Aquatic Toxicology 97 (2010) 212–225

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Gene expression profiling and environmental contaminant assessment of migrating Pacific salmon in the Fraser River watershed of British Columbia

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ARTICLE INFO

Article history:
Received 13 June 2009
Received in revised form
13 September 2009
Accepted 14 September 2009

Keywords:
Gene expression
mRNA
Oncorhynchus
Environmental contaminant
Real-time quantitative PCR
Migration

ABSTRACT

The health and physiological condition of anadromous salmon is of concern as their upriver migration requires navigation of human-impacted waterways and metabolism of stored energy reserves containing anthropogenic contaminants. Such factors may affect reproductive success of fish stocks. This study investigates chemical contaminant burdens and select gene expression profiles in Pacific Sockeye (*Oncorhynchus nerka*) and Chinook (*Oncorhynchus tshawytscha*) salmon which traverse the Fraser River watershed during their spawning migration. Chemical analyses of muscle tissue and eggs of salmon collected from the lower Fraser River (pre-migration) and from upstream spawning grounds (post-migration) during the 2007 migration revealed the presence of numerous chemical contaminants, including PCBs, dioxins/furans, pesticides, and heavy metals. However, muscle tissue residue concentrations were well below human health consumption guidelines and 2,3,7,8 TCDD toxic equivalents (ΣTEQs) in salmon eggs, calculated using WHO toxic equivalency factors (WHO-TEFs) for fish health, did not exceed the 0.3 pg g^{-1} wet weight toxicological threshold level previously associated with 30% egg mortality in salmon populations. Quantitative real-time PCR probes were generated and used to assess differences in abundance of key mRNA transcripts encoding nine gene products associated with reproduction, stress, metal toxicity, and exposure to environmental contaminants. Gene expression profiles were characterized in liver and muscle tissue of pre- and post-migration Sockeye and Chinook salmon. The results of stock-matched animals indicate that dynamic changes in mRNA levels occur for a number of genes in both species during migration and suggest that Sockeye salmon exhibit a greater level of biological stress compared to the Chinook salmon population. Using a male-specific genotypic marker, we found that out of the 154 animals examined, one Sockeye was genotypically male but phenotypically female. This individual's gene expression profile in liver and muscle was reminiscent of, but not identical to, the female expression profile. These studies provide the first glimpse of the dynamic yet common nature of changes in the transcriptome that are shared between species during in-migration and highlight differences that may relate to population success. Continued longitudinal assessment will further define the association between contaminant burden, physiological stress, and modulation of gene expression in migrating Pacific salmon.

1. Introduction

The Fraser River is one of Canada's largest rivers, spanning 1375 km from its headwaters in eastern British Columbia (BC) to its mouth in the Strait of Georgia (Canadian Heritage Rivers System, 2009). This extensive drainage basin encompasses approximately 25% of the area of BC and contains a population of 2.7 million people (Fig. 1). The river represents a major transportation corridor between the Pacific Ocean and inland Canada, accounting for 80% of the economic prosperity of BC. The Fraser River watershed is a key migratory route for various Pacific salmon species, including several populations of Chinook (*Oncorhynchus tshawytscha*) and Sockeye (*Oncorhynchus nerka*) (Canadian Heritage Rivers System, 2009).

Adult salmon returning to the Fraser River to spawn can, in some cases, travel several hundred kilometres upstream to reach natal spawning grounds (Fig. 1). This arduous journey requires a high degree of an individual's overall energy requirement (Groot

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doi:10.1016/j.aquatox.2009.09.009

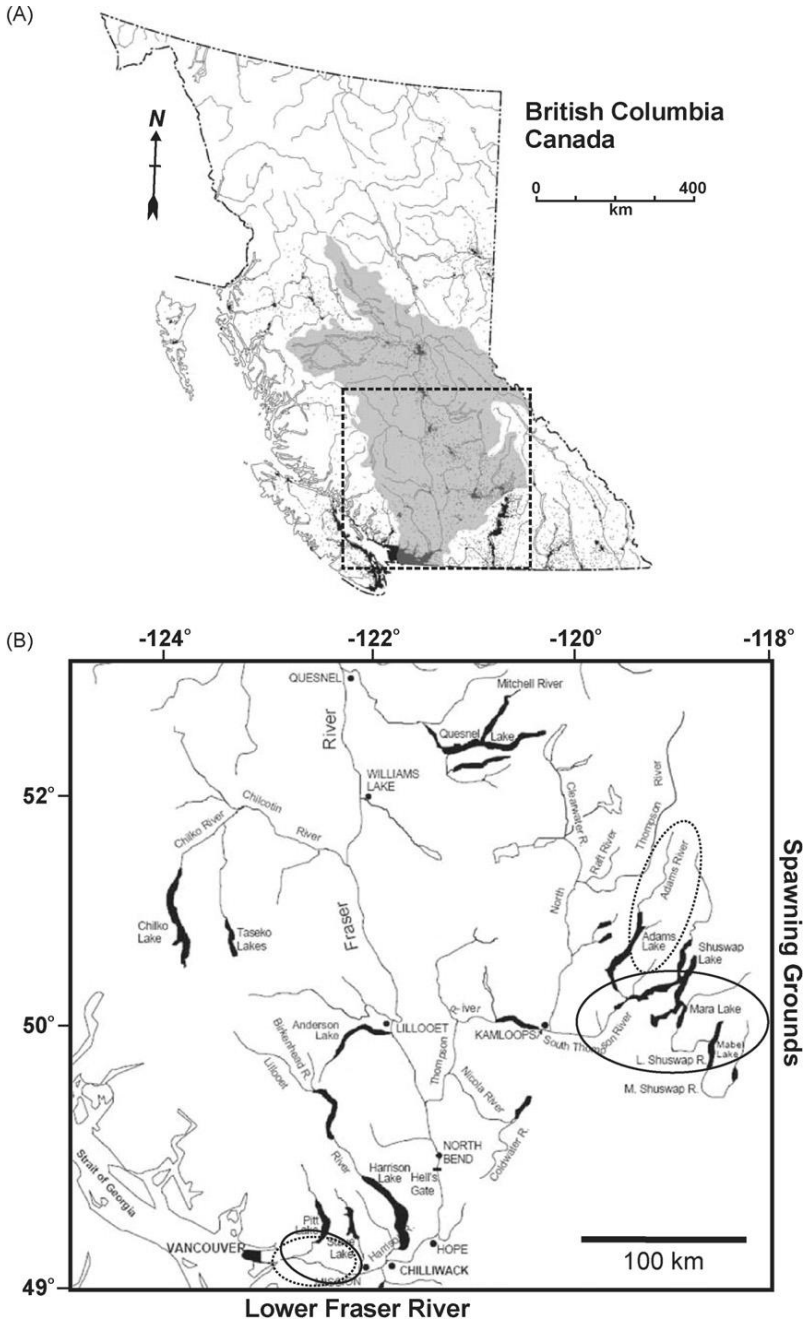

Fig. 1. The Pacific salmon migratory route within the Fraser River drainage system of British Columbia. (A) Map of British Columbia, Canada showing the Fraser River watershed shaded in grey. Highly populated areas are indicated by black dots (one dot equals 50 persons) (BC Stats; http://www.bcstats.gov.bc.ca/data/pop/georef/geopage.asp). The dashed region is enlarged in panel (B) to highlight the pre-migration (Lower Fraser River) and post-migration (Spawning Grounds) collection sites. Latitude and longitude are indicated. The solid ovals represent Chinook salmon sampling locations whereas the dashed ovals denote the Sockeye collection sites. Details on the specific animals are presented in Supplementary Table S1.

and Margolis, 1991; Ewald et al., 1998). There is increasing concern regarding health and environmental fitness of several Canadian Pacific salmon stocks. Weaver Creek Sockeye returning to the Fraser River have exhibited abnormal migration behavior, elevated in-river mortality and reduced spawning success in recent years (Pacific Salmon Commission, 2001). Climatic changes, industrialization, and/or chemical pollution may have a negative impact on biological processes involved in the environmental fitness and reproductive success of salmon stocks. In particular, environmental contaminants such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs), can impact olfactory function and migration behavior (Moore and Waring, 1996) and reproductive success in fish (Guiney et al., 1979; Zitko and Saunders, 1979; Niimi, 1983; Walker and Peterson, 1991; Miller, 1993; Giesy et al., 2002). Since Pacific salmon are an important commercial and wildlife food source, it is important to establish effective contaminant monitoring programs of these pivotal sentinel species. Such initiatives will help to identify geographic regions with elevated contaminant exposure risks and charac-

terize temporal stress patterns related to their cyclic migration pattern.

A powerful tool in evaluating biological effects is based upon genomics applications. The interaction between a living organism and its surrounding environment is dynamic and adaptive responses in biological processes are required for continued survival. At the cellular level, a number of defence pathways exist to help protect living systems following exposure to potentially damaging chemicals or deleterious environmental conditions. Such responses originate from changes in the expression of genes and techniques, such as quantitative real-time polymerase chain reaction (QPCR) analysis, can be employed to effectively detect and quantify these changes at the level of the transcriptome. Thus, alterations in specific targeted gene mRNA transcripts found within the transcriptome can be identified which may shed light on the presence and nature of a chemical exposure event or lend insight into a specific developmental process within a species under investigation.

The present study involves an evaluation of environmental contaminant exposure and gene expression status in two Pacific salmon species, Sockeye and Chinook, during their 2007 migration through the industrialized lower Fraser River delta region to upstream spawning grounds (Fig. 1). Specifically, we measured concentrations of several organohalogen contaminants including PCBs, PCDDs, PCDFs, polybrominated diphenyl ethers (PBDEs) and several organochlorine pesticides (OCPs), as well as Hg and several other trace elements. Specific gene transcripts were targeted for isolation from each species and used to develop QPCR detection tools. The selected genes encode products related to (1) estrogen exposure/response indicators: vitellogenin A (*VTG*), vitelline envelope protein gamma (*VEP*Δ), and estrogen receptor alpha (*ERα*); (2) polyaromatic hydrocarbon exposure: aryl hydrocarbon receptor alpha (*AhR*) and 3-methylcholanthrrene responsive cytochrome P450 CYP1A (*CYP1A*); and (3) metal/oxidative/general stress: metallothionein A (*MT*), heat shock protein 27 alpha (*HSP27*), and catalase (*CAT*). Changes in the expression of these gene targets have been associated in a number of different species with exposure to estrogenic substances, exposure to aryl hydrocarbon-containing congeners, metal toxicity, and oxidative as well as other environmental stressors. These QPCR probes were subsequently used to generate tissue-specific gene expression profiles of Sockeye and Chinook salmon collected in the lower Fraser River and at upstream spawning grounds. Both liver and muscle tissue were investigated in stock-matched males and females. A comparison of mRNA expression phenotype of both salmon species to QPCR-based sex genotyping was also performed to screen for indications of sex reversal. Information obtained from gene expression profiling, sex phenotype to genotype comparison, and measurement of chemical contaminant body burdens will help in providing a broader view of the status of migratory salmon species in the Fraser River watershed.

2. Materials and methods

2.1. Sample collection and handling

Individual fish from two returning Fraser River Sockeye stocks (Adams River and Weaver Creek Sockeye) and two Fraser River Chinook salmon stocks (Thompson River and Shuswap River Chinook) were collected from two general locales within the Fraser River watershed that included sites near the mouth of the Fraser River (pre-migration group in the lower Fraser River) and upstream spawning grounds (post-migration group in the upper Fraser River watershed) (Fig. 1; Table 1 and Table S1). These fish were part of the Fisheries & Oceans Canada fisheries program or the First Nations fishery. Male and female fish were caught by seine, gill-net, or dip-net between June and October, 2007. Due to conservation concerns, our team coordinated sampling with existing fisheries to limit the amount of salmon being taken out of the river systems. Attention was given to obtaining an equal number of males and females, where available, for each species and stock matching the samples collected from the lower Fraser River and spawning ground sites (Supplementary Table S1).

The animals were aged by scale growth by Birkenhead Scale Analyses, Lone Butte, British Columbia using both the Gilbert-Rich and European methods. Fish were 4 or 5 years of age. DNA fingerprinting was carried out for stock group identification at the Pacific Biological Station Molecular Genetics Laboratory in Nanaimo, British Columbia according to published procedures (Beacham et al., 2003, 2004). Tissue samples were excised from individual fish for DNA fingerprint-based stock identification, contaminant analysis, and gene expression analysis. Dorsal muscle and roe samples were collected from individual fish for contaminant analysis and were processed into several tissue-specific composites per sampling location. Composite samples were stored in solvent-rinsed amber glass jars at −80 °C prior to contaminant analysis. Tissue samples for gene expression analyses (liver and dorsal muscle) were excised from individual fish and placed in RNA*later* tissue preservation solution (Applied Biosystems Inc., Foster City, CA, USA) for a minimum of 24 h at 4 °C. The preserved tissues were subsequently stored at −20 °C prior to processing.

2.2. Contaminant analyses

Organohalogen contaminant analyses in salmon muscle (males and females grouped together) and roe samples were conducted at the Institute of Ocean Sciences, Sidney, BC, Canada. Procedures and quality assurance/quality control (QA/QC) protocols employed for simultaneous extraction, cleanup and quantification of major organohalogen contaminants (i.e. PCBs, PCDD/Fs, PBDEs and pesticides) by gas chromatography/high resolution mass spectrometry (GC-HRMS) have been previously described in detail (Ikonomou et al., 2001, 2007). Tissue lipid contents were determined gravi-

Table 1
Salmon collected for gene expression analysis.

Species	Sample group	Sex	Number	Sampling location
O. nerka	Lower Fraser River	Female	16	Fort Langley-Kwantlen
	Lower Fraser River	Male	20	Fort Langley-Kwantlen
	Spawning Ground	Female	10	Adams River
	Spawning Ground	Female	10	Weaver Creek
	Spawning Ground	Male	10	Adams River
	Spawning Ground	Male	10	Weaver Creek
O. tshawytscha	Lower Fraser River	Female	20	Albion Test Fishery
	Lower Fraser River	Male	16	Albion Test Fishery
	Lower Fraser River	Male	2	Rosedale
	Lower Fraser River	Male	1	Lytton-Siska Fishwheel
	Spawning Ground	Female	19	Shuswap Falls Hatchery
	Spawning Ground	Male	20	Shuswap Falls Hatchery

Table 2
DNA Primers used in the isolation of specific expressed gene sequences from Sockeye and Chinook salmon.

Gene	Primer name	Cloning primer pair	Predicted DNA (base pairs)	NCBI GenBank accession number
VTG	ONC1 ONC2	5′-TCCATAGAGGTATCCTGAACA-3′ 5′-ATCAGAGCACCATTGKCA-3′	304–311	Chinook: FJ226365 Sockeye: FJ226375
VEPΔ	ONC3 ONC4	5′-ACCCAGGCYAAGCAGAAG-3′ 5′-ACCATATCACCCAACACG-3′	575	Chinook: FJ226366 Sockeye: FJ226376
ERα	ONC5 ONC6	5′-CCGCCTCAGAAAGTGTTA-3′ 5′-ATCTCAGCCATACCCTCC-3′	567	Chinook: FJ226367 Sockeye: FJ226377
AhR	ONC9 ONC10	5′-CTGTCAARAAGCGGAAGA-3′ 5′-ATGGAAGCCCAGGTAGTC-3′	410	Chinook: FJ226368 Sockeye: FJ226379
CYP1A	ONC11 ONC12	5′-GCACAACAACCCTCACCT-3′ 5′-CCACCTGCCCAAAYTCAT-3′	518	Chinook: FJ226369 Sockeye: FJ226380
MT	ONC13 ONC14	5′-ATCTTGCAACTGCGGTGG-3′ 5′-GACAGCAGKCGCAGCAAC-3′	80–83	Chinook: see text Sockeye: see text
HSP27	ONC15 ONC16	5′-GATCACTGGCAAGCATGA-3′ 5′-GCTCATCATRATGGTGCC-3′	272	Chinook: FJ226370 Sockeye: FJ226381
CAT	ONC17 ONC18	5′-GAGATGGCCCACTTCGAC-3′ 5′-ATGAAGGATGGGAACAGC-3′	317	Chinook: FJ226371 Sockeye: FJ226382
CBA	ONC19 ONC20	5′-CACGGCATCGTCACCAAC-3′ 5′-CATACCGAGGAAGGAGGG-3′	591	Chinook: FJ226372 Sockeye: FJ226383
rpL8	ONC21 ONC22	5′-GCCGCTAAACTCAGACAC-3′ 5′-CTTCAGGATGGGCTTGTC-3′	468	Chinook: FJ226373 Sockeye: FJ226384
GAPDH	GAPDH-f GAPDH-r	5′-GTAATGCATCTTGCACGACT-3′ 5′-AAGGCCATGCCAGTCAGCT-3′	250	Chinook: FJ226374 Sockeye: FJ226385

metrically using an extracted 5 g sub-sample (wet weight). Lipid contents (% lipid) were expressed as a percentage of the original wet tissue weight. Analyses for the determination of Hg and thirty three other trace elements (Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, S, Sb, Se, Sn, Sr, Ti, Tl, U, V, Zn, Zr) were conducted at CANTEST Ltd. (Burnaby, BC, Canada), using inductive coupled plasma mass spectrometry, following standard methods (U.S. Environmental Protection Agency Method 6020 (U.S. Environmental Protection Agency, 1986)). A list of all chemicals monitored in salmon muscle and roe are shown in Supplementary Table S2.

2.3. Total RNA isolation and preparation of total cDNA

Total RNA was isolated from each tissue sample using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). Care was taken to minimize carryover of RNAlater solution into the TRIzol by blotting each tissue sample onto a Kimwipe prior addition of the tissue sample to the TRIzol reagent. Mechanical disruption of tissue utilized 700 μl TRIzol reagent, a 3 mm diameter tungsten-carbide bead, and safelock Eppendorf 1.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific Ltd, Ottawa, ON, Canada) at 20 Hz for 9 min. Mixing chambers were stopped and rotated 180° every 3 min through the homogenization procedure. Glycogen (20 μg) was added as a nucleic acid carrier to the muscle homogenate samples just prior to RNA precipitation as described in the TRIzol protocol. Isolated total RNA was subsequently resuspended in 120 μl or 30 μl diethyl pyrocarbonate (DEPC)-treated RNase-free water for liver or muscle samples, respectively, and then stored at −70 °C. Total RNA isolated from each tissue sample (1 μg for muscle and 3 μg for liver) was annealed with random hexamer oligonucleotide and cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit as described by the manufacturer (Fermentas Life Sciences, Burlington, ON, Canada). All cDNA reactions were diluted 20-fold prior to QPCR amplification.

2.4. Isolation of liver genomic DNA

Genomic DNA was isolated from liver tissue of the 154 study animals using the DNeasy Blood and Tissue Kit (QIAGEN Inc., Mississauga, ON, Canada) as per the manufacturer's protocol including overnight proteinase K treatment and the optional RNase A (QIAGEN) addition. DNA samples were isolated from the spin columns with sequential elutions of 100 μl and 50 μl Buffer AE and the nucleic acid concentrations determined by A_{260} spectrophotometry. Genomic DNA from Sockeye salmon number S46 was prepared independently twice from liver tissue and once from muscle for sex genotype analysis to ensure correct classification.

2.5. QPCR assay development

Before evaluation of fish stocks could be carried out, the molecular tools required for species-specific QPCR had to be developed. In this instance, eight genes were selected, based upon available literature, for their potential to provide evidence of exposure to environmental stressors and/or chemical contaminants. These included markers for: (1) estrogen exposure/response: vitellogenin A (*VTG*), vitelline envelope protein gamma (*VEPΔ*), and estrogen receptor alpha (*ERα*); (2) polyaromatic hydrocarbon exposure: aryl hydrocarbon receptor alpha (*AhR*) and 3-methylcholanthrrene responsive cytochrome P450 CYP1A (*CYP1A*); and (3) metal/oxidative/general stress: metallothionein A (*MT*), heat shock protein 27 alpha (*HSP27*), and catalase (*CAT*). Three additional genes were selected as potential invariant normalizers for baseline correction: cytoplasmic beta-actin (*CBA*), ribosomal protein L8 (*rpL8*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Expressed gene sequence from related salmonid species or other teleosts were collected from NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/) and aligned by Clustal W (http://align.genome.jp/). These cDNA sequences were used to design degenerate or specific DNA primers that would amplify targeted gene sequences from both salmonid

Table 3
Gene-specific DNA primers for QPCR.

Gene	Primer pair name	Primer up	Primer down	DNA Size (base pairs)	PCR Annealing Temp (°C)
VTG	ONQ1	5′-GTCTATGAGTTGCAGGAGG-3′	5′-TGAGGTAKTTGTAAGTGGC-3′	223	60
*VEP*Δ	ONQ2	5′-AGCCAGAGCCCAAGATTA-3′	5′-GGTGTTTGCCAGAGGTTT-3′	308	60
ERα	ONQ3	5′-AGGATAAGCGGTATTGKGG-3′	5′-AMCTGTTCAGGAGGCATG-3′	166	60
AhR	ONQ5	5′-GCTCCAGATGTGGTCAAGT-3′	5′-GAGTTTGTCCAGGCGAGA-3′	123	62
CYP1A	ONQ6	5′-TCATCAACGACGGCAAGA-3′	5′-GTTCACCAAGCCCAACAG-3′	317	60
MT	ONQ7	5′-ATCTTGCAACTGCGGTGG-3′	5′-GACAGCAGTCGCAGCAAC-3′	83	60
HSP27	ONQ8	5′-CTGACGCTGAGAAGGTGA-3′	5′-TAGGGCTTGGTCTTGCTG-3′	136	60
CAT	ONQ9	5′-GCCAAGGTGTTTGAGCAT-3′	5′-GCGTCCCTGATAAAGAAGAT-3′	185	60
CBA	ONQ10	5′-CCAAAGCCAACAGGGAGA-3′	5′-AGGAAGGAGGGCTGGAAG-3′	466	60
rpL8	ONQ11	5′-TTGGTAATGTTCTGCCTGTG-3′	5′-GGGTTGTGGGAGATGACTG-3′	130	60
GAPDH[a]	ONQ12	5′-CCRCCAGAACATYATCCC-3′	5′-GTCAGCTTGCCRTTSAGC-3′	81	62
OTY2-WSU[a]	OTY2	5′-CTGGTTCGAGCCTAAGTAG-3′	5′-GATGCAGTAGGAGCAGATG-3′	260	64

[a] OTY2 primers were used in QPCR-based sex genotyping of genomic DNA isolated from the Chinook and Sockeye salmon analyzed in this study. In addition to their use in determination of mRNA abundance, the GAPDH primers were employed in normalization of genomic DNA input in the sex genotype QPCR analysis.

species in a polymerase chain reaction (PCR) (Table 2). DNA products of the correct predicted size were isolated and cloned for sequence identification. The identity of each isolated cDNA was confirmed through DNA sequencing followed by NCBI BLASTn analysis against gene sequences from related salmonid species or other teleosts. All 20 cDNA sequences were deposited in NCBI GenBank with the accession numbers FJ226365–FJ226374 (Chinook) and FJ226375–FJ226377 and FJ226379–FJ226385 (Sockeye) (Table 2). Due to their small size, cDNA sequence obtained for metallothionein A (Chinook, ATCCTGCAAGTGCTCCAACTGCGCATGCACCAGTTGTAAGAAAGCAA; Sockeye ATCCTGCAAGTGCTCCAACTGCGCATGCACTAGTTGTAAGAAAGCAA) were not submitted to NCBI GenBank. The sequences obtained from the two salmonid species were used to design gene-specific QPCR DNA primers (Table 3). DNA primers for the male-specific *OTY2* genomic marker were also prepared for QPCR-based sex genotyping analysis of Chinook and Sockeye salmon (Table 3).

Each QPCR primer pair was assessed in a three-tier quality control process on a MX3005P Real-Time PCR System (Stratagene, LaJolla, CA) for their specificity, sensitivity, and a robust signal to noise comparison using amplification reactions containing a mixed cDNA template representing a combination of the liver and muscle samples. In addition, an amplification reaction containing no DNA template was generated for each primer pair in order to establish non-specific background amplification. Positive selection criteria for each primer pair included amplification of a cDNA-specific DNA fragment of the correct predicted size and reasonable separation of DNA template-associated signal compared to background noise. The specificity of each QPCR primer pair was further confirmed by purification of the amplified DNA and restriction enzyme mapping. To satisfy the requirements for application of the comparative C_t (C_t) method (http://www.dorak.info/genetics/realtime.html), standard curve analysis was performed for each gene-specific primer pair against twofold serially diluted cDNA template (range: twofold to 256-fold dilution of neat cDNA preparation). The slope of C_t (test gene minus the *rpL8* normalizer) versus the log of cDNA template dilution was <0.1 for all primer pairs examined.

2.6. QPCR assay methodology

Quadruplicate reactions were performed for each cDNA sample and cycle threshold (C_t) data obtained. Each 15 μl QPCR amplification reaction consisted of 10 mM Tris-HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Invitrogen, Burlington, ON, Canada), 69.4 nM ROX (Invitrogen), 5 pmol of each primer, 200 μM dNTPs (Fermentas), 2 μl of 20-fold diluted cDNA, and one unit of Hot Start Taq DNA polymerase (Fermentas). Amplification reactions were subject to the following general thermocycle conditions: an initial activation step of 9 min at 95 °C followed by 40 cycles of 15 s denaturation at 95 °C, 30 s annealing at 60 °C, and 45 s polymerization at 72 °C. Modifications to this thermocycle profile were done for specific gene target and tissue combinations as follows: *AhR* and *GAPDH* used a 62 °C annealing step for both tissue samples, *ERα* and *CYP1A* used 45 cycles for both tissues while *HSP27* used 45 cycles for liver, and *VTG* and *VEP*Δ cycles for muscle samples were increased to 45 and 48, respectively. Specificity of target amplification was measured by the inclusion of reactions lacking cDNA (no DNA template control) or Taq DNA polymerase (no amplification control) and by subjecting completed runs to thermodenaturation analysis. Reactions that failed to provide the requisite thermodenaturation profile were removed from further data analysis. An additional QPCR run control comprising a universal cDNA sample present on all QPCR runs was included to assess inter-plate variation for each gene target (14 sample plate runs were performed per gene to complete the entire sample set). Variation between plate runs was observed at less than one C_t value for all gene targets. C_t values for each gene target and salmonid cDNA sample examined from all collection sites were adjusted to the same threshold value across multi-plate runs and the resulting data were normalized to selected invariant expressed genes using the comparative C_t method (C_t).

Each prepared cDNA sample analyzed by QPCR was examined for the absence of genomic DNA contamination that could potentially contribute to amplified DNA signal in QPCR. This was done with the GAPDH primer pair (Table 3) that can detect an approximately 720 base pair DNA amplicon product originating from genomic DNA in addition to the 81 base pair cDNA product. Inspection of the thermodenaturation profile of each QPCR run in combination with random checks on amplified QPCR products by agarose gel electrophoresis confirmed the lack of genomic DNA contamination in both Chinook and Sockeye liver and muscle samples undergoing analysis of mRNA abundance.

Normalization of gene expression data between sexes and sampling sites must be performed for data derived from each tissue type to remove any effects due to differences in sample handling, sample processing, and QPCR performance that may negatively bias data analysis and interpretation. Three candidate genes were examined for their ability to provide data that would be used to create geometric mean values for normalization of the other gene expression data examined. These included *CBA*, *rpL8*, and *GAPDH*. Due to variation in the nature and/or levels of mRNA transcript detected across the data sets, differences in the applicability of these gene targets as QPCR normalization factors in salmonid liver and muscle samples existed. Of the three, *CBA* was the least robust with respect to signal quality and was used following careful scrutiny of the data in the Sockeye tissue analyses as well as the Chinook liver but was not used to normalize the Chinook muscle gene expression data set. This exclusion was due to *CBA* presenting as a doublet thermode-

naturation profile with sample to sample variation on which peak predominated. Expression of the *rpL8* transcript is the least variant between sexes and sampling sites and, therefore, represented the stronger gene for use in data normalization. This gene was used in all data set normalization either with *CBA* or, in the case of Chinook muscle analysis, as the sole normalization factor. *GAPDH* failed to represent an invariant gene expression target and displayed significant changes between the lower Fraser River and spawning ground sampling locations in both salmonid species examined. It was subsequently assessed along with the other gene targets under study. The Ct method was applied to the resultant normalized data sets and fold difference in mRNA abundance determined between the sample groups under study.

For sex genotype analysis, QPCR was performed on each study animal as described above with the following modifications. Primers directed towards the male-specific *OTY2-WSU* genomic sequence (OTY2-f2; CTGGTTCGAGCCTAAGTAG and OTY2-r2; GATGCAGTAGGAGCAGATG) that generate a DNA amplicon of 259–287 base pairs in length in Chinook and Sockeye species (Brunelli and Thorgaard, 2004) were used at 5 pmol each in a 15 μl QPCR reaction containing 2 μl of prepared genomic DNA (40–160 ng/μl). A *GAPDH* QPCR was also performed as an internal quality control on the genomic DNA sample set using the ONQ12 primer pair at 5 pmol each per reaction. This QPCR reaction produces a DNA amplicon of approximately 720 base pairs in length. Both *OTY2* and *GAPDH* QPCR reactions were performed in duplicate for each genomic DNA sample with an annealing temperature of 64 °C. All other reagents used and thermocycle conditions were as described above for determination of mRNA abundance. Genotypic males displayed an *OTY2* signal range of C_t 17–22 while genotypic females showed *OTY2* signals much later at C_t 30–40. Sex genotype data was presented as the difference in C_t value (C_t) between *OTY2* and *GAPDH*.

2.7. QPCR data quality control

The amplified DNA signals for all QPCR reactions performed were assessed for specificity based upon their thermodenaturation profiles. Data that failed to provide a profile indicative of gene target-specific detection were either removed (poor sample quality) or a limit of detection (LOD) applied. Expression profiles of the *CBA* and *rpL8* normalizer genes were used to aid in the identification of poor quality samples which resulted in six out of 308 tissue samples excluded from determination of gene expression profiles while the mRNA abundance profile information for an additional Sockeye salmon (S46) was excluded from male-female group analysis due

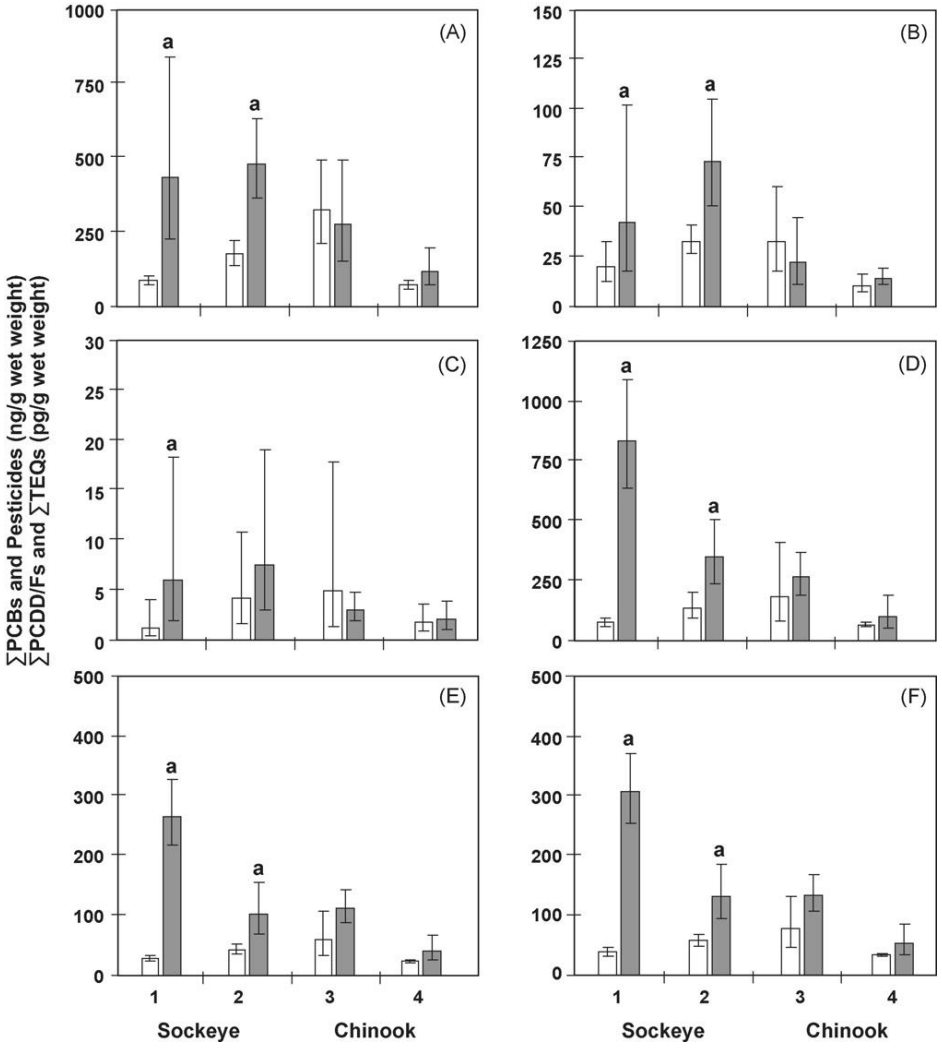

Fig. 2. Concentrations of various environmental contaminants, including (A) ΣPCBs, (B) ΣPCDD/Fs, (C) ΣTEQs, (D) ΣDDTs, (E) ΣChlordanes, (F) ΣCyclodienes, observed in muscle tissue of Sockeye and Chinook salmon collected in the lower Fraser River (white bars) and at their respective spawning grounds (grey bars). Data are expressed on a per wet weight basis. Stock groups include Adams River (1) and Weaver Creek (2) for Sockeye and Shuswap River (3) and Thompson River (4) for Chinook.

to the identification of sex reversal characteristics (confirmed by genotype analysis).

Although all QPCR primer pairs used in the data collection passed critical QC selection criteria, it should be noted that each will display different levels of experimental robustness dependent on the tissue source of the cDNA sample examined and whether sufficient levels of a given gene mRNA exist within that tissue type. Therefore, there are applicable limits of detection (LOD) at which point specific signal is replaced by non-specific signal resulting from QPCR side-reactions. This was evident for *VTG* and *ERα* in liver tissue of both species at the spawning grounds where a LOD of $C_t = 30$ was applied. Thus, the data presented as relative fold change of both sexes between the lower Fraser River and spawning ground sites represents the minimum value expected. In muscle tissue, *VTG*, *VEPΔ*, *ERα*, and *CYP1A* could not be reliably detected and quantified due to low abundance in Chinook salmon, while *VTG* and *VEPΔ* were omitted from the Sockeye muscle data analysis due to the same low abundance issues.

2.8. Data treatment and statistical analyses

Measured contaminant concentration data were reported as geometric means (GM) in units of ng g^{-1} lipid or wet weigth for PCBs, PBDEs and pesticides, pg g^{-1} lipid or wet weight for PCDDs, PCDFs and 2,3,7,8 TCDD toxic equivalents (TEQs) and μg g^{-1} wet weight for Hg and other trace elements. Asymmetric errors were calculated as the range of 1 standard deviation (SD) and 95% confidence limits (CI_{95}). TEQs were determined for individual PCDD/Fs and dioxin-like PCBs using World Health Organization toxic equivalency factors (WHO-TEFs), developed for assessing 2,3,7,8 TCDD toxicity in fish (Van den Berg et al., 1998) (Tables S2 and S3). One-Way Analyses of Variance (ANOVA) and Tukey's HSD comparison tests were performed to evaluate differences between contaminant concentrations in pre-migration (lower Fraser River) and post-migration (upstream spawning grounds) fish. For each tissue type, fold difference mRNA abundance data derived from Sockeye versus Chinook, male versus female, and between site comparisons for each species were initially assessed for normality using the Shapiro–Wilk test using SPSS version 12 software (Chicago, IL, USA). All data sets displayed non-normal distribution and were further evaluated using non-parametric analyses. Kruskal–Wallis test showed significance within each data set and subsequent pairwise analysis were performed using the Mann-Whitney U test with significance set at a 95% confidence.

3. Results

3.1. Contaminant residue concentrations in migrating Pacific salmon

A wide variety of organohalogens and metals were observed in muscle and roe of returning Fraser River Sockeye (Adams River and Weaver Creek runs) and Chinook salmon (Thompson and Shuswap River runs) (Tables S4 and S5). The overall mean ΣPCBs in the Fraser River salmon examined ranged between approximately 75–500 ng g^{-1} lipid in muscle tissue and between 60 and 130 ng g^{-1} lipid in roe. Organochlorine pesticides, including octachlorostyrene, chlorobenzenes, DDTs, HCHs, chlordanes and other cyclodienes (mirex, dieldrin, endosulfans), were detected at concentrations between 1 and 800 ng g^{-1} lipid in muscle and roe. PBDEs, which were only measured in pre-spawning Thompson River Chinook, exhibited comparable concentrations in muscle (mean = 13.4, CI_{95} = 5.35–33.4) and roe (mean = 8.8, CI_{95} = 3.47–22.3).

Organohalogen residue concentrations (ΣPCBs, ΣPCDDs and ΣPCDFs and organochlorine pesticides) in muscle tissue were often significantly higher ($p < 0.05$) in Sockeye at spawning grounds, compared to pre-spawning locations in the lower Fraser River (Fig. 2). Chinook did not show these location differences (Fig. 2). These observations were further supported by the data obtained based upon lipid content (Tables S4 and S5). Moreover, ΣPCBs in Adams River Sockeye at the spawning grounds (mean = 437 ng g^{-1} lipid, CI_{95} = 113–1700) were three times higher than Adams River Sockeye entering the lower Fraser River (mean = 86.8 ng g^{-1} lipid, CI_{95} = 36.6–206). This magnification of hydrophobic contaminants during migration is consistent with previous observations (deBruyn et al., 2004; Kelly et al., 2007), and is attributable to the combination of rapid lipid reserve depletion and slow chemical elimination kinetics in these salmon during this period.

Mean 2,3,7,8 TCDD toxic equivalents (i.e. ΣTEQs, representing the sum of PCB, PCDD and PCDF TEQs) ranged between 0.7 and 4.22 pg g^{-1} lipid in muscle and 0.34 and 1.77 pg g^{-1} lipid in roe for Fraser River salmon. Organohalogen levels were generally higher in Sockeye compared to Chinook salmon (Tables S4 and S5). For example, ΣTEQs (pg g^{-1} lipid) observed in muscle tissue of spawning Adams River Sockeye (mean = 2.83, CI_{95} = 0.40–20.1) and Weaver Creek Sockeye (mean = 4.22, CI_{95} = 0.92–19.4) were significantly higher ($p < 0.05$) compared to ΣTEQs in spawning Thompson River Chinook (mean = 1.0, CI_{95} = 0.31–3.18) and Shuswap River Chinook (mean = 1.47, CI_{95} = 0.41–5.20).

Concentrations of Hg in muscle and roe (range = 0.004–0.07 μg g^{-1} wet weight) were well below Health Canada's human health consumption guidelines (0.5 μg g^{-1} wet weight) (Health Canada, 2007). Similarly, concentrations of As observed in Fraser River salmon tissues (range = 0.05–2.2 μg g^{-1} wet weight) were well below the U.S. Food and Drug Administration action level of 76 μg g^{-1} wet weight (U.S.F.D.A., 2001). Concentrations of other metals of concern, including Pb, Cu, Ni, ranged between 0.1 and 20 μg g^{-1} wet weight (Tables S4 and S5). No significant differences in metal tissue concentrations were apparent between the two salmon species studied. Previous studies have shown Chinook salmon tend to exhibit higher Hg concentrations compared to other Pacific salmon species, due to their relatively high trophic position and longevity (Ikonomou et al., 2007).

3.2. Sex-dependent gene profile differences

We first compared transcript levels of males to females within a species at each of the two collection sites, lower Fraser River and spawning grounds. The two species showed a large degree of similarity in transcript profiles for liver and muscle tissues (Figs. 3 and 4, respectively). This was particularly true of the liver and muscle profiles in animals from the lower Fraser River. The gene expression profiles in liver of both species displayed a much greater overall dynamic range in mRNA levels than that observed in muscle tissue.

Transcripts whose products were involved in egg production were present in greater abundance in female liver compared to males from both species. *VTG* and *VEPΔ* transcripts showed 3–5 orders of magnitude higher levels in females compared to males in either species in the lower Fraser River animals (Fig. 3). This sex difference was maintained at the spawning grounds (Fig. 3). *ERα* mRNA was significantly increased in the liver of both Chinook and Sockeye females from the lower Fraser River compared to their respective males, but this difference was absent at the spawning grounds (Fig. 3). Other gene mRNA targets investigated in liver (*AhR*, *CYP1A*, *MT*, *CAT*, and *GAPDH*) showed reduced levels in both Chinook and Sockeye females compared to their respective males from the lower Fraser River (Fig. 3). At the spawning grounds, these sex differences disappeared for *AhR* and *MT* in both species (Fig. 3).

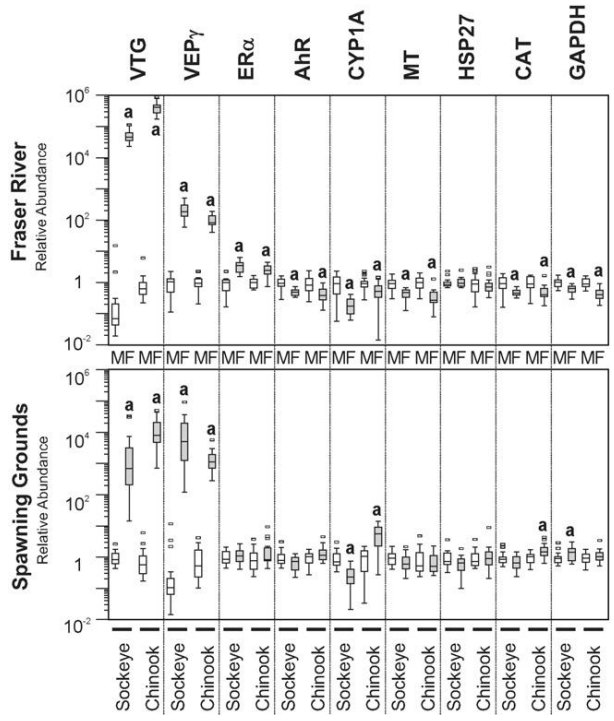

Fig. 3. Comparison of mRNA expression profiles in the liver between males and females within a salmon species and collection site. Specific mRNA abundance levels determined within liver tissue of female salmon were compared to those obtained for male salmon in the lower Fraser River and spawning grounds. For each gene, the Sockeye data are presented first (males (M) with white bars and females (F) with grey bars) followed by the Chinook data (males (M) with white bars and females (F) with grey bars). Data are expressed as the fold difference relative to the males within a species. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$).

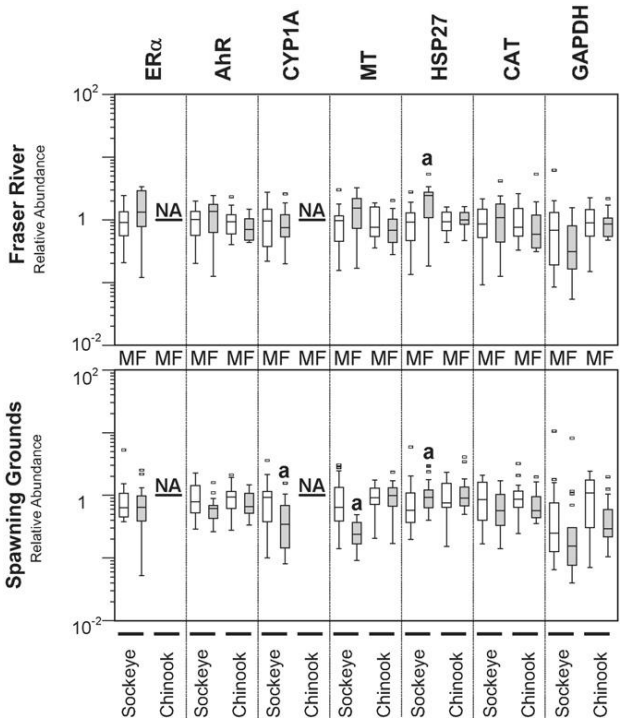

Fig. 4. Comparison of mRNA expression profiles within muscle tissue between males and females within a salmon species and collection site. Specific mRNA abundance levels determined within muscle tissue of female salmon were compared to those obtained for male salmon in the lower Fraser River and spawning grounds. For each gene, the Sockeye data are presented first (males (M) with white bars and females (F) with grey bars) followed by the Chinook data (males (M) with white bars and females (F) with grey bars). Data are expressed as the fold difference relative to the males within a species. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$). NA; no assessment due to gene-specific limit of detection surpassed in Chinook salmon muscle tissue.

CYP1A, *CAT*, and *GAPDH* transcripts showed species differences in the relative relationship of these transcripts between males and females at the spawning grounds. *CYP1A* transcripts in Sockeye were lower in female livers compared to Sockeye male livers whereas Chinook female livers showed higher *CYP1A* transcript levels compared to Chinook male livers (Fig. 3). Sex differences observed in the lower Fraser River for *CAT* transcripts were not evident in Sockeye salmon at the spawning grounds whereas *CAT* transcript levels in Chinook female livers were higher than in male livers at this location. Finally, *GAPDH* transcript levels were elevated in Sockeye female livers compared to male livers of the same species whereas the sex differences observed in Chinook in the lower Fraser River were not apparent at the spawning grounds (Fig. 3).

Transcript levels in muscle tissue showed some sex-related differences in Sockeye although most transcript levels did not differ between sexes at either site (Fig. 4). Females had lower levels of *CYP1A* and *MT* transcripts at the spawning grounds and higher levels of *HSP27* transcripts at both the lower Fraser River and the spawning grounds compared to Sockeye males at the respective sites (Fig. 4). No differences were observed between sexes for Chinook salmon at the lower Fraser River or the spawning grounds (Fig. 4).

3.3. Collection site-associated gene profile differences

The two sexes in both salmonid species underwent a dynamic alteration in the levels of many of the target gene transcripts during migration from the lower Fraser River to the spawning grounds (Figs. 5 and 6). Sockeye salmon showed changes in more transcripts than Chinook in the liver tissue (nine versus four; Fig. 5). In Sockeye, *VTG* and *VEP*Δtranscripts were markedly reduced in the livers of females at the spawning grounds compared to the lower Fraser River (Fig. 5). *VTG* in male salmon liver remained at low, unchanging levels between the two sites (Fig. 5). Livers from both male and female Sockeye showed a significant decrease in *ERα* transcript levels during migration, an increase in stress markers (*MT*, *HSP27*, and *CAT*), and an increase in *GAPDH* transcript levels (Fig. 5). Female Sockeye showed evidence of an enhanced hepatic detoxification response (*AhR* and *CYP1A*) while males only showed an elevation of *AhR* transcripts with invariant levels of *CYP1A* (Fig. 5).

Like in Sockeye, *VTG* transcripts were markedly reduced in the livers of Chinook females at the spawning grounds compared to the lower Fraser River and remained unchanged in Chinook males between these sites (Fig. 5). A decrease in *VEP*Δ transcript levels at the spawning ground compared to the lower Fraser River was also detected in male Chinook. However, Chinook females did not exhibit the change in *VEP*Δ transcripts observed in Sockeye (Fig. 5). A significant decrease in *ERα* transcript levels during migration for each sex was observed similar to that seen in Sockeye (Fig. 5). Chinook females showed a significant increase in *MT* transcripts similar to Sockeye (Fig. 5).

In contrast to Sockeye, the livers of Chinook females showed no change in other stress or detoxification indicators (*AhR*, *CYP1A*, *HSP27*, *CAT*) between sites while Chinook males showed a marked

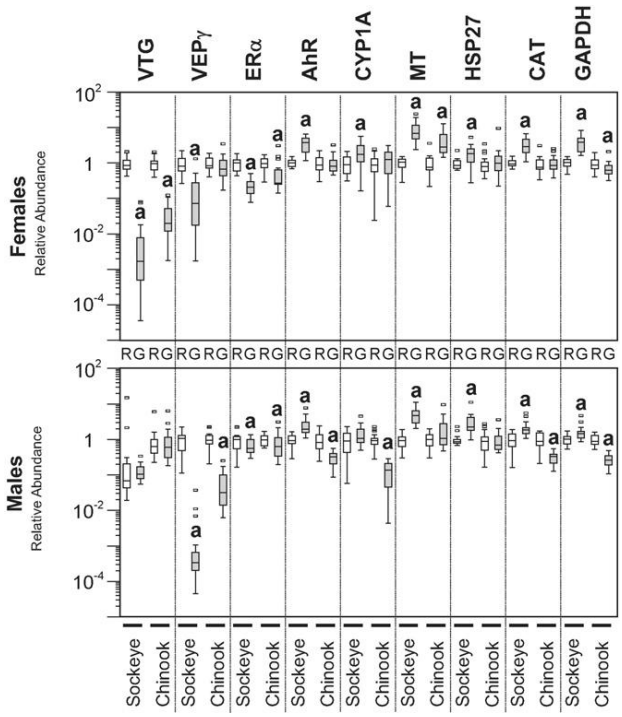

Fig. 5. Comparison of mRNA expression profiles in liver tissue of in-migrating Sockeye and Chinook salmon. Specific mRNA abundance levels determined within liver tissue of each salmon species were compared between the lower Fraser River and spawning grounds. Sexes are shown separately. For each gene, the Sockeye data are presented first from the lower Fraser River (R; white bars) then spawning ground (G; grey bars) followed by the Chinook data (lower Fraser River (R) with white bars then spawning ground (G) with grey bars). Expression data are presented as fold difference relative to lower Fraser River salmon. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$).

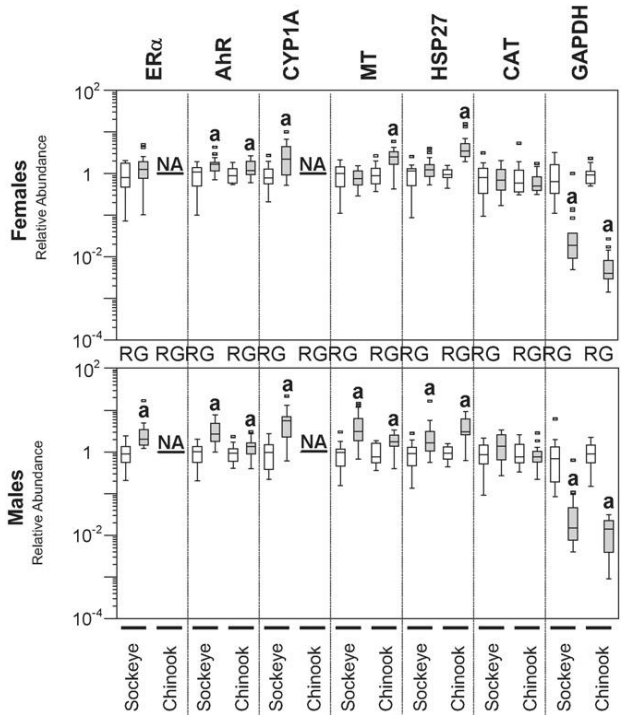

Fig. 6. Comparison of mRNA expression profiles in muscle tissue of in-migrating Sockeye and Chinook salmon. Specific mRNA abundance levels determined within muscle tissue of each salmon species were compared between the lower Fraser River and spawning grounds. Sexes are shown separately. For each gene, the Sockeye data are presented first from the lower Fraser River (R; white bars) then spawning ground (G; grey bars) followed by the Chinook data (lower Fraser River (R) with white bars then spawning ground (G) with grey bars). The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$). NA; no assessment due to gene-specific limit of detection surpassed in Chinook salmon muscle tissue.

decrease in *AhR*, *CYP1A*, and *CAT* transcripts (Fig. 5). *MT* and *HSP27* transcripts were unchanged in male Chinook livers during migration (Fig. 5). *GAPDH* transcript levels decreased significantly in the livers of both sexes of Chinook salmon (Fig. 5).

In muscle tissue, female Sockeye showed an increase in *AhR* and *CYP1A* transcript levels, no change in *CAT* transcripts, and approximately 100-fold decrease in *GAPDH* transcripts during migration (Fig. 6). These levels were also observed in Sockeye males. While no change was observed in *ERα*, *MT*, and *HSP27* transcripts in female Sockeye muscle tissue, male Sockeye muscle tissue showed significant increases in these transcripts (Fig. 6).

Chinook muscle tissue from females and males showed identical expression profiles (Fig. 6) and they will be discussed together. The measurement of *ERα* or *CYP1A* transcripts was not determinable from either sex because the levels were below the limit of detection. The levels of *AhR*, *MT*, and *HSP27* transcripts were significantly elevated in the spawning grounds compared to the lower Fraser River whereas a decrease comparable to that observed in Sockeye was observed for *GAPDH* transcripts and *CAT* transcript levels remained unchanged (Fig. 6).

3.4. Species-dependent differences in mRNA abundance profiles

We then performed an inter-species comparison at each site with males and females separately to ascertain the relative transcript abundances (Figs. 7 and 8). At the lower Fraser River, liver tissue from Sockeye females showed elevated levels of *VEPΔ*, *ERα*, *CYP1A*, and *HSP27* transcripts compared to Chinook females (Fig. 7). *AhR*, *CAT* and *GAPDH* mRNAs were reduced while *VTG* and *MT* transcripts did not differ (Fig. 7).

A substantial change in these relationships were observed at the spawning grounds where *VTG* and *VEPΔ*transcript levels were approximately 10-fold lower in Sockeye compared to Chinook females. Moreover, *ERα* and *AhR* transcript levels were not different between species at the spawning grounds. Like the lower Fraser River, *MT* transcript levels did not differ between species in the female livers. *CYP1A* and *HSP27* transcript levels remained elevated in the livers of Sockeye females compared to Chinook at the spawning grounds (Fig. 7). *CAT* and *GAPDH* transcript levels showed an increase in Sockeye female livers relative to Chinook female livers contrasting the results observed in the lower Fraser River above (Fig. 7).

Livers from Sockeye males did not show differences in the expression of *VTG*, *VEPΔ HSP27* or *CAT* transcripts compared to Chinook males at the lower Fraser River (Fig. 7) whereas *ERα* and *CYP1A* transcripts were elevated in Sockeye relative to Chinook, and *AhR*, *MT*, and *GAPDH* transcripts were reduced (Fig. 7).

This expression profile changed dramatically in males sampled from the spawning grounds. *CYP1A* transcript levels were the only ones that remained elevated in the Sockeye male livers compared to Chinook at the spawning grounds relative to the lower Fraser River (Fig. 7). All other transcript relationships between species changed at the spawning grounds relative to the lower Fraser River. *VTG*, *ERα*, *MT*, *HSP27*, *CAT*, and *GAPDH* transcripts were all elevated in Sockeye male livers compared to Chinook (Fig. 7). *VEPΔ*transcripts

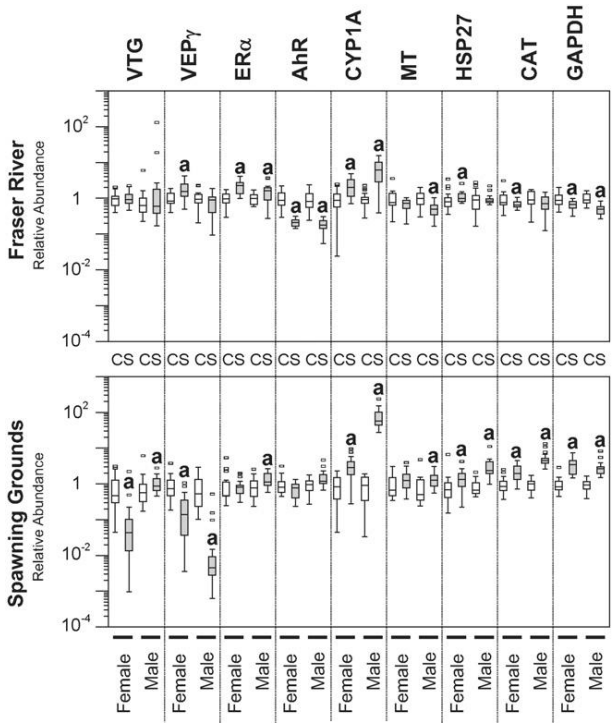

Fig. 7. Comparison of mRNA expression profiles in liver tissue between Sockeye and Chinook salmon at each of the two collection sites. Specific mRNA abundance levels determined within liver tissue of Sockeye salmon were compared to those obtained for Chinook salmon in the lower Fraser River and spawning grounds. For each gene, the female data are presented first (Chinook (C) with white bars and Sockeye (S) with grey bars) followed by the male data (Chinook (C) with white bars and Sockeye (S) with grey bars). Expression data is presented as fold difference relative to Chinook salmon. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$).

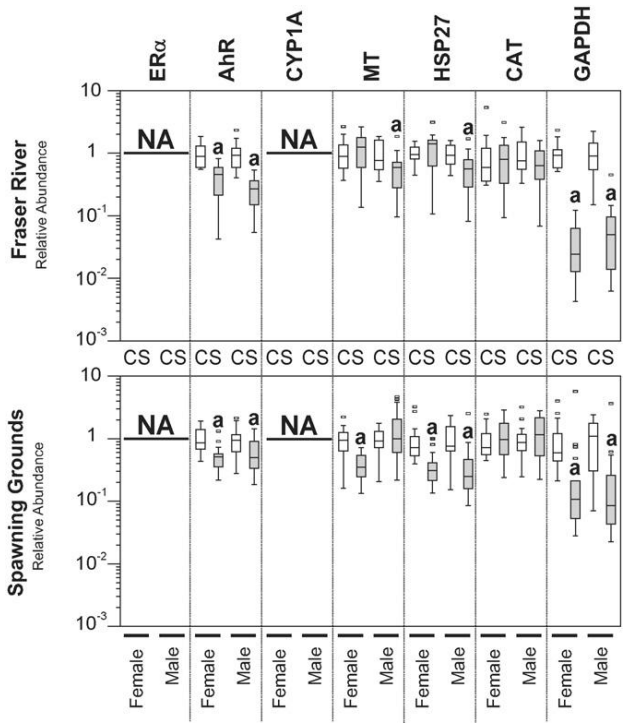

Fig. 8. Comparison of mRNA expression profiles in muscle tissue between Sockeye and Chinook salmon at each of the two collection sites. Specific mRNA abundance levels determined within muscle tissue of Sockeye salmon were compared to those obtained for Chinook salmon in the lower Fraser River and spawning grounds. For each gene, the female data are presented first (Chinook (C) with white bars and Sockeye (S) with grey bars) followed by the male data (Chinook (C) with white bars and Sockeye (S) with grey bars). Expression data is presented as fold difference relative to Chinook salmon. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range with outliers indicated by the small open rectangles. Significant differences are identified by an 'a' ($p < 0.05$). NA; no assessment due to gene-specific limit of detection surpassed in Chinook salmon muscle tissue.

were decreased and *AhR* transcripts did not differ between species (Fig. 7).

The female muscle gene expression profiles comparing species to one another at each site had little overall similarity to the liver gene expression profiles (Fig. 8). *ERα* and *CYP1A* transcript levels were below the limit of detection in muscle tissue. Like the results at the lower Fraser River for female livers, female muscle *AhR* and *GAPDH* transcripts were significantly lower in Sockeye compared to Chinook and *MT* transcripts were not different (Fig. 8). However, here the similarity ended with *HSP27* and *CAT* transcripts showing no difference between species at this site (Fig. 8).

The reduced levels of *AhR* and *GAPDH* transcripts and no difference in *CAT* transcripts were maintained at the spawning grounds (Fig. 8). *MT* and *HSP27* transcripts were reduced in Sockeye female muscle compared to Chinook at this site (Fig. 8).

The male muscle gene expression profiles comparing species to one another at each site showed better concurrence (4/5 transcripts) with the liver results at the lower Fraser River site, but no concurrence at the spawning grounds. *ERα* and *CYP1A* transcript levels were below the limit of detection in muscle tissue. Decreased transcript levels in Sockeye compared to Chinook male muscle were observed for *AhR*, *MT*, *HSP27*, and *GAPDH* at the lower Fraser River site (Fig. 8). *CAT* transcript levels did not differ (Fig. 8).

At the spawning grounds, *AhR*, *HSP27*, and *GAPDH* transcript levels remained lower in Sockeye and *CAT* transcripts remained the same between Sockeye and Chinook male muscle samples (Fig. 8). *MT* transcript levels were found to be the same between these species at the spawning ground (Fig. 8).

3.5. Comparison of mRNA expression profile phenotype with sex genotype

The elevated levels of transcripts associated with the estrogen signalling pathway in Sockeye salmon compared to Chinook suggested that Sockeye may be more sensitive to exposure to xenoestrogens. Following the characterization of mRNA abundance profiles in liver and muscle tissues Sockeye and Chinook study animals, a comparison was made between the sex-associated transcriptome profiles (phenotype) and the genotypic sex of each animal (Fig. 9A). Of the 154 animals investigated, 153 showed a match between the sex phenotype and genotype while a single Sockeye individual (S46) sampled from the lower Fraser River displayed sex reversal (Fig. 9A). This animal contained the *OTY2* male-specific marker (examined in liver and muscle) but produced roe and expressed a distinctive pattern of mRNA expression in liver and muscle tissues reminiscent of, but not identical to, the female expression pattern compared with other Sockeye collected from the Fraser River (Fig. 9B).

4. Discussion

The majority of toxicogenomic work on salmonid species presently available is based upon studies in Atlantic salmon (*Salmo*

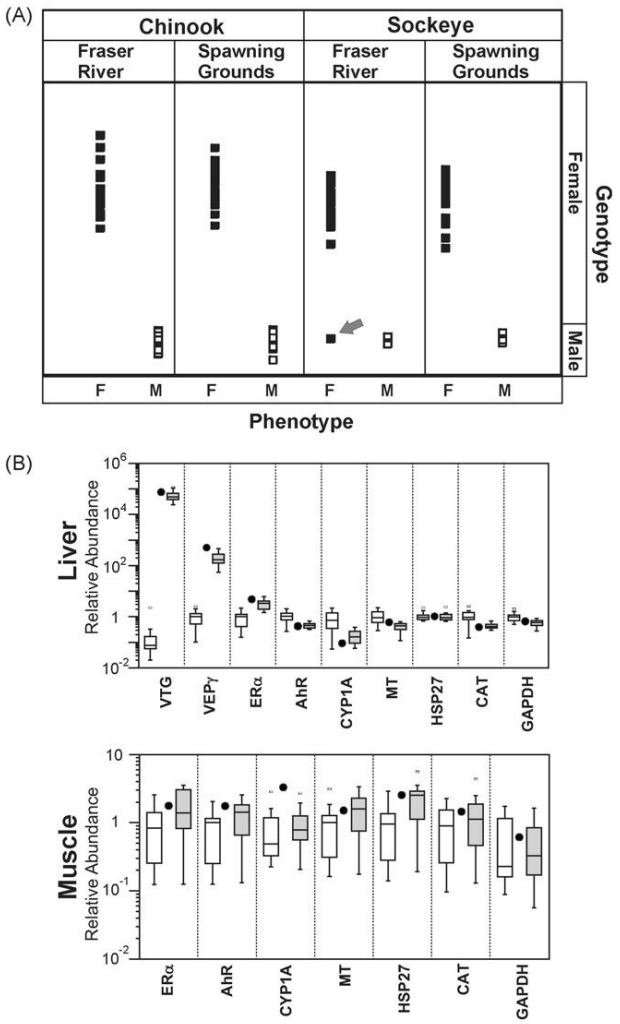

Fig. 9. Comparison of mRNA expression phenotype and male-specific genotype analysis. (A) Chinook and Sockeye salmon collected from the lower Fraser River and upstream spawning grounds ($n = 154$) were sexed based upon expression of the male-specific *OTY2* genomic marker with values representing the difference in cycle threshold (dC_t) value between *OTY2* and the endogenous control *GAPDH* gene marker. Phenotypic females (F) are denoted by closed squares while phenotypic males (M) are represented by open squares. A single sex-reversed Sockeye salmon is shown by a grey arrow. (B) Comparison of average mRNA expression profiles in liver and muscle tissue for female (grey bars) and male (white bars) Sockeye salmon located in the lower Fraser River with that obtained from the individual animal S46 (closed circles). Expression data is presented as relative mRNA abundance. The medians are shown as solid black lines within the box and the box indicates the 25th and 75th percentiles. Whiskers indicate the range.

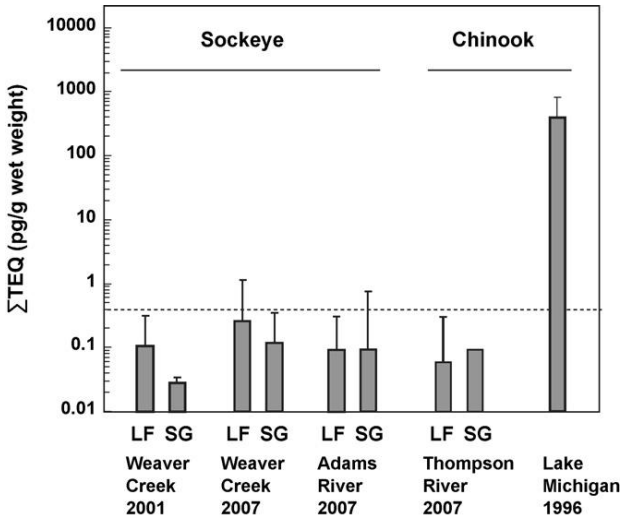

Fig. 10. ΣTEQs in egg from female Sockeye salmon (Adams River and Weaver Creek stock groups) and Chinook salmon (Thompson River stock group) sampled in the lower Fraser River (LF) and at their respective spawning grounds (SG) in 2007. The data are expressed as pg g^{-1} wet weight. Comparative egg ΣTEQ data are shown for Weaver Creek Sockeye sampled in 2001 (deBruyn et al., 2004; Kelly et al., 2007) and for Chinook sampled from Lake Michigan in 1996 (Williams and Giesy, 1992; U.S. Geological Survey, 1999; Hickey et al., 2006). Error bars represent upper bound 95% confidence limits. Horizontal line represents ΣTEQs in salmonid eggs associated with 30% egg mortality (Giesy et al., 2002).

salar) and the non-anadromous Rainbow trout (*O. mykiss*) and has focused on immune function, lipid metabolism, and infectious or chemical agents that may negatively impact fish health. However, comparatively little QPCR-related tools currently exist for Pacific salmon species. This is highlighted by our need to de novo target and clone eight environmentally relevant gene transcripts from both Chinook and Sockeye prior to development and validation of the QPCR assay. In addition, genomics-based analyses have focused mostly on smoltification during outmigration of salmon species while there is a paucity of gene expression profiling information of spawning salmon runs. We were interested in investigating the dynamic changes that occur during migration of Fraser River salmon to their spawning locations relating changes in gene expression with contaminant load.

Observed contaminant profiles of upstream migrating Fraser River salmon revealed the presence of a wide variety of environmental contaminants, including PCBs, PCDD/Fs, PBDE flame retardants, pesticides, as well as Hg, As and many other trace metals. Organohalogen and metal concentrations in salmon muscle were well below human health consumption guidelines. In eggs of spawning females from various Pacific salmon runs, concentrations of 2,3,7,8 TCDD toxic equivalents were close to levels observed to negatively impact egg development and survival (Williams and Giesy, 1992; U.S. Geological Survey, 1999; Hickey et al., 2006) (Fig. 10). Fourteen percent of the Sockeye roe samples measured in 2007 ($n = 14$) exhibited TEQs above the 0.3 pg g^{-1} wet weight TEQ concentration previously shown to cause 30% egg mortality in salmonids (Giesy et al., 2002). None of the Chinook roe samples ($n = 5$) surpassed this limit.

The relatively high TEQs in these Fraser River salmon is primarily due to the presence of 2,3,4,7,8 PeCDF, which typically contributed 50–80% of ΣTEQ burdens in these fish. The data indicate that the degree of accumulation/retention of relatively more toxic PCDD/F congeners (such as 2,3,4,7,8 PeCDF exhibiting high TEFs) in eggs of upstream migrating salmon may be a contributing determinant of reproductive success of Fraser River salmon stocks. The ΣTEQ concentrations identified in Fraser River salmon eggs are substantially lower than previous observations in Chinook from the Lake Michigan (Fig. 10), which may have suffered dioxin-related population declines during the 1980s and 1990s (Niimi, 1983).

With these contaminant loads present during this demanding life stage, we undertook to evaluate the transcriptome in both pre-migration populations and later in salmon that had successfully completed their journey to upstream spawning locations. To our knowledge, this study is the first to examine gene transcript profiles using real-time quantitative PCR analysis of two salmon species traversing the Fraser River during the same migration year. One recent study reported the gene expression profiles of Sockeye white muscle during migration from a 16,006 feature microarray and did extensive comparisons along the migration route of a selected stock (Miller et al., 2009). Like the present study, Miller et al. found

dynamic changes in gene expression during migration but the numbers of males and females varied from site to site and sexes were not separated in their analyses.

Our study suggests that there are distinctive sex-dependent differences in gene expression profiles. We compared the expression profiles of up to nine gene transcripts representing exposure to estrogens, xenobiotics, and stress in the liver and muscle tissues in males to females within each test species at the lower Fraser River. Eight transcripts showed distinctive sex-related differences in the liver and these differences were similar in both species. Muscle tissue showed no sex-dependent differences in Chinook whereas Sockeye females had higher levels of *HSP27* compared to males. With the exception of *VTG* and *VEP*Δ transcripts, the sex differences observed in the hepatic transcripts at the lower Fraser River were no longer evident at the spawning ground. Moreover, the sex-dependent relationship of hepatic *CYP1A*, *CAT*, and *GAPDH* transcripts showed species-specific patterns of change at the spawning ground often revealing a significant difference in transcript levels opposite to those observed at the lower Fraser River. Sex-dependent muscle transcript levels were also different at the spawning grounds compared to the lower Fraser River, but only in Sockeye, not Chinook. These data indicate that sex-dependent differences in gene expression exist in both species and that these relationships are distinctive at the two locations, often in a species-specific fashion. Miller et al. observed two highly differentiated profiles in the Savona sampling location; a freshwater location that was along the Sockeye migration route (Miller et al., 2009). It is highly likely that these profiles correspond to male/female differences. Unfortunately comparison with the spawning ground location (Lower Adams) is not possible, since only females were examined at this location in the Miller study.

The liver and muscle gene expression profiles of both Sockeye and Chinook underwent significant changes during their migration to the spawning grounds, but the nature of the changes were distinctive depending upon the tissue, sex, and species. Sockeye migration was associated with a reduction of egg producing protein transcripts and estrogen receptor in female livers which was recapitulated in male livers (with the exception of *VTG*).

Given that magnification of hydrophobic contaminants during migration was observed in this and other studies (deBruyn et al., 2004; Kelly et al., 2007), we would expect that transcripts encoding detoxification enzymes or transcription factors controlling the expression of those enzymes to increase during migration. *CYP1A* encodes an enzyme that plays a central role in the oxidative metabolism or biotransformation of xenobiotics and is regulated by the aryl hydrocarbon receptor, AhR (Nelson et al., 1996). We found that *CYP1A* and *AhR* transcripts were elevated in muscle samples at the spawning ground. Elevation of these two transcripts was also evident in Sockeye female livers. Male Sockeye livers showed an increase only in *AhR* and not in *CYP1A*. In contrast, no change in these two transcripts was observed in Chinook female livers and a decrease was observed in Chinook male livers. The differences observed between species may be related to contaminant loads and biomagnification observed in the tissues and species (Fig. 2, Tables S4 and S5).

The fact that the levels of these two transcripts do not covary in all cases highlights an important caveat to the QPCR method of analysis of the steady-state level of transcripts. We selected for analysis transcripts known to encode products related to pathways or processes involved in response to metal/chemical exposure and biological stress. Regulation of these responses may include a transcriptional component, but multiple levels of regulation can also be present that involve protein translational modulation and activation of protein function. Our QPCR analyses do not allow us to directly address the potential contributions of these non-transcriptional events.

The QPCR data indicate that migration induced stress responses in the liver within the sexes more strongly in Sockeye than in Chinook. *MT*, *HSP27*, and *CAT* transcripts were all increased at the spawning grounds compared to the lower Fraser River in Sockeye livers. While *MT* transcripts did increase in Chinook livers, the other stress-related transcripts showed no change or decreased. Muscle had a profile distinct from liver in that female Sockeye had no change in these transcripts, whereas male Sockeye and both sexes of Chinook showed elevated levels of *MT* and *HSP27* transcripts but not *CAT*. While we are unable to directly compare the sexes, our observations are consistent with previous observations in Sockeye muscle of an increase in *MT* and *HSP*-related transcripts during maturation and starvation (Salem et al., 2006; Miller et al., 2009).

It is possible that some of the apparent species differences observed at the two sites may be due to different relative transcript levels. Comparison of estrogen signalling transcripts *VTG*, *VEP*Δ and *ERα* do not yield a clear picture of a relationship between responsiveness to migration and relative levels of transcripts for a given species. In liver, Sockeye showed lower *AhR* transcript levels compared to Chinook with concurrently higher *CYP1A* transcript levels in the lower Fraser River. At the spawning ground, Sockeye maintained higher levels of *CYP1A* transcript while exhibiting the same levels of *AhR* transcripts compared to Chinook. The lower levels of *AhR* transcripts in Sockeye were also evident in muscle, but a comparison with *CYP1A* transcripts was not possible in this tissue. The observed relationship between *AhR* and *CYP1A* transcripts in the liver may be somewhat counterintuitive to the expected relationship between these two proteins (AhR up-regulation of the *CYP1A* gene). However, it is possible that the production of AhR protein may be more efficient or AhR protein may be more active in Sockeye tissues. Regardless, these data suggest that Sockeye may be more stressed by xenobiotics than Chinook; a fact supported by contaminant load data and the expression patterns of other stress-related gene transcripts particularly at the spawning grounds.

A sharp reduction in *GAPDH* mRNA levels in muscle of both species during migration is suggestive of changes in bioenergetic metabolism as a consequence of migration. A similar reduction was observed previously in Sockeye white muscle along with evidence of a shift from anaerobic to aerobic carbohydrate metabolism (Miller et al., 2009). Sockeye had lower amounts of *GAPDH* transcripts compared to Chinook tissues with the exception of higher levels at the spawning ground in the liver. This latter observation concurs with the significant increase of hepatic *GAPDH* transcripts observed in both sexes at the spawning grounds compared to the lower Fraser River. It is not clear whether or not the transcript levels correspond directly to the protein and enzyme activities of GAPDH in this tissue. Indeed, there are indications in muscle that these factors do not covary with each other (refer to Miller et al., 2009 for an extensive discussion of this point). The pronounced changes observed with *GAPDH* transcripts may have greater implications than simply those on bioenergetics. In addition to GAPDH's role in glycolysis, recent reports have ascribed an important role of this protein in modulating apoptosis and transcription (Sen et al., 2008).

Measured concentrations of several endocrine disrupting chemicals of concern (PCBs, PCDD/Fs and organochlorine pesticides) were generally higher in Sockeye salmon, compared to Chinook and it is possible that the elevated contaminant burdens apparent in Fraser River Sockeye salmon may contribute to observed gene expression differences in those animals. As many of the gene targets selected were chosen for their ability to indicate exposure to environmental contaminants such as anthropogenic chemical substances or industrial by-products (e.g. heavy metals, pesticides, estrogenic pharmaceuticals or personal care products), it is tempting to ascribe a simple cause-effect relationship for the stocks examined. However, it must be noted that, although there is general

concordance between exposure to contaminants and significant alterations in mRNA abundance, the normal biological stress effects of migration, late life stage, and reproduction cannot be excluded as being contributory factors towards the observed gene profiles.

Sex reversal in wild salmon populations is of growing concern within the Pacific Northwest and it is interesting to observe only a single sex-reversed Sockeye originating from the Mitchell River stock group within the salmon examined (Sockeye $n = 76$; Chinook $n = 78$). The low frequency of sex reversal observed in Fraser River salmon stocks was in sharp contrast to evidence obtained from the late wild Chinook runs of the Columbia River in 1999 where 84% of Hanford Reach stock phenotypic females ($n = 50$) carried genomic markers for the male Y-chromosome (Nagler et al., 2001). Genetically similar fish located at a nearby hatchery with a well water source were not found to display significant levels of sex reversal suggesting river-associated environmental factors, such as temperature fluctuations or contaminant exposure, may contribute to altered sex determination and feminization of this Chinook stock during early development. Contaminant exposure in estuarine juvenile Chinook salmon in the Columbia River was found to occur at significantly high levels and a number of the chemicals present have been shown to display estrogenic activity in a Rainbow trout (*Oncorhynchus mykiss*)-based bioassay (Petit et al., 1997; Johnson et al., 2007). Similar factors that contribute to sex reversal or lesser aspects of feminization within the male population do not appear to be present to a great extent in the late run Sockeye and Chinook salmon stocks examined in the Fraser River watershed in the present study. This is supported by the absence of increased estrogen-regulated gene transcript targets in male liver following migration (see Fig. 5).

Contaminant and gene expression profiles indicated that inmigrating Sockeye salmon were experiencing a greater degree of biological stress than Chinook salmon in the same migration year. However, the changes experienced as a result of the migration process were similar between species reflective of a shared biological process. Many of the mRNA transcripts evaluated in the present study provide an additional gene expression endpoint in the determination of physiological fitness and reproductive status of Pacific salmon stocks. Information related to the 2007 migration provide benchmark data from which longitudinal analyses can be established which will help further discriminate between effects related to normal biology and those associated with a deleterious change in the aquatic environment within the Fraser River watershed. Continued longitudinal assessment of Pacific salmon stocks will help elucidate the causal nature of contaminant burden on modulation of gene expression and subsequent migratory and reproductive success in the Fraser River watershed.

Acknowledgements

We greatly appreciate the efforts of D. Patterson from DFO and the staff of Environmental Watch Program for their expertise in salmon migration timing and sampling logistics. We also thank those who were involved with the sampling logistics: A. Baker, Albion Test Fishery; P. Matthew, Secwepemc Nation Tribal Council; L. Antone, Kwantlen First Nation; J. Pooley, Adam's Lake Band; I. Alexander, Spallumcheen First Nation; R. Bailey, DFO Escapement Assessment; L. Stephan, Shuswap Falls Hatchery; G. Zwack, DFO Escapement Assessment; and the Siska Traditions Society Fish Wheel Staff. Many thanks to all the chemists, technicians, and co-op students of the DFO Regional Contaminants Laboratory, located at the Institute of Ocean Sciences, for the contaminant analyses. We are grateful to DFO management for providing the opportunity to conduct this project in collaboration with Health Canada and the Siska Traditions Society, Nlaka'pamux First Nation. The authors gratefully acknowledge funding support to the Siska Traditions Society, Nlaka'pamux First Nation through the National First Nations Environmental Contaminants program (Health Canada). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.09.009.

References

Beacham, T.D., Candy, J.R., Supernault, K.J., Wetklo, M., Deagle, B., Labaree, K., Irvine, J.R., Miller, K.M., Nelson, R.J., Withler, R.E., 2003. Evaluation and application of microsatellites for population identification of Fraser River chinook salmon (*Oncorhynchus tshawytscha*). Fish. Bull. 101, 243–259.

Beacham, T.D., Lapointe, M., Candy, J.R., McIntosh, B., MacConnachie, C., Tabata, A., Kaukinen, K., Deng, L., Miller, K.M., Withler, R.E., 2004. Stock identification of Fraser River sockeye salmon (*Oncorhynchus nerka*) using microsatellites and major histocompatibility complex variation. Trans. Am. Fish. Soc. 133, 1106–1126.

Brunelli, J.P., Thorgaard, G.H., 2004. A new Y-chromosome-specific marker for Pacific salmon. Trans. Am. Fish. Soc. 133, 1247–1253.

Canadian Heritage Rivers System, 2009. Fraser River, Retrieved 15 April, 2009.

deBruyn, A.M., Ikonomou, M.G., Gobas, F.A., 2004. Magnification and toxicity of PCBs, PCDDs, and PCDFs in upriver-migrating Pacific salmon. Environ. Sci. Technol. 38, 6217–6224.

Ewald, G., Larsson, P., Linge, H., Okla, L., Szarzi, N., 1998. Biotransport of organic pollutants to an inland Alaska lake by migrating sockeye salmon (*Oncorhynchus nerka*). Arctic 51, 40–47.

Giesy, J.P., Jones, P.D., Kannan, K., Newsted, J.L., Tillitt, D.E., Williams, L.L., 2002. Effects of chronic dietary exposure to environmentally relevant concentrations to 2,3,7,8-tetrachlorodibenzo-p-dioxin on survival, growth, reproduction and biochemical responses of female rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 59, 35–53.

Groot, C., Margolis, L., 1991. In: Groot, C., Margolis, L. (Eds.), Pacific Salmon Life Histories. UBC Press, Vancouver.

Guiney, P.D., Melancon Jr., M.J., Lech, J.J., Peterson, R.E., 1979. Effects of egg and sperm maturation and spawning on the distribution and elimination of a polychlorinated biphenyl in rainbow trout (*Salmo gairdneri*). Toxicol. Appl. Pharmacol. 47, 261–272.

Health Canada, 2007. Human Health Risk Assessment of Mercury in Fish and Health Benefits of Fish Consumption. Technical Report, Ottawa, ON. H164-54/2007E, http://www.hc-sc.gc.ca/fn-an/pubs/mercur/merc_fish_poisson-eng.php.

Hickey, J.P., Batterman, S.A., Chernyak, S.M., 2006. Trends of chlorinated organic contaminants in great lakes trout and walleye from 1970 to 1998. Arch. Environ. Contam. Toxicol. 50, 97–110.

Ikonomou, M.G., Fraser, T.L., Crewe, N.F., Fischer, M.B., Rogers, I.H., He, T., Sather, P.J., Lamb, R.L.A., 2001. A comprehensive multiresidue ultra-trace analytical method, based on HRGC/HRMS, for the determination of PCDDs, PCDFs, PCBs, PBDEs, PCDEs, and organochlorine pesticides in six different environmental matrices. Can. Tech. Rep. Fish. Aquat. Sci. 2389, vii+95.

Ikonomou, M.G., Higgs, D.A., Gibbs, M., Oakes, J., Skura, B., McKinley, S., Balfry, S.K., Jones, S., Withler, R., Dubetz, C., 2007. Flesh quality of market-size farmed and wild British Columbia salmon. Environ. Sci. Technol. 41, 437–443.

Johnson, L.L., Ylitalo, G.M., Arkoosh, M.R., Kagley, A.N., Stafford, C., Bolton, J.L., Buzitis, J., Anulacion, B.F., Collier, T.K., 2007. Contaminant exposure in outmigrant juvenile salmon from Pacific Northwest estuaries of the United States. Environ. Monit. Assess. 124, 167–194.

Kelly, B.C., Gray, S.L., Ikonomou, M.G., Macdonald, J.S., Bandiera, S.M., Hrycay, E.G., 2007. Lipid reserve dynamics and magnification of persistent organic pollutants in spawning sockeye salmon (*Oncorhynchus nerka*) from the Fraser River, British Columbia. Environ. Sci. Technol. 41, 3083–3089.

Miller, K.M., Schulze, A.D., Ginther, N., Li, S.R., Patterson, D.A., Farrell, A.P., Hinch, S.G., 2009. Salmon spawning migration: metabolic shifts and environmental triggers. Comp. Biochem. Physiol. D: Genom. Proteom. 4, 75–89.

Miller, M.A., 1993. Maternal transfer of organochlorine compounds in salmonids to their eggs. Can. J. Fish. Aquat. Sci. 50, 1405–1413.

Moore, A., Waring, C.P., 1996. Sublethal effects of the pesticide Diazinon on olfactory function in mature Atlantic salmon. J. Fish Biol. 48, 758–775.

Nagler, J.J., Bouma, J., Thorgaard, G.H., Dauble, D.D., 2001. High incidence of a male-specific genetic marker in phenotypic female chinook salmon from the Columbia River. Environ. Health Perspect. 109, 67–69.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6, 1–42.

Niimi, A.J., 1983. Biological and toxicological effects of environmental contaminants in fish and their eggs. Can. J. Fish. Aquat. Sci. 40, 306–312.

Pacific Salmon Commission, 2001. Executive summaries of 2001 studies on potential causes of early upstream migration of late-run Fraser River Sockeye salmon. http://www.psc.org/info_laterunsockeye.htm.

Petit, F., Le Goff, P., Cravedi, J.P., Valotaire, Y., Pakdel, F., 1997. Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. J. Mol. Endocrinol. 19, 321–335.

Salem, M., Kenney, P.B., Rexroad, C.E., Yao, J., 2006. Molecular characterization of muscle atrophy and proteolysis associated with spawning in rainbow trout. Comp. Biochem. Physiol. D: Genom. Proteom. 1, 227–237.

Sen, N., Hara, M.R., Kornberg, M.D., Cascio, M.B., Bae, B.I., Shahani, N., Thomas, B., Dawson, T.M., Dawson, V.L., Snyder, S.H., Sawa, A., 2008. Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. Nat. Cell Biol. 10, 866–873.

United States Environmental Protection Agency, 1986. United States Environmental Protection Agency Method 6020: Inductively Coupled Plasma-Mass Spectrometry. SW-846. National Technical Information Service, Springfield, VA.

United States Food and Drug Administration, 2001. Fish and Fishery Products Hazards and Controls Guide, 3rd ed. Food and Drug Administration, Washington, DC.

United States Geological Survey, 1999. Lake Trout Early Life Stage Mortality: interactions of the nutrient thiamine and dioxin-like PCBs and their mixtures mound in Green Bay. Final Report of Interagency Agreement.

Van den Berg, M., Birnbaum, L., Bosveld, A.T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X., Liem, A.K., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Perspect. 106, 775–792.

Walker, M.K., Peterson, R.E., 1991. Potencies of polychlorinated dibenzo-p-dioxin, dibenzofuran, and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin, for producing early life stage mortality in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 21, 219–238.

Williams, L.L., Giesy, J.P., 1992. Relationships among concentrations of individual PCB congeners, 2,3,7,8-TCDD equivalents and rearing mortality of Chinook salmon (Oncorhynchus tshawytscha) eggs from Lake Michigan. J. Great Lakes Res. 18, 108–124.

Zitko, V., Saunders, R.L., 1979. Effect of PCBs and other organochlorine compounds on the hatchability of Atlantic salmon (*Salmo salar*) eggs. Bull. Environ. Contam. Toxicol. 21, 125–130.