)	Length (mm)	K-factor	HSI (%)
Control	3.10 ± 0.17	56.4 ± 2.2	1.74 ± 0.12	2.14 ± 0.24
Exposed	2.87 ± 0.24	58.8 ± 0.9	1.40 ± 0.04	2.30 ± 0.32

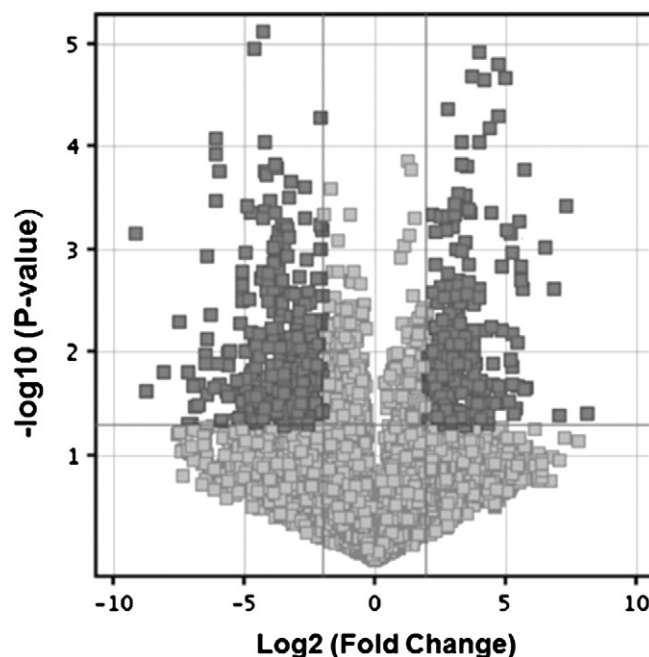


Fig. 1. Volcano plot of genes whose expression in the liver of control and MWWE-exposed minnows was found to be statistically significant (unpaired *t*-test) compared with their *p*-value. A total of 309 genes were differentially expressed. Of these genes, 118 were up-regulated and 191 were down-regulated. Spots in dark gray were found to have a greater than 2-fold change and a *p*-value of less than 0.05.

to controls (Fig. 1). Of the 309 differentially expressed genes, 118 were up-regulated and 191 were down-regulated. The data discussed in this publication have been deposited in NCBI's GEO (<http://www.ncbi.nlm.nih.gov/geo> (access number GSE52693)).

Mammalian homologues were identified for the differentially expressed genes and found to be associated with metabolism, development, signaling, cellular organization and assembly, and cell death (Fig. 2). Further analysis was conducted using differentially expressed genes with a greater than 4-fold change: 17 genes were up-regulated and 18 genes were down-regulated (Tables 2 and 3). A large number of the up-regulated genes were implicated in cellular signaling,

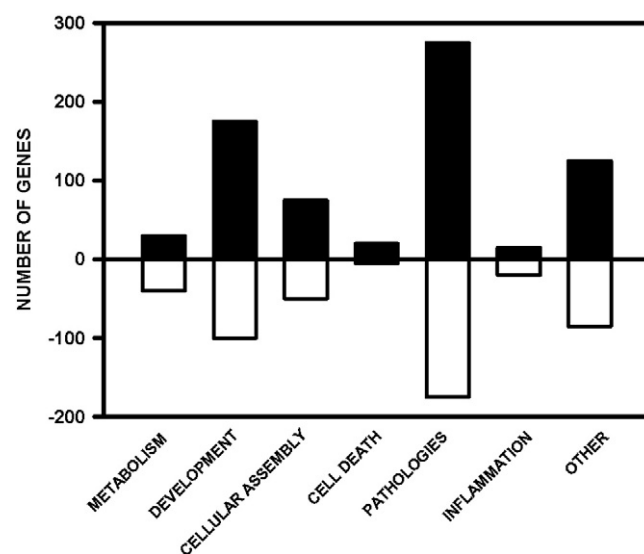


Fig. 2. Functional classification of differentially expressed genes by at least 2-fold in the liver of control and MWWE-exposed minnows. The genes were grouped based on their biological function. The bars in black represent the up-regulated genes whereas the bars in white represent the down-regulated genes.

Table 2
List of up-regulated genes with a greater than 4-fold change.

Gene ID	Gene symbol	Fold change	Gene function	Pubmed accession number
EA_Pp_62041a	MCAM	10.15	Cell adhesion, anatomical structure morphogenesis	NC_018922.1
EA_Pp_56813a	SIPA1L2	8.28	Regulation of small GTPase mediated signal transduction	NM_020808.3
EA_Pp_52712a	SASH3	6.55	Proliferation	NM_018990.3
EA_Pp_69607a	CPEB4	6.52	Nucleotide binding	NM_030627.2
EA_Pp_58287a	MAGEA10	6.51	May play a role in embryonal development and tumor transformation	NM_001011543.2
EA_Pp_64305a	SLC40A1	5.82	Ion transport, anatomical structure morphogenesis	NM_014585.5
EA_Pp_54032a	ATP6V0A2	5.53	Ion transport, immune response, ATP synthesis coupled proton transport	NM_012463.3
EA_Pp_70960a	PDZRN3	5.13	Protein ubiquitination	NM_015009.1
EA_Pp_57661a	PNOC	5.09	Signal transduction, neuropeptide signaling pathway, synaptic transmission, sensory perception	NM_006228.3
EA_Pp_56645a	DNAJC5	4.96	Protein folding, negative regulation of neuron apoptosis	NM_025219.2
EA_Pp_69299a	KIAA0748	4.88	Protein coding	NM_001261844
EA_Pp_70787a	PHF13	4.63	Transcription regulator	NM_153812.2
EA_Pp_59744a	TLCD1	4.62	Integral to the membrane	NM_001160407.1
EA_Pp_52997a	UBQLN4	4.46	Cell death, negative regulation of transcription	NM_020131.3
EA_Pp_67710a	LCMT2	4.40	tRNA processing	NM_014793.4
EA_Pp_57375a	C10orf27	4.37	Multicellular organismal development, cell differentiation	NM_152710.2
EA_Pp_69299a	KIAA0319	4.31	Cell adhesion	NM_001168374.1

organization and development (Fig. 2). MCAM (melanoma cell adhesion molecule), SIPA1L2 (signal-induced proliferation-associated 1 like 2), SASH3 (SAM and SH3 domain containing 3) and MAGEA10 (melanoma antigen family A, 10) were the most highly up-regulated genes (Table 2). Of all of the differentially expressed genes, three functional groups, each containing a large number of altered genes were identified: These groups contained genes associated with cellular adhesion, inflammation and kinase activity (Fig. 3).

Altered genes involved in cellular adhesion included the down-regulation of PCDH7 (protocadherin beta 7), ARHGAP17 (Rho GTPase activating protein 17), HEPACAM (hepatic and glial cell adhesion molecule), RIKEN 2009E01, and the up-regulation of MCAM, PTK2 (protein tyrosine kinase 2), and L1CAM (L1 cell adhesion molecule) (Fig. 3). Down-regulated genes associated with inflammatory activities included MAVS (mitochondrial antiviral signaling protein), CXCR2 (chemokine (C-X-C motif) receptor 2), SMAD3 (SMAD family member 3), TNFSF11 (tumor necrosis factor (ligand) superfamily, member 11), AMBP (alpha-1-microglobulin/bikunin precursor), ADAMTS (ADAM metalloproteinase with thrombospondin), and ATG16L1 (autophagy related 16-like 1; Fig. 3). Finally, the majority of genes involved in kinase activity were down-regulated, including ADPGK (ADP-dependent glucokinase), NME1 (NME/NM23 nucleoside diphosphate kinase 1), Raf-like, Aromatase (CYP19A1; cytochrome P450, family 19, subfamily A, polypeptide 1), AK8 (adenylate kinase 8), AKAP6 (A kinase anchor protein 6), PAK6 (p21 protein (Cdc42/Rac)-activated kinase 6), GUCY2C (guanylyl cyclase

2C), EPHA2 (EPH receptor A2), PHKA1 (phosphorylase kinase, alpha 1) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) with only one gene up-regulated, PFKL (phosphofructokinase) (Fig. 3).

At least ten genes that are known to be associated with the estrogen receptor signaling pathway were identified (Table 4). The majority of these genes were found to be down-regulated following exposure to MWW. These genes included: SPOCK2 (spare/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2), HSPA5 (heat shock 70 kDa protein 5), BNIP2 (BCL2/adenovirus E1B 19 kDa interacting protein 2), NME1, PHKA1 (phosphorylase kinase alpha 1), CEP152 (centrosomal protein 152 kDa), and CBF2T2 (core-binding factor, runt domain, alpha subunit 2; translocated to 2). Three of these genes were up-regulated including: DNAJC5 (DNACJ (Hsp40) homologue subfamily C member 5), NELL2 (NEL-like 2), and ENPEP (glutamyl aminopeptidase). One gene involved in this pathway, VTG, was shown to have no change in expression levels.

Some genes (13) implicated in the Wnt/ β -catenin signaling pathway were up-regulated in exposed fish as compared to controls. These included Wnt4 (Wingless-type MMTV integration site family, member 4), LRP6 (low density lipoprotein receptor-related protein 6), AES (amino-terminal enhancer of split), and PP2A/PP2R5E (ariadne homologue 2 ARIH2 and protein phosphatase 2, regulatory subunit B, epsilon isoform). Several other genes involved in this pathway were down-regulated in treated fish when compared to controls. These included dapper antagonist of CTNNB1 homologue 1 (DACT1), KREMEN1 (kremen

Table 3
List of down-regulated genes with a greater than 4-fold change.

Gene ID	Gene symbol	Fold change	Gene function	Pubmed accession number
EA_Pp_54861a	FOXB1	11.63	DNA dependent transcription regulation	NM_012182.2
EA_Pp_58527a	ADPGK	11.47	Carbohydrate metabolic process, glycolysis	NM_031284.4
EA_Pp_70091a	RACGAP1	10.71	Transporter	NM_001126103.1
EA_Pp_52153a	DCLRE1A	8.57	Nucleotide-excision repair	NM_001271816.1
EA_Pp_50170a	TMEM63B	7.15	Protein binding	NM_018426.1
EA_Pp_53694a	TNFSF13	5.92	Immune response, signal transduction, positive regulation of cell proliferation	NM_001198622.1
EA_Pp_68848a	NME1	5.72	Nervous system development, negative regulation of cell proliferation, cell differentiation, regulation of apoptosis	NM_000269.2
EA_Pp_68213a	DACT1	5.39	Multicellular organismal development, Wnt receptor signaling pathway	NM_001079520.1
EA_Pp_54650a	KREMEN1	5.33	Cellular communication, Wnt receptor signaling pathway	NM_001039570.2
EA_Pp_68856a	TIMM50	5.10	Mitochondrial membrane organization, protein transport	NM_001001563.1
EA_Pp_63441a	SKOR1	4.88	Regulation of transcription, negative regulation of BMP signaling pathway	XM_236329.4
EA_Pp_55978a	UCHL3	4.80	Ubiquitin-dependent protein catabolic process	NM_001270952.1
EA_Pp_52942a	AMBP	4.64	Cell adhesion, negative regulation of immune response	NM_001633.3
EA_Pp_59061a	HEPACAM	4.604	Cell cycle, cell arrest, cell adhesion, regulation of growth	NM_152722.4
EA_Pp_55468a	TMEM151A	4.55	Integral to the membrane	NM_153266.3
EA_Pp_68927a	SGCD	4.40	Cytoskeleton organization, muscle organ development	NM_000337.5
EA_Pp_58058a	TTPAL	4.38	Transporter activity	NM_001145527.1
EA_Pp_51446a	PBX3	4.08	Transcription regulator	NM_001134778.1

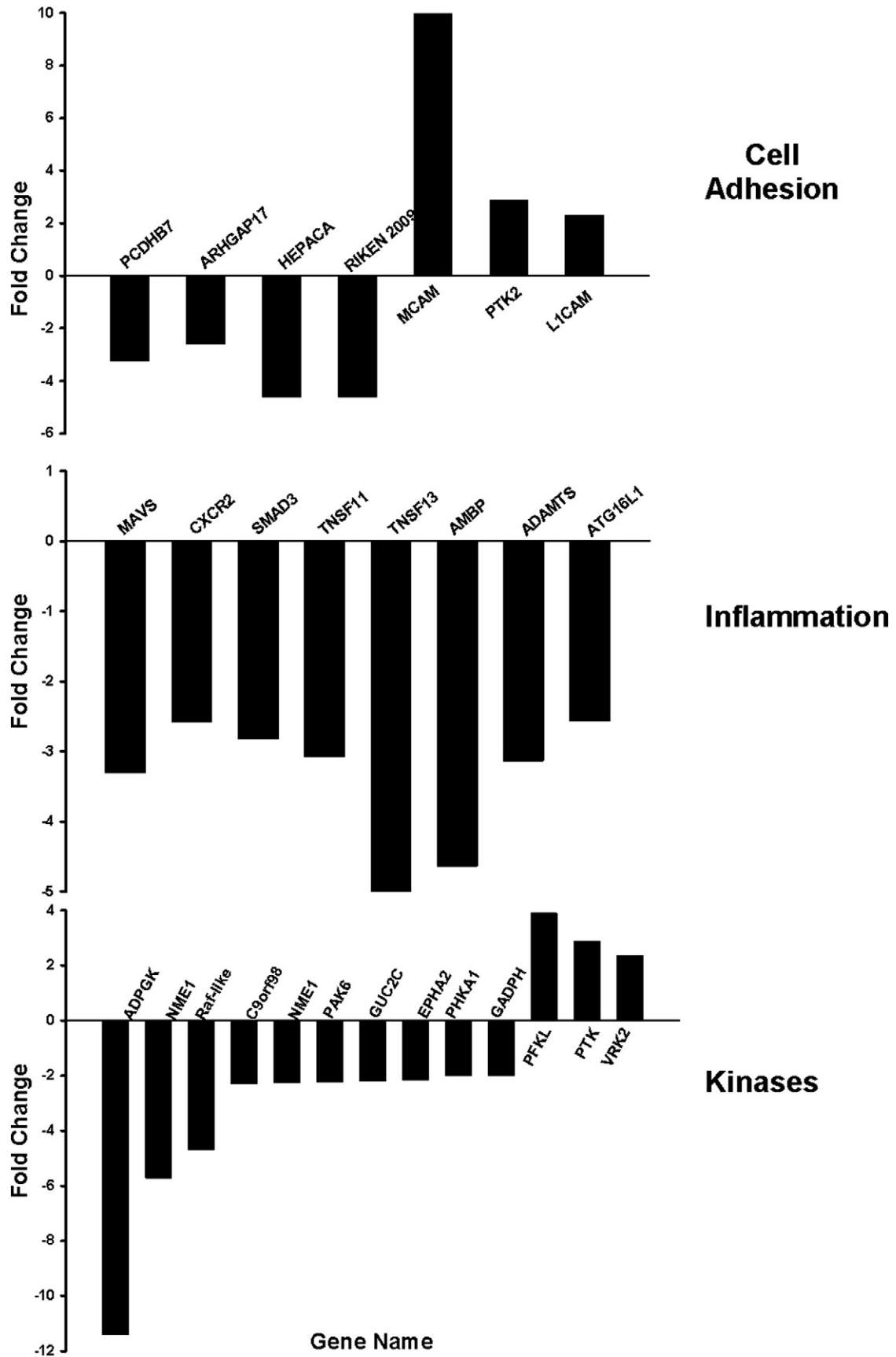


Fig. 3. Differentially expressed genes in the liver of control and MWWE-exposed minnows grouped according to their function in cell adhesion, inflammation and kinase activity. The upper panel represents mRNA levels for genes associated with cell adhesion; the middle panel B shows mRNA levels for genes associated with inflammation; the bottom panel shows mRNA levels for genes coding for different kinases.

Table 4
List of differentially expressed genes associated with specific pathways.

Pathway	Gene ID	Gene symbol	Fold change	Gene function	Pubmed accession number
Estrogen Receptor	EA_Pp_58527a	ADPGK	-11.47	Carbohydrate metabolic process, glycolysis	NM_031284.4
	EA_Pp_68848a	NME1	-5.71	Nervous system development, negative regulation of cell proliferation, cell differentiation, regulation of apoptosis	NM_000269.2
	EA_Pp_50367a	SPOCK2	-3.89	Binds with glycosaminoglycans to form part of the extracellular matrix	NM_001134434.1
	EA_Pp_53119a	HSPA5	-3.71	facilitating the assembly of multimeric protein complexes inside the ER	NM_005347.4
	EA_Pp_55993a	CBFA2T2	-2.56	tumor suppressor gene candidate	NM_001032999.2
	EA_Pp_55342a	BNIP2	-2.19	Implicated in the suppression of cell death	NM_004330.2
	EA_Pp_52335a	CEP152	-2.08	Regulator of genomic integrity and cellular response to DNA damage acting through ATR-mediated checkpoint signaling	NM_001194998.1
	EA_Pp_63001a	PHKA1	-2.05	Phosphorylase b kinase catalyzes the phosphorylation of serine in certain substrates	NM_001122670.1
	EA_Pp_69607a	CPEB4	6.52	Nucleotide binding	NM_030627.2
	EA_Pp_56645a	DNAJC5	4.96	Protein folding, negative regulation of neuron apoptosis	NM_025219.2
	EA_Pp_69541a	UBQLN4	4.46	Cell death, negative regulation of transcription	NM_020131.3
	EA_Pp_52652a	NELL2	2.99	The encoded protein acts as a homotrimer and is found in the cytoplasm	NM_001145107.1
	EA_Pp_68848a	NME1	-5.72	Nervous system development, negative regulation of cell proliferation, cell differentiation, regulation of apoptosis	NM_000269.2
	EA_Pp_68213a	DACT1	-5.39	Multicellular organismal development, Wnt receptor signaling pathway	NM_001079520.1
WNT/ β -Catenin Signaling	EA_Pp_54650a	KREMEN1	-5.33	Cellular communication, Wnt receptor signaling pathway	NM_001039570.2
	EA_Pp_53009a	SKOR1	-4.88	Regulation of transcription, negative regulation of BMP signaling pathway	XM_236329.4
	EA_Pp_70693a	SMAD3	-2.82	intracellular signal transducer and transcriptional modulator	NM_001145102.1
	EA_Pp_64316a	CPRI28	-2.21	Orphan receptor	NM_032787.2
	EA_Pp_55342a	BNIP2	-2.19	Implicated in the suppression of cell death	NM_004330.2
	EA_Pp_69307a	PP2A/PP2R5E	3.71	Regulates tau phosphorylation directly and also indirectly via activating GSK-3beta	NM_006246.2
	EA_Pp_50988a	ARIH2	3.32	E3 ubiquitin-protein ligase	NM_006321.2
	EA_Pp_69288a	AES	3.03	Inhibits NF-kappa-B-regulated gene expression	NM_001130.5
	EA_Pp_56793a	Wnt4	2.60	WNT genes implicated in chemical signaling pathways implicated in various physiological systems	NM_030761.4
	EA_Pp_55010a	LRP6	2.54	Complex that triggers beta-catenin signaling through inducing aggregation of receptor-ligand complexes into ribosome-sized signalosomes	NM_002336.2

protein 1), GPR128 (G protein-coupled receptor 128), SKOR1 (SKI family transcriptional corepressor 1), SMAD3 (SMAD family member 3), FOXB1, SNIP2 (BCL2/adenovirus E1B 19 kDa interacting protein), and NME1 (nucleoside diphosphate kinase A; Table 4).

To determine if the Wnt/ β -catenin signaling pathway was altered at the protein level, we examined the levels of nuclear CTNNB1 in the liver of treated and control fish, since CTNNB1 is the key mediator in the canonical Wnt signaling pathway. CTNNB1 protein levels in both the cytosolic and nuclear fractions tended to be lower in MWWE-treated fish as compared to controls (Fig. 4).

4. Discussion

Exposure of fish to MWWE has been associated with a variety of physiological alterations, including reproductive and immune dysfunction. Pathway analysis software predicted at least 5 signaling pathways that were altered by treatment. These included pathways implicated in cell adhesion, inflammation, numerous kinases, estrogen receptor signaling and WNT signaling.

In vertebrates, adherens junctions, tight junctions and desmosomes, are responsible for epithelial integrity and the formation of immune-privileged sites within the body (Battle et al., 2006; Dubé and Cyr, 2012). In the present study, seven transcript coding for proteins implicated in cell adhesion were altered. Three of these genes (MCAM, PTK2 and L1CAM) were up-regulated, while four others were down-regulated (PCDHB7, ARHGAP17, HEPACAM and Riken 2009E01). Among these genes, PCDHB7 is a protocadherin which has been implicated in the establishment and function of specific cell-cell neural connections (Yagi and Takeichi, 2000). ARHGAP17 encodes for a protein involved in cell polarity and the maintenance of tight junctions by regulating the activity of CDC42. In mammals, ARHGAP17 is induced by GTPase-activating proteins (Kobayashi et al., 2013). HEPACAM has been shown to be down-regulated in many cancer cell lines and therefore may act as a tumor suppressor gene, due to its role in cell motility and cell-matrix interactions (Moh and Shen, 2009). In this study HEPACAM was down-regulated by almost 6-fold, suggesting that normal developmental functions that require cell motility and cell interactions were being inhibited (Moh and Shen, 2009). Another cell adhesion protein, RIKEN 209E01, responsible for hepatocyte cell adhesion and motility processes in the liver of MWWE-exposed fish were impaired. L1CAM, which was upregulated, is normally induced during development because of its role in axon guidance and cell migration (Dou et al., 2013). MCAM is a cell adhesion molecule that acts as a surface receptor that can trigger tyrosine phosphorylation of PTK2 and causes a transient increase in intracellular Ca²⁺ concentrations. Its induction has been shown to allow melanoma cells to interact with cellular elements of the vascular system, potentially increasing the spread of tumors (Chen et al., 2013; Ward et al., 2013). While the function of these genes in fish liver is largely unknown, alteration in cell adhesion suggests that epithelial function in the liver may be impaired and may predispose fish to other pathologies, including tumor development.

Studies have shown that fish exposed to mixtures of toxicants, similar to those found in MWWE, display altered immune function and an induction of the stress-related pathways (Hebert et al., 2008, b; Muller et al., 2009; Ings et al., 2011). Ings et al. (2011) sampled juvenile rainbow trout placed downstream from a municipal wastewater discharge pipe for 14 days (Ings et al., 2011). Using a cDNA microarray, a number of genes involved in immune response, including low molecular mass protein 2, major histocompatibility complex 1, Mx2 and Sox24 were down-regulated, suggesting an inhibition of the immune system (Ings et al., 2011). A second study showed that juvenile rainbow trout exposed to 1% wastewater showed an increase in phagocytic activity after 28 days but a decrease in phagocytic activity after 90 days, suggesting that accumulation of toxicants can inhibit the immune response (Hebert et al., 2008). In this study, a number of genes implicated in the

inflammatory response were decreased by exposure to municipal effluent. These included: MAVS, CXCR2, SMAD3, TNFSF11, TNFSF13, AMBP, ADAMTS, and ATG16L1. Several studies have reported that the immune system of aquatic organisms exposed to municipal wastewater effluent mixtures was altered (Hoole et al., 2003; Gagné et al., 2005, 2007, 2008; Bouchard et al., 2009; Gagné et al., 2012; Tetreault et al., 2012). In the present study, a number of cell-derived mediators of the inflammation response were down-regulated, including MAVS, which is required for the activation of transcription factors which regulate the expression of beta-interferon. During a stress response, leukocytes are mobilized towards toxicants, reducing the levels available within the organism. As well, CXCR2, a receptor for the cytokine interleukin 8 and chemokine ligand 1, mediates neutrophil migration to sites of inflammation, suggesting a reduction of key mediators in the inflammatory pathway response. The inhibition of AMBP, a glycoprotein and ADAMTS, a peptidase, both play important roles in the regulation of inflammatory processes including tissue organization, coagulation, and cell migration. Finally, three genes involved in the T cell-dependent immune response, TNFSF11, TNFSF13 and ATG16L1, which play a role in cell-mediated immunity, were down-regulated. Overall, the present study showed a reduction in various aspects of the inflammation response, as shown by a reduction in proteins expressed by T cells, as well as by a decrease in cytokines and other key mediators. This decrease in immune/inflammation response points to a pre-disposition to infection and possibly other pathogenic attack, thus reducing the overall health of both the individual fish, and more alarmingly, pointing to a reduction in the health of the fish population.

Several gene transcripts for protein kinases were also altered by exposure to MWWE. Ten genes in the present study were down-regulated, including ADPGK, NME1, Raf-like, AK8, AKAP6, PAK6, GUCY2C, EPHA2, PHKA1, and GAPDH, while PFKL, PTK and VRK2 were

upregulated (Table 4; Fig. 4). ADPGK is responsible for the catalyzing ADP-dependent phosphorylation of glucose to glucose-6-phosphate in the glycolytic cycle. GAPDH catalyzes the breakdown of glucose for energy and carbon molecules (Tarze et al., 2007). It has also recently been implicated in several non-metabolic processes, including transcription activation and initiation of apoptosis (Tarze et al., 2007). AK8 is a phosphotransferase enzyme that catalyzes the interconversion of adenine nucleotides that play a role in cellular energy homeostasis. The PAK6 gene encodes for an enzyme implicated in the regulation of a number of cellular processes, including cytoskeleton rearrangement, apoptosis, and MAP kinase signaling (Ye and Field, 2012; Furnari et al., 2013). As well, PAK6 interacts with the androgen receptor, which is important for male sexual differentiation and development (Lee et al., 2002), although this is unlikely involved in liver function. An overall inhibition of kinase activity observed in this study suggests that various intracellular signaling pathways may be directly targeted by exposure to MWWE, again negatively impacting various metabolic processes, as well as sexual differentiation and development. It is likely that the alterations in genes comprising these pathways may be responsible, in part, for some of the effects previously reported in fish or hepatocytes exposed to MWWE (Gagné and Blaise, 1995; Aravindakshan et al., 2004; de Montgolfier et al., 2008).

Analyses of gene expression data indicated that 13 estrogen (E2)-regulated genes were altered after exposure to MWWE. These genes are involved in numerous cellular functions and have been shown in mammals to be regulated either directly or indirectly by estradiol. Genes that are estrogen-dependent and whose expression was decreased by exposure WWWE included NELL2, HSPA5, SPOCK2 and BNIP2 (Belcredito et al., 2001; Watanabe et al., 2003; Ivanga et al., 2007; Choi et al., 2010; Guzel et al., 2011). Furthermore, eight genes shown to be regulated downstream of the E2 receptor including

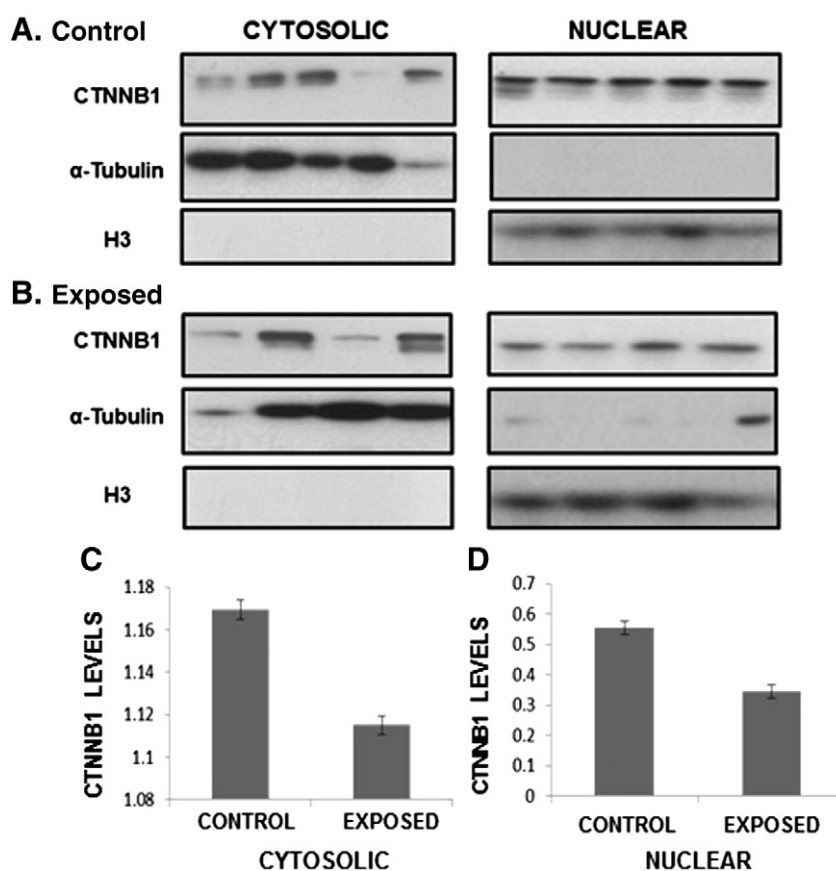


Fig. 4. Western blot analysis of CTNNB1 protein expression in fathead minnow exposed to MWWE as compared with controls. Cytosolic protein loading was standardized using α -tubulin as a protein loading control (Panels A and B) and nuclear protein loading was standardized using H3 as a protein loading control (Panels C and D). Data are expressed as the mean \pm SEM.

ENPEP, DNAJC5, PLXNA3, and UBQLN4 were also down-regulated. Aromatase, a member of the cytochrome P450 superfamily, and a key enzyme in the biosynthesis of estrogens, was down-regulated in the liver of MWWE-exposed fish in the present study. Inhibition of aromatase suggests a reduction in the synthesis of the hormone estrogen, a key hormone for both sexual differentiation and morphogenesis (Cooke and Naaz, 2004; McCarthy and Knokle, 2005).

While the data suggest an inhibition of the estrogenic pathway, it should be noted that estradiol levels in these immature fish are likely to be very low, and thus the significance of this is unknown, nor can we draw any conclusions at this time on the potential response of these fish to E2. Previous studies have shown that in brook trout, VTG mRNA induction in male and juvenile fish occurred after only 12 weeks of exposure (de Montgolfier et al., 2008) suggesting the need for contaminants to accumulate in these fish prior to the induction of an estrogenic response. The present data would support these conclusions.

A number of genes involved in the Wnt/CTNNB1 signaling pathway were differentially expressed in MWWE-exposed fathead minnows. Wnt signaling is implicated in numerous critical cellular functions including cell–cell signaling during embryogenesis, cellular differentiation, cell adhesion, cell cycle progression and differentiation (Hendriks and Reichmann, 2002; Monga and Michalopoulos, 2005).

Wnt signaling is also involved in liver development, cell proliferation, apoptosis and tissue homeostasis (Logan and Nusse, 2004; Lu et al., 2011). Several genes in the canonical Wnt pathway, including KREMEN1, Frizzled, DACT1, and DVL2 (disheveled dsh homologue 2), were dramatically down-regulated. The Wnt pathway can be activated either when the Fz-LRP complex is formed or with the autophosphorylation of LRP6; both actions send a signal through intermediate proteins to activate Dsh (disheveled) (Veeman et al., 2003; Monga and Michalopoulos, 2005; Lade and Monga, 2011). Dsh then blocks CTNNB1 degradation by recruiting GSK3B, allowing the axin APC complex to form and inactivating CTNNB1 ubiquitination (Veeman et al., 2003; Monga and Michalopoulos, 2005; Lade and Monga, 2011). Dkk-1 interacts with LRP5/6 and the co-receptor KREMEN1 to prevent the formation of the LRP5/6–Wnt–Frizzled complex (Osada et al., 2006). In the present study, KREMEN1 was down-regulated, suggesting a reduction in the formation of the LRP5/6 Wnt–Frizzled complex (Cadoret et al., 2002; Davidson et al., 2002; Mao et al., 2002; Osada et al., 2006). DACT1 binds to Dsh and impedes the degradation of CTNNB1, enhancing the transcriptional activity of genes targeted by the Wnt pathway. In this study, fathead minnows exposed to MWWE exhibited a down-regulation of DACT1, suggesting that one mechanism of controlling CTNNB1 levels was inhibited. Dsh signaling through the Wnt pathway can be transduced by one of two routes depending on the target gene and end function. Several other genes involved in the non-canonical Wnt pathway, such as Wnt4, LRP6, and PPP2R5E (Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit epsilon isoform), which are known to inhibit the canonical Wnt pathway, were also increased. Wnt4 has been shown to inhibit Wnt signaling to the nucleus by redirecting CTNNB1 to the plasma membrane (Bernard et al., 2008). Thus the increase in Wnt4 may decrease overall Wnt signaling via the canonical Wnt signaling pathway. Exposed minnows in this study showed higher levels of LRP6 mRNA. The role of LRP6 in Wnt signaling has been reported to be either stimulatory or inhibitory. Activation of Wnt signaling can occur when LRP6 inhibits GSK3 β (Metcalf and Bienz, 2011), resulting in increased CTNNB1 in the cytosol and subsequent translocation to the nucleus, where it can act as a transcription factor (Fang et al., 2000). However, LRP can also interact with DKK1, which can then inhibit Wnt signaling. Since LRP6 was increased in MWWE-exposed fish, this may also contribute to the inhibition of the WNT pathway.

In the absence of Wnt signaling, APC, axin and GSK3 phosphorylate CTNNB1 marking it for degradation by an E3 ubiquitination protein

(Polakis, 2001; Hendriks and Reichmann, 2002). In this study fish exposed to MWWE showed an increase in three E3 ubiquitination proteins: ARIH2, TOPORS (topoisomerase I binding arginine serine-rich) and UBQLN4 (ubiquilin 4). A lack of Wnt signaling in the nucleus could act as a repressor of target gene transcription in conjunction with the co-repressor Groucho/AES (Monga and Michalopoulos, 2005). Two Wnt pathway inhibitors, AES and PPP2R5E (phosphatase 2A) were also up-regulated in these fish, further supporting an inhibition of the canonical Wnt signaling pathway. The decrease in Wnt signaling was supported by the results of the Western blot, which suggested a decrease in both cytosolic and nuclear Wnt protein levels in the livers of fish exposed to MWWE.

Other studies have reported that components of the Wnt/ β -catenin signaling pathway may be altered following exposure to environmental contaminants. Ung et al. (2010) reported that in zebrafish, HgCl₂ exposure induced GSK3 mRNA and decreased levels of cadherin1 (also known as E-cadherin) immunostaining. In another study, Mirbahai et al. (2011) collected dab from five sampling sites along the Irish Sea and Bristol Channel and compared healthy livers with those showing lesions and/or hepatocellular carcinoma. Livers of fish sampled from two of the sampling sites, which contained elevated levels of polychlorinated biphenyl, polybrominated diphenyl ethers, and heavy metals such as cadmium and lead (Mirbahai et al., 2011), displayed altered DNA methylation of genes implicated in the Wnt/ β -catenin signaling pathway (Mirbahai et al., 2011). These included Dsh, Frz, and the tumor suppressor gene APC (adenomatous polyposis coli) and c-myc, a TCF/LEF regulated gene. Previous studies from our laboratory have shown that in rats treated with hexachlorobenzene, there is an activation of WNT signaling and increased translocation of CTNNB1 to the nucleus (Plante et al., 2005). This is associated with decreased levels of Cx43, Cx32 and Cx26 (Plante et al., 2002). While we have shown that short-term exposure of brook trout to MWWE can result in a slight decrease in testicular Cx43 and Cx43.1 (de Montgolfier et al., 2008), further studies will be needed to establish a link between Wnt signaling and changes in Cx levels in the testis. The present study is the first to suggest an alteration in Wnt/ β -catenin signaling in fish exposed to MWWE.

Together, results from the present study suggest that short-term exposure to MWWE results in an inhibition of estrogenic signaling and Wnt signaling pathways.

Signaling pathway analyses suggest that other pathways mediated by several kinases and inflammation are also targeted following exposure to MWWE, and likely result in a variety of altered physiological functions. The effects on Wnt signaling are particularly interesting and suggest that in addition to effects in juveniles and adults, the effluent may exert significant effects during embryonic development or in the development of pathological conditions, when Wnt signaling plays an important role in cellular differentiation and development. This would also suggest the potential for abnormal development as well as leading to embryonic death, parameters that have not been examined in MWWE-exposed fish. Such effects would have significance both for individual, as well as for the fish populations living downstream from the MWWE point of discharge.

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