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Proteomic analysis of sockeye salmon serum as a tool for biomarker discovery and new insight into the sublethal toxicity of diluted bitumen

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ABSTRACT

Pipelines carrying diluted bitumen (dilbit) from Canada's oil sands traverse North America, including the freshwater habitat of Pacific salmon, posing a risk of environmental release and aquatic exposure. Swimming performance is impacted in juvenile sockeye (*Oncorhynchus nerka*) exposed to dilbit; therefore biomarkers of dilbit exposure will be valuable for monitoring at-risk salmon stocks. This study characterized changes in the serum proteome of sockeye exposed to a sub-lethal and environmentally relevant concentration of dilbit using isobaric tags for relative and absolute quantitation (iTRAQ), and included a range of experimental conditions to permit identification of biomarkers that are robust across time (1 and 4 wk) and exercise level (at rest and following a swim test). Over 500 proteins were identified and quantified in sockeye serum, with dilbit exposure significantly altering the abundance of 24 proteins irrespective of time and exercise, including proteins associated with immune and inflammatory responses, coagulation, and iron homeostasis. An increase in creatine kinase (CK) activity in serum of dilbit-exposed salmon confirmed the higher CK protein abundance measured using iTRAQ. The combination of 4 wk dilbit exposure and a swim test had a greater effect on the serum proteome than either treatment alone, including a marked increase in tissue leakage proteins, suggesting that aerobic exercise exacerbates the serum proteome response to dilbit, and the increased cellular damage could impede exercise recovery. This study provides a foundation for the development of bio-monitoring tools for salmon stock assessments, and offers new insights into the sub-lethal toxicity of crude oil exposure in fish.

1. Introduction

Pacific salmon including sockeye (*Oncorhynchus nerka*) are of major cultural, economic, and ecological significance in the Northwest coast of North America (Gende et al., 2002; Levy, 2009; Schindler et al., 2003). Salmon have a biphasic anadromous lifecycle that includes a rearing phase in coastal freshwater lakes and streams, and a multi-year maturation phase in the Pacific Ocean (Hinch et al., 2006). These two life stages are connected by long and challenging migrations that culminate in a single lifetime spawning event (Burgner, 2003). However, approximately 40% of the sockeye populations currently monitored by the Canadian government are of conservation concern (Fisheries and Oceans Canada, 2016). The unpredicted and historically low sockeye return in 2009 (McKinnell et al., 2012) highlights the sensitivity of sockeye stocks to many factors including anthropogenic stressors (*e.g.* habitat encroachment; pollution), pathogen outbreaks, and high predation rates during outmigration (Cohen, 2012), underscoring the need for early action on issues that impact salmon populations. In particular, spawning escapement is a major causative factor contributing to future sockeye returns (Henderson and Graham, 1991); therefore issues that directly affect juvenile salmon are a high priority concern. Among such issues are increases in the transport of crude oil products through salmon bearing watersheds, including the Fraser River, which contributes the majority of annual sockeye production in Canada (Henderson and Graham, 1991).

Bitumen is a heavy type of crude oil found in rich supply in the oil sands deposits in the Western Canada Sedimentary Basin. Extracted bitumen is mixed with natural gas condensate or synthetic oils to reduce viscosity for ease of transport, and the diluted product (dilbit) is transported by rail and pipeline across North America for refinement and sale on global markets. Impending pipeline expansion to ports on the Pacific coast of North America will triple the volume of dilbit

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carried through salmon habitat and increase the risk of environmental release and exposure of juvenile salmonids (Levy, 2009). At present there is a paucity of information regarding the fate of spilled dilbit in freshwater ecosystems and on species-specific sensitivities to sub-lethal exposures (Dew et al., 2015). Japanese medaka (*Oryzias latipes*; Madison et al., 2015) and zebrafish (*Danio rerio*; Philibert et al., 2016) exposed to dilbit during ontogeny develop deformities consistent with those observed in other fish species exposed to conventional crude oils (Collier et al., 2014), including pericardial edema. It was recently shown that cardiac morphology is altered and swimming performance is impacted in juvenile sockeye exposed to the dissolved fraction of dilbit (Alderman et al., 2017). This adds to a growing body of evidence that crude oils are cardiotoxic in fish (Incardona et al., 2011) and suggests that there is potential for reduced migratory success in exposed juveniles. Accordingly, establishing bio-indicators of dilbit exposure will be valuable for current and future monitoring of at-risk salmon stocks especially if they can be used in large-scale, non-lethal sampling initiatives.

Adult sockeye salmon are regularly biopsied and radio-tagged to follow their migration (Cooke et al., 2012; Donaldson et al., 2013; Farrell et al., 2001a, 2001b; Jeffries et al., 2012). The tissue and plasma samples have provided valuable information on their genotype, tissue specific gene expression, and physiological status, and in some cases these have been used to predict survival (Donaldson et al., 2010; Miller et al., 2011). In the meantime, the use of "omics" technologies in environmental toxicology and ecosystem health management is gaining momentum (Martyniuk and Simmons, 2016; Simmons et al., 2015), and the comprehensive data sets gained from such approaches are ideal for biomarker discovery and can inform us in novel ways about the sublethal effects of toxicants (Martyniuk et al., 2012a, 2012b). Thus an in-depth proteomic analysis of serum samples from dilbit-exposed sockeye is an important first step towards the development of biomonitoring tools for population assessments in the event of accidental dilbit release into salmon habitat.

The major objective of the present study was to determine whether or not the serum proteome of dilbit-exposed sockeye can be distinguished from unexposed fish, and if so, to identify candidate biomarkers of dilbit exposure. Importantly, many internal and external factors can influence the serum proteome of a fish, and in a field scenario it is nearly impossible to know the pre-capture conditions that could be simultaneously affecting blood proteins along with environmental exposure to dilbit. Therefore, this study incorporated serum samples from a complex experimental background to identify robust changes in the serum proteome induced by a controlled exposure to an environmentally-relevant concentration of dilbit that were independent of time (1 or 4 wk dilbit exposure) and exercise (fish sampled at rest or following exhaustive swimming). In addition, since we observed reduced performance in a critical swimming speed test in the sockeye exposed to dilbit for 4 wk (Alderman et al., 2017), a second objective of this study was to identify potential toxic mechanisms by which swimming performance is compromised following dilbit exposure in fish.

2. Methods

2.1. Animals and experimental design

This study used archived serum samples from a previous experiment that characterized the consequence of dilbit exposure on the swimming performance and cardiac morphology of sockeye salmon (Alderman et al., 2017). Briefly, 1-year old sexually immature sockeye salmon (*O. nerka*; $n = 96$, average mass 99.0 ± 3.3 g, average fork length 20.7 ± 0.2 cm) were distributed among 8 experimental tanks ($n = 12$ per tank) supplied with either clean water (control, C), or the water-soluble fraction of dilbit (DB) at an initial total PAH (TPAH) concentration of 66.7 μg/l (sum of 75 individual PAH). Dilbit was not replenished during the exposure period; therefore initial TPAH declined by approximately 60% during the first week and then remained relatively stable (Fig. S1). After either 1 wk or 4 wk of dilbit exposure, 6 fish per tank were removed. Half of the fish were immediately sacrificed as described below (no swim; NS), and the other half were exercised (swim; S) using a standard ramp critical swimming speed test (Jain et al., 1997). The swim tests were approximately 2.5 h in duration and included a 45-min acclimation period at low water velocity, and 20 min intervals at defined velocity increments until the fish became physiologically fatigued, at which point they were euthanized as described below. Thus the experimental design included 3 factors each with 2 levels: exposure (Control *vs* DilBit), time (1 wk *vs* 4 wk), and exercise (No Swim *vs* Swim), resulting in 8 treatment groups each with 12 individual fish. Care and use of animals was approved by the Simon Fraser University Animal Care Committee, according to the guidelines of the Canadian Council for Animal Care.

2.2. Serum

Free-flowing blood was collected from severed caudal vessels immediately after euthanasia in a 2-phenoxyethanol overdose. It is worth noting that euthanasia was necessary to satisfy the aims of our previous study (Alderman et al., 2017), but would not otherwise have been required to collect a useable volume of blood by caudal puncture in these fish. Other tissues were also harvested at this time, including skeletal muscle and head kidney, and either snap frozen or minced and preserved in RNAlater (Life Technologies, Carlsbad, CA) before storing at − 80 °C. Blood was allowed to clot at ambient temperature (11 °C) for 1 h. Serum was separated by centrifugation and then immediately snap-frozen and stored at − 80 °C. Serum was chosen over plasma because it is collected without prior preparation of syringes and collection vials with anti-coagulants (ex. EDTA), nor does it require immediate centrifugation and freezing, and thus lends itself well to field applications. Total protein concentration was quantified in serum samples thawed on ice using the Pierce BCA Protein Assay Kit (Thermo-Fisher, Whitby, ON) and bovine serum albumin as a standard.

2.3. iTRAQ labeling

In keeping with the primary objective of this study, and given the technical constraint of an 8-plex iTRAQ kit, labeling reactions were assigned to permit maximum treatment diversity within the main variable of exposure (dilbit *vs* control; $n = 4$) while maintaining substantial biological input. To accomplish this, an equal amount of total protein from the serum of all fish within a treatment ($n = 12$) was pooled under the assumption of biological averaging given the high number of individuals represented within each pool (Kendziorski et al., 2005). Approximately 500 μg total protein from each of the 8 pooled sera samples was diluted 20-fold in SDS buffer (4% *w/v* SDS, 100 mM HEPES, 0.1 M DTT, pH 7.6) containing 1 × protease inhibitor (Roche, Mississauga, ON) and incubated for 30 min at room temperature. Proteins were precipitated using the Calbiochem Protein Precipitation Kit (EMD Millipore, Billerica, MA) according to the manufacturer protocols. The protein pellet was dissolved in HEPES buffer (1 M HEPES, 8 M urea, 2 M thiourea, 4% CHAPS *w/v*; pH 8.5) and re-quantified. For each sample, 200 μg of protein was transferred to a pre-equilibrated Amicon centrifugation filter and washed 3 times with UA buffer (8 M urea in 0.1 M HEPES, pH 8.5; EMD Millipore). Samples were then incubated for 30 min in the dark with UA buffer containing 0.05 M of iodoacetamide (IAA, Sigma-Aldrich, Oakville, ON). Following incubation with IAA, samples were washed 3 times with 0.5 M of triethylammonium bicarbonate (TEAB, Sigma-Aldrich). Sequence-modified trypsin (Thermo-Fisher) was dissolved in 0.5 M TEAB and was added to each sample at a 1:50 enzyme:protein ratio. Samples were digested with trypsin overnight (approximately 18 h) at 37 °C. Digested peptides were labeled for 2 h at room temperature with an 8-plex

iTRAQ kit (Thermo-Fisher), as outlined in the manufacturer's protocol; all labeled samples were then pooled and purified through a C18 column (Sigma-Aldrich). Labeled peptides were eluted with 70% acetonitrile and 0.1% formic acid.

2.4. Mass spectrometry and protein identification

Mass spectrometry was carried out at SickKids Proteomics Analysis Robotics & Chemical Biology Centre (SPARC BioCentre; Toronto, ON). Peptides were loaded onto a 50 cm × 75 μm ID column with RSLC 2 μm C18 resin (EASY-Spray, Thermo-Fisher) with an integrated emitter. The Easy-Spray nLC 1000 chromatography system was used to elute peptides onto a Q-Exactive hybrid mass spectrometer (Thermo-Fisher) using a solvent gradient (0 to 35% acetonitrile in 0.1% formic acid) over 4 h. The mass spectrometer was operated in the data-dependent mode with 1 MS followed by 10 MS/MS spectra. Resolution of MS scans was either 70,000 (MS) or 17,500 (MS/MS) FWHM, with a target of 1×10^6 ions and maximum scan time of 120 ms. A relative collision energy of 27% was used for MS/MS. First mass was fixed at 80 Da with a dynamic exclusion of 15 s for MS/MS scans. Raw data files were acquired with XCalibur 2.2.

Identification and quantification of serum proteins was performed by PEAKS Studio 7 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). The mass spectra were searched against the ray-finned fishes (*Actinopterygii*) NCBI non-redundant protein database with iTRAQ as a fixed modification on N-termini and lysine residues, and carbamidomethylation as a variable modification as this can be induced by high molar urea incubations. PEAKS database and *de novo* sequencing parameters allowed up to one missed trypsin cleavages, 10.0 ppm parent mass error tolerance and 0.02 Da fragment mass error tolerance. A high confidence in the peptide-protein identifications was assured by using a false detection rate (FDR) cut-off of 1% and by requiring at least two unique peptides for protein identification. Only proteins with reporter ions detected from all 8 iTRAQ labels were used in the analysis, and unnamed proteins were identified using Blast2GO (Conesa et al., 2005). Protein quantification by PEAKS is calculated from the centralized peak intensities for reporter ion intensities of all proteotypic peptides with a significant peptide-spectrum match ($p < 0.01$), with outliers removed prior to protein abundance calculation (Lacerda et al., 2008).

2.5. Creatine kinase activity

Since iTRAQ studies tend to underestimate the fold-change in protein abundance (Mahoney et al., 2011), and because serum CK enzyme activity is a widely available diagnostic tool that does not require species-specificity or antibody-based quantification, serum CK enzyme activity was directly quantified in unpooled sera samples to cross-validate the near-significant increase in CK protein abundance detected using iTRAQ. Total creatine kinase (CK) activity in sera of 4 wk exposed fish was quantified at the Animal Health Laboratories (University of Guelph, Guelph, ON) using the CKL assay kit and Cobas 6000 c501 biochemistry analyzer (Roche Diagnostics, Indianapolis, IN) following standard procedures. A total of 42 sera samples were analyzed, representing nearly all fish in the 4 wk time category (3 samples each from control and dilbit-exposed fish were removed due to insufficient serum volume; therefore $n = 10–11$ per concentration × exercise treatment). The assay detection range is 7–20,000 U/l.

2.6. Reverse transcription quantitative PCR

For a post-hoc validation of a cellular response to the dilbit exposure in skeletal muscle and head kidney, the transcript abundance of cytochrome P450 1a (*cyp1a*) was quantified by RT-qPCR as previously described (Alderman et al., 2017). Briefly, frozen tissue pieces were homogenized in Trizol (Life Technologies) using a Precellys 24 (Bertin Instruments, Markham, ON) with 2 × 25 s cycles, and then cleared by centrifugation ($n = 8$ for muscle, 1 wk only as all 4 wk muscle samples were lost; $n = 6$ for head kidney, 1 wk and 4 wk samples). Total RNA was extracted from the supernatant using chloroform and precipitated with isopropanol, and then used to synthesize cDNA with the High Capacity cDNA Synthesis Kit (Life Technologies) following standard procedures. The transcript abundances of *cyp1a* and the reference gene ribosomal protein L8 (*rpl8*) were measured in duplicate by RT-qPCR using gene specific primers exactly as previously described (Alderman et al., 2017). The quantification cycle (Cq) of each reaction was determined by default settings and was used to calculate transcript abundances from standard curves generated from serially diluted cDNA. The transcript abundance of *cyp1a* was standardized to the abundance of *rpl8*, and data was normalized to the mean expression in control fish at 1 wk for each tissue.

2.7. Statistical analyses

Differences in creatine kinase activity in serum samples were detected, following log2 data transformation, with a two-way ANOVA (concentration × exercise) and a Bonferroni post-hoc test ($n = 10–11$, $p < 0.05$). A difference in muscle *cyp1a* abundance between control and dilbit exposed fish was determined by a one-tailed *t*-test ($n = 8$, $p < 0.05$). Differences in head kidney *cyp1a* abundance were assessed using a two-way ANOVA (concentration × time; $n = 6$), but separate post-hoc pairwise comparisons were not pursued ($p > 0.05$).

The reporter ion intensity values across the 8 iTRAQ labels were globally normalized using variance stabilization within the 'VSN' bioconductor package in the R statistical interface (Huber et al., 2002). To address our main objective, proteins that were significantly altered by dilbit exposure (irrespective of the level of time or exercise) were identified by grouping the VSN normalized abundance values within the single main factor, concentration (control and dilbit), such that each group contained 4 data points (1 wk, 4 wk, no swim, swim) for each identified protein. A univariate receiver operator characteristics (ROC) curve analysis was then conducted within Metaboanalyst (http://www.metaboanalyst.ca), to help determine which proteins could distinguish between dilbit-exposed fish *vs* control fish based on relative abundance. Significant differences in protein abundances were determined with a Student's *t*-test (two-tailed; $p < 0.05$). An ROC curve assesses the sensitivity (true positive classification) and specificity (true negative classification) of a biomarker throughout the range of the response variable, such as protein abundance (Xia et al., 2015). The overall performance of a biomarker is reflected by the area under the ROC curve (AUC), with a value of 1.0 indicating complete separation of groups (100% sensitivity and specificity).

A separate analysis was conducted on ungrouped VSN normalized data to address the second objective of identifying potential toxic mechanisms by which dilbit exposure impairs swimming performance. Pairwise comparisons (fold-change) between experimental groups were used to isolate interaction effects among the main factors (concentration × exercise × time). An outlier test ('Significance A') was then executed in Perseus software (Tyanova et al., 2016) to identify differentially abundant proteins (< 0.54 or > 1.5 fold change; Benjamini-Hochberg FDR $p < 0.05$) within each pairwise comparison. For completeness, all relevant pairwise comparisons were analyzed (Supplemental Data Table S1); however, since swimming performance was only impaired at 4 wk in these fish (Alderman et al., 2017), only results from the 4 wk comparisons are considered herein. Importantly, interpretation of this analysis relies on the assumption of biological averaging from pooled samples of a large number of individuals (Kendziorski et al., 2005), and is included here more to prompt future studies than to offer concrete conclusions. A z-score heat map was generated to visualize the interaction of concentration and exercise at 4 wk on the serum proteome using complete-link hierarchical clustering within the open source InfernoRDN statistical software analysis

(Polpitiya et al., 2008).

3. Results

3.1. Characterization of the sockeye serum proteome

A total of 513 proteins were quantified by iTRAQ in all 8 sera pools (Supplemental Data Table S1). Among these proteins, 188 were "unnamed" or "uncharacterized" protein entries in NCBI, accounting for 37% of the quantified portion of the proteome. All but 5 of these unnamed entries were successfully identified in Blast2GO (Supplemental Data Table S1), and are used throughout the main text and tables. The average protein sequence coverage was 20.4% and ranged from 1 to 84%. The average number of identified peptides per protein was 15.8 and ranged from 2 to 184. The average number of unique peptides per hit was 6.9 and ranged from 2 to 58. To best present the relative protein abundances in the serum proteome of a 'typical' juvenile sockeye, the sum of each protein's intensity score for the two control non-exercised groups (C1NS + C4NS) was ranked and the 513 identified proteins are presented in descending order of relative abundance (Supplemental Data Table S1). The top 30 most abundant proteins are listed in Table 1, and these proteins constituted 78% of the quantified proteome, while the top 100 proteins comprised 89% of the quantified proteome. Of note, there was little variation in the list of 100 topmost abundant proteins (97% similar) when the sum of all 8 treatments was used for the ranking.

3.2. Effects of dilbit exposure on the serum proteome

The abundances of 24 serum proteins were significantly altered by dilbit exposure (Table 2; $n = 4$; $p < 0.05$). Of the significantly altered proteins, 12 had an area under the ROC curve (AUC) equal to 1.0, indicating complete separation between dilbit-exposed and not exposed groups (Table 2). The majority of differentially abundant proteins (62.5%) were constituent serum proteins, including components of the complement system (ex. complement components C7 and C3), regulators of blood coagulation (ex. antithrombin-III, coagulation factor IX), and transport proteins (ex. warm temperature acclimation protein, WAP65; hemopexin, HPX). In total, 7 proteins increased and 17 proteins decreased in the serum of dilbit-exposed fish relative to control fish. The 3 proteins with the largest increase following dilbit exposure were C7, WAP65, and HPX (Fig. 1A), and the 3 proteins with the largest decrease were folliculin (FCLN), pyruvate kinase (PK), and plasminogen (PLG; Fig. 1B). All of these top altered proteins had an AUC of 1.0 except WAP65 (AUC = 0.94). Of note, 4 unique C3-like proteins were identified in this analysis, all decreasing by a similar magnitude with dilbit exposure. A multiple sequence alignment of the corresponding NCBI entries confirmed low sequence identity among the C3-like proteins (38–59% identity; data not shown).

The protein abundance of creatine kinase (CK) measured using iTRAQ was 1.26 ± 0.12 fold higher in the serum of dilbit-exposed fish relative to control fish, but this change did not reach statistical significance (Fig. 2A; $n = 4$; $P = 0.056$). However, CK enzyme activity quantified in unpooled sera samples confirmed a significant 2.04-fold increase in the serum CK activity for dilbit-exposed fish relative to control fish when expressed as units of activity per sample volume (U/ml; $n = 10–11$; $p < 0.01$; Fig. 2B), and 1.72-fold higher when standardized to serum protein concentration (U/mg; $n = 10–11$; $p < 0.001$; Fig. 2C). Neither exercise nor the interaction of exercise and dilbit exposure significantly affected serum CK activity ($p > 0.05$).

3.3. Effects of dilbit and exercise on the serum proteome

Both exercise and dilbit exposure affected the serum proteome, with the combined treatment producing the most pronounced changes. A total of 93 proteins were identified as outliers across the 8 pairwise treatment comparisons (Supplemental Data Table S2; FDR adjusted $p < 0.05$), with 9 to 41 differentially abundant (DA) proteins present within each pairwise comparison. In keeping with the second objective of this study to inform on potential toxic mechanisms by which

Table 1
Top 30 most abundant proteins in sockeye serum, ranked in decreasing order of abundance.

#	Accession	Protein name	Function
1	gi|185132771	apolipoprotein A-I-1 precursor	Lipid transport
2	gi|5837767	transferrin	Iron transport
3	gi|185132822	apolipoprotein A-I-2 precursor	Lipid transport
4	gi|1848139	hemopexin-like protein	Heme transport
5	gi|95931876	serum albumin	Non-specific carrier protein
6	gi|617439215	collagen alpha-1(VII) chain-like isoform X12	Cell adhesion
7	gi|642094847	alpha-2-HS-glycoprotein-like	Non-specific carrier protein
8	gi|642099235	warm-temperature-acclimation-related 65-kDa	Thermal acclimation
9	gi|1352103	complement C3	Immune/inflammatory response
10	gi|185132174	alpha-1-antiproteinase-like protein precursor	Serine protease inhibitor
11	gi|642093623	salarin precursor	Cysteine peptidase
12	gi|642116634	complement C3-like	Immune/inflammatory response
13	gi|642086397	apolipoprotein C-I-like	Lipid transport
14	gi|642076289	antihemorrhagic factor cHLP-B-like	Coagulation
15	gi|74096001	hemopexin precursor	Heme transport
16	gi|641949962	14 kDa apolipoprotein	Lipid transport
17	gi|641985655	complement C3	Immune/inflammatory response
18	gi|642123352	apolipoprotein B-100-like	Lipid transport
19	gi|350537315	complement factor B/C2 precursor	Immune/inflammatory response
20	gi|642116635	complement C3-like	Immune/inflammatory response
21	gi|185133875	precerebellin-like protein precursor	Precursor, neuromodulatory
22	gi|742106867	complement C3-like	Immune/inflammatory response
23	gi|642075161	complement component C8 beta chain	Immune/inflammatory response
24	gi|642123102	carboxylesterase 5A-like	Xenobiotic detoxification
25	gi|185133428	apolipoprotein E precursor	Lipid transport
26	gi|641970226	alpha-2-macroglobulin 1	Anti-protease
27	gi|742122380	serum albumin 2-like	Non-specific carrier protein
28	gi|642048510	apolipoprotein C-I	Lipid transport
29	gi|642097655	haptoglobin-like	Binds free hemoglobin
30	gi|11055326	chemotaxin	Immune/inflammatory response

Table 2
Proteins significantly altered by dilbit exposure in sockeye serum. Differences were determined using a univariate receiver operator characteristics (ROC) curve analysis and Student's *t*-test ($n = 4$; $p < 0.05$). Results are indicated as the area under the ROC curve (AUC) and as fold-change from control with standard error (FC ± SE).

Accession	Protein name	FC ± SE	AUC	Function
gi|742191606	complement component C7	1.39 ± 0.08	1.00	Immune/inflammatory
gi|237512664	warm temperature acclimation-related protein	1.28 ± 0.11	0.94	Iron homeostasis
gi|657798968	hemopexin	1.26 ± 0.07	1.00	Iron homeostasis
gi|742083337	alpha-2-macroglobulin-like	1.22 ± 0.07	1.00	Anti-protease
gi|185135501	chitinase precursor	1.19 ± 0.08	0.88	Chitin breakdown
gi|642101935	Beta-Ala-His dipeptidase	1.14 ± 0.06	1.00	Serum protease
gi|642096909	vitamin K-dependent protein C	1.11 ± 0.04	0.94	Coagulation
gi|642082470	thrombospondin-1-like	0.94 ± 0.02	1.00	Cell adhesion
gi|597760976	titin-like	0.91 ± 0.04	0.81	Muscle protein
gi|768942557	complement C3-like	0.89 ± 0.04	0.88	Immune/inflammatory
gi|185135824	carbonic anhydrase II	0.87 ± 0.04	0.94	Acid/base; CO_2 transport
gi|597746045	inter-alpha-trypsin inhibitor heavy chain H2	0.84 ± 0.06	0.94	Anti-protease
gi|642113141	antithrombin-III isoform X2	0.84 ± 0.04	0.94	Coagulation
gi|642099637	ependymin precursor	0.82 ± 0.04	1.00	Cell adhesion
gi|573884304	rho guanine nucleotide exchange factor 11-like	0.81 ± 0.05	0.94	Intracellular signaling
gi|742106867	complement C3-like	0.78 ± 0.06	1.00	Immune/inflammatory
gi|742166032	coagulation factor VII-like	0.78 ± 0.07	1.00	Coagulation
gi|765156826	complement C3-like	0.77 ± 0.08	0.88	Immune/inflammatory
gi|742093634	coagulation factor IX	0.76 ± 0.08	0.88	Coagulation
gi|768951276	DEP domain-containing protein 5 isoform X5	0.76 ± 0.06	1.00	Intracellular signaling
gi|742250586	complement C3-like	0.76 ± 0.07	1.00	Immune/inflammatory
gi|47222714	plasminogen	0.69 ± 0.07	1.00	Coagulation
gi|213512270	pyruvate kinase	0.65 ± 0.06	1.00	Glycolysis
gi|188536034	folliculin-interacting protein 1	0.63 ± 0.11	1.00	Cellular energy sensing

swimming performance is impaired in dilbit-exposed fish, comparisons between 4 wk exposed fish are the focus hereafter. A heat-map was generated to visualize the global changes in serum protein abundance at 4 wk for fish exposed to dilbit relative to controls, and for fish exercised relative to resting fish (Fig. 3), and illustrates clearly that the largest shift in the proteome occurred with the combined treatments of dilbit-exposure and swimming test. Potential indicators of dilbit-induced performance impairment were identified from the lists of DA proteins based on the following 3 criteria: (1) proteins that were significantly altered by swimming in dilbit-exposed fish (S *vs* NS, 4 wk dilbit); (2) proteins that were also significantly altered by dilbit in exercised fish (DB *vs* C, 4 wk swim); and (3) excluding proteins that were significantly altered by swimming in control-exposed fish (S *vs* NS, 4 wk control). This rigorous approach ensures that only the interacting effects of dilbit and exercise were considered, and proteins altered by dilbit alone or by swimming alone were excluded. A total of 18 proteins met all 3 criteria, and in all cases were present at higher levels in dilbit-exposed exercised fish, with fold-changes ranging from 1.72 to 16.9 (Table 3). A striking feature of this list is the high proportion of proteins originating from tissue leakage and/or cell damage (77.8%), as opposed to proteins that are serum constituents (22.2%). These included proteins involved in tissue integrity, cellular processes, and muscle contraction. The highest fold-changes were for hephaestin-like protein 1 (HPHL1; 16.9-fold increase), protein piccolo (PCLO; 12.1-fold increase), and dynein heavy chain 8 axonemal (DNAH8; 11.5-fold increase).

As a secondary approach to confirm a direct effect of dilbit on peripheral tissues, the expression of *cyp1a* was quantified in both skeletal muscle and head kidney tissue to indicate PAH-induced activation of the aryl hydrocarbon receptor (AhR). Muscle *cyp1a* was 1.7-fold higher in fish exposed to dilbit for 1 wk relative to control fish ($n = 8$, $p < 0.05$; Fig. 4A). Head kidney *cyp1a* was also elevated after 1 wk of dilbit exposure but did not reach statistical significance, and returned to control levels by 4 wk ($n = 6$, $p > 0.05$; Fig. 4B).

4. Discussion

The present study is the first to characterize a salmonid serum proteome and to quantify the changes in this proteome following exposure to the water-soluble fraction of dilbit. A range of experimental conditions was used to acquire the sera samples used in the analysis, including 2 exposure durations (1 and 4 wk) and 2 relevant physiological states (rest and swimming fatigue), allowing for the identification of robust changes in the serum proteome caused by an environmentally relevant concentration of dilbit. The fact that the observed changes were independent of these potentially confounding variables supports their application to field-based post-spill scenarios where pre-exposure conditions are unknown and/or uncontrolled. In addition, since the swimming performance of the fish exposed to dilbit for 4 wk was reduced (Alderman et al., 2017), the data were also analyzed to gain insight into the observed performance impairment. Combined, these data yield important insights into the organismal response of fish to crude oil exposure, including the identification of several candidate serum biomarkers that could be used to assess population exposure in the event of a pipeline failure in salmon habitat.

4.1. Serum proteome characterization

Over 500 proteins were detected and quantified in juvenile sockeye serum. The most abundant proteins detected were constituent plasma proteins, including apolipoproteins, complement component proteins, and hemopexin (HPX); these are primary hepatic secretions and routinely function within the circulatory system. Multiple entries for several of the identified proteins were detected. This result was expected as multiple isoforms of paralogs of human serum proteins exist in fish plasma (Li et al., 2016; Simmons and Sherry, 2015), owing to a whole genome duplication event in the Actinopterygian lineage. Salmonids are tetraploid, having undergone a fourth whole genome duplication event, and many duplicated genes remain in the genome and show unique expression profiles (Berthelot et al., 2014).

Three notable differences were apparent when the analysis of sockeye serum is compared with the plasma proteomes recently described in adult zebrafish (Li et al., 2016) and in mature white sucker (Simmons and Sherry, 2015). First, albumin is among the top 30 most abundant proteins in sockeye serum, but albumin was not detected in either zebrafish (Li et al., 2016) or white sucker (Simmons and Sherry, 2015). In humans, albumin constitutes half of the protein content of plasma (Anderson and Anderson, 2002), which

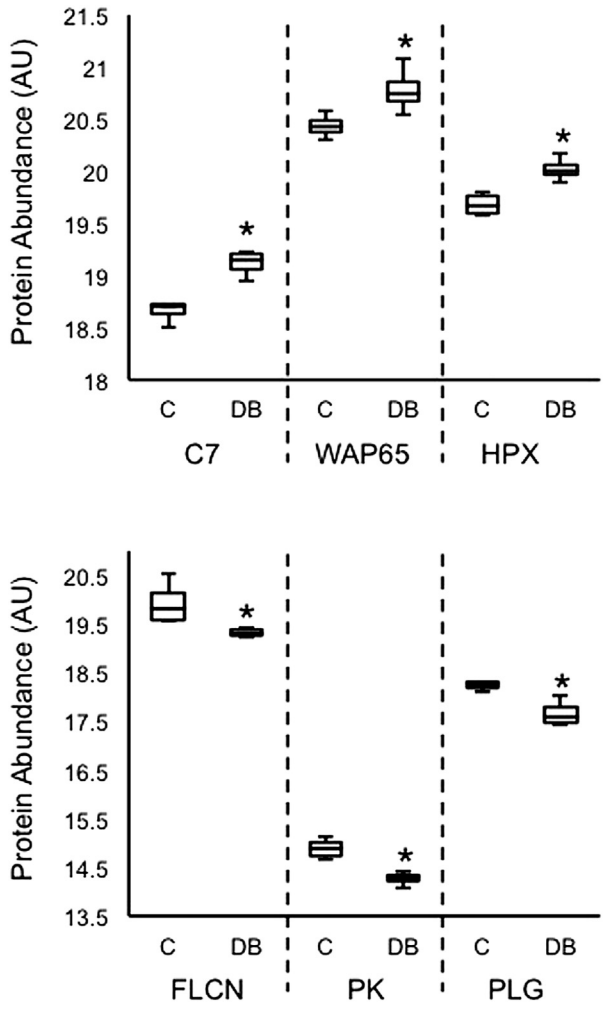

Fig. 1. Serum biomarkers of dilbit exposure. Box plots represent protein abundances for the 3 proteins with the largest significant increase (A) and decrease (B) in the serum of dilbit-exposed fish (DB), relative to control-exposed fish (C). Data is expressed as arbitrary units (AU) from VSN normalized data (n = 4 pools of 10–11 fish; $p < 0.004$). C7, complement component C7; WAP65, warm temperature acclimation-related protein; HPX, hemopexin; FLCN, folliculin; PK, pyruvate kinase; PLG, plasminogen.

means that mammalian studies typically deplete this protein (and abundant immune proteins) prior to MS analysis to facilitate detection of low abundance proteins (Parker and Borchers, 2014). Non-depleted samples were used in the present study, as in the above fish studies. While albumin is known to circulate at high concentrations in salmonids, its presence and abundance varies greatly among fishes depending on the osmotic and transport needs of the species, and also varies between environments and life history stages within species (Andreeva, 2010). A second major difference in the present study was the absence of vitellogenin proteins. Vitellogenins are yolk proteins synthesized by the liver for incorporation into developing oocytes, and are abundant in the circulation of sexually mature females and in males exposed to environmental estrogens (Li et al., 2016; Simmons and Sherry, 2015). Therefore, we interpret this result to confirm that the sockeye salmon were sexually immature, as determined by post-mortem gonad analysis. Finally, fibrinogen was detected at comparatively lower levels than is typical of vertebrate plasma samples (Anderson and Anderson, 2002; Li et al., 2016). This result was expected given the role of fibrinogen in blood clot formation. In fact, a recent study comparing paired human plasma and serum samples showed very strong correlation in the quantified proteomes, with the singular exception of fibrinogen depletion in serum (Zimmerman et al., 2012).

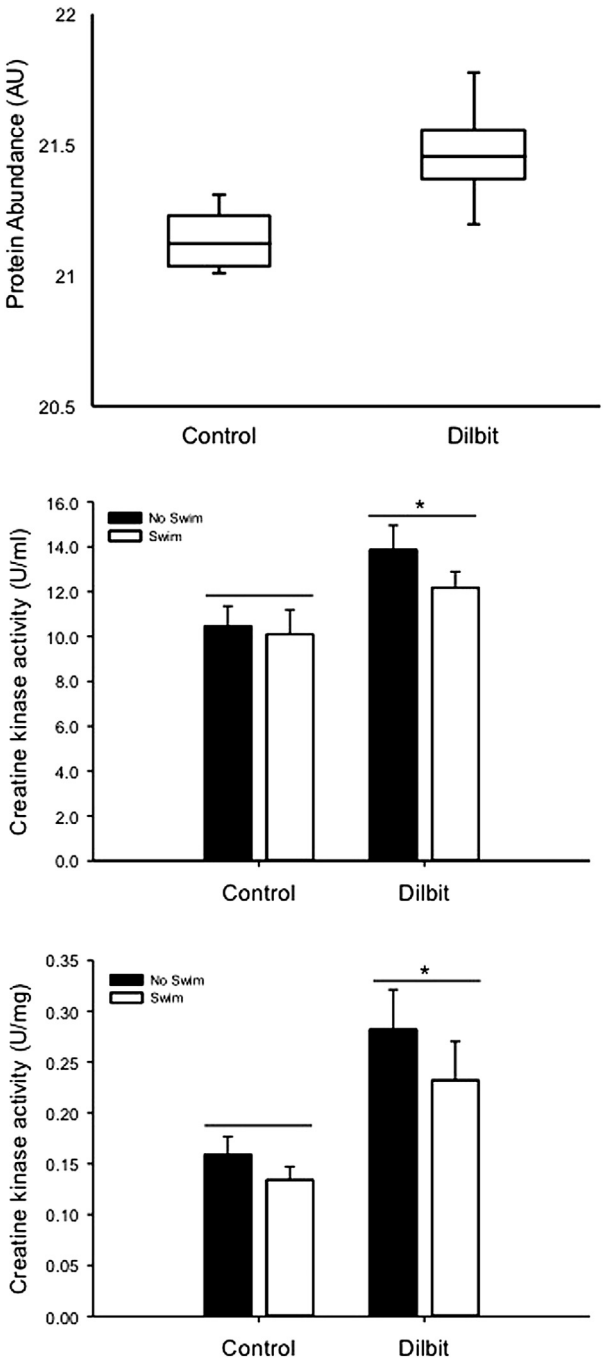

Fig. 2. Effect of dilbit on serum creatine kinase (CK). (A) CK protein abundance was quantified in pooled sera samples (n = 4 pools of 10–11 fish) using iTRAQ paired with LC-MS/MS. The increase in CK protein abundance did not reach statistical significance (t-test; $P = 0.056$). (B–C) CK enzyme activity was quantified in sera from individual fish that were exposed to dilbit or control conditions for 4 wk, and were either sampled at rest (No Swim; black bars) or following a critical swimming speed test (Swim; white bars). Serum CK activity was significantly higher in dilbit-exposed fish when expressed as activity per volume of serum (U/ml; $p < 0.01$; panel B), and when normalized to serum protein concentration (U/mg; $p < 0.001$; panel C), as determined using a two-way ANOVA and Bonferroni multiple comparison test (n = 10–11 individual fish).

4.2. Candidate serum biomarkers of dilbit exposure

The application of omics technologies to the field of aquatic toxicology over the last two decades has significantly advanced our understanding of biological effects and adverse outcomes of chemical exposure, and this topic has been reviewed extensively by others (ex. Martyniuk et al., 2012a; Martyniuk and Simmons, 2016; Sanchez et al.,

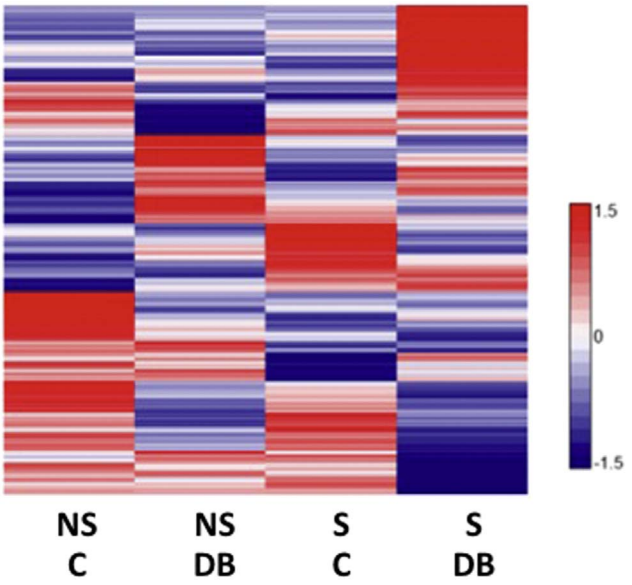

Fig. 3. Global effects of dilbit exposure and exercise on the serum proteome. Heat map showing hierarchical clustering of the serum proteomes for sockeye showing the effects of exercise and/or dilbit exposure on protein abundance. The exercise treatment involved a critical swimming speed test and serum was collected at physical exhaustion (S) or from non-exercised fish (NS). Fish were exposed to the water-soluble fraction of dilbit for 4 wk (DB), or were held in clean water for 4 wk (C).

2011; Simmons et al., 2015). The potential for biomarker discovery remains a prime motivation for the continued study of proteomic changes in response to a toxicant exposure. Blood is an ideal tissue for biomarker discovery and development due to its ease of collection from live specimens, the minimal effect of sampling on animal health, and lower downstream processing time required for analysis relative to solid tissues. In addition, blood consists not only of its own core protein complement but also secreted and leaked contributions from any and all other body tissues, and therefore offers a comprehensive and organismal perspective on adverse effects of environmental contamination when analyzed on an omic-scale. Blood biopsy has been extensively used with adult sockeye salmon (Donaldson et al., 2013; Farrell et al., 2001a, 2001b; Jeffries et al., 2012), and there is no reason why such biopsy could not be used with salmon fry as well, even though fish were sacrificed in the present study. For example, the average serum protein concentration in the present study was 64 mg/ml, which means that an individual protein of interest could easily be quantified using standard techniques from a single drop of blood.

Dilbit exposure had a large effect on the serum proteome, but proteins that were altered irrespective of time and exercise treatment present the strongest biomarker potential. For example, several components of the complement system were altered by dilbit exposure, including a relatively large increase in C7, suggesting that dilbit exposure impairs the immune/inflammatory response in fish. This is supported by the observed immunotoxicity of conventional crude oil and its constituents (Kennedy and Farrell, 2008; Reynaud and Deschaux, 2006). There were also several regulators of coagulation affected by dilbit exposure, including a robust decrease in PLG. Combined, these changes imply an impaired injury response in dilbit-exposed fish. Two orthologous serum glycoproteins, HPX and WAP65, were significantly elevated in dilbit-exposed fish. Both proteins bind free heme with high affinity, and thus function in iron homeostasis and as antioxidants following hemolysis (Machado et al., 2014; Tolosano et al., 2010). A2M, a circulating glycoprotein that functions broadly as a protease inhibitor (Rehman et al., 2013), was also higher in dilbit-exposed fish. A2M is used as a diagnostic in clinical pathology for a number of conditions, including nephrotic syndrome ("leaky kidney"). Of note, complement components, HPX, and A2M were all identified as

Table 3
Proteins that were significantly increased in the serum of exercised fish following 4 wk of dilbit exposure, expressed as fold-change. Two pairwise comparisons were considered: the effect of exercise in dilbit-exposed fish (Dilbit S *vs* NS), and the effect of dilbit on exercised fish (Swim DB *vs* C). Only proteins that significantly increased in both comparisons are shown, and none of the proteins listed were found to increase with exercise in control-exposed fish (4 wk S *vs* NS C; Supplemental Data Table S2). Classic serum proteins are in regular lowercase font; proteins originating from tissue leakage and/or cellular damage are in italicized lowercase font. C, control; DB, dilbit; NS, no swim; S, swim.

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Accession	Protein name	Function	Dilbit S *vs* NS	Swim DB *vs* C
gi|736223836	*protein piccolo*	*Synaptic trafficking*	*12.1*	*4.15*
gi|658846088	*dynein heavy chain 8 axonemal*	*Cilia motility*	*4.31*	*11.5*
gi|498981474	*hephaestin-like protein 1*	*Iron absorption*	*3.94*	*16.9*
gi|498952961	*sacsin-like isoform X1*	*Proteasome component*	*3.40*	*4.83*
gi|831280882	*SCO-spondin*	*Cell adhesion*	*3.17*	*2.53*
gi|573876316	apolipoprotein B-100-like	Lipid transport	2.92	2.67
gi|688568447	*hemicentin-1*	*Cell adhesion*	*2.79*	*3.23*
gi|808884183	*myosin-7*	*Muscle contraction*	*2.41*	*2.65*
gi|336087815	alpha-2-macroglobulin	Anti-protease	2.36	3.50
gi|642100192	coagulation factor IX	Coagulation	2.27	2.49
gi|6686389	apolipoprotein A1	Lipid transport	2.24	4.03
gi|551504003	*vacuolar protein sorting-associated protein 13B*	*Golgi trafficking*	*2.23*	*1.89*
gi|548375018	*small subunit processome component 20*	*Protein synthesis*	*2.09*	*2.32*
gi|47221067	*heterogeneous nuclear ribonucleoprotein*	*Transcription*	*2.02*	*2.28*
gi|734605711	*fibronectin-like*	*Cell adhesion*	*1.91*	1.87
gi|734616630	*F-box only protein 10*	*Protein ubiquitination*	*1.89*	2.49
gi|498976641	*myosin heavy chain fast skeletal muscle*	*Muscle contraction*	*1.86*	*2.83*
gi|734639152	*vacuolar protein sorting-associated protein 13C*	*Golgi trafficking*	*1.72*	*2.77*

candidate plasma biomarkers in juvenile cod exposed to North Sea crude oil (Bohne-Kjersem et al., 2010), supporting further validation studies of these proteins as crude oil biomarkers in fish.

Serum CK activity, quantified in individual sera samples, was significantly elevated in dilbit-exposed fish after 4 wk, which validates the marginally significant increase in CK protein abundance ($P = 0.056$) detected in pooled sera samples using iTRAQ. Therefore CK activity may also be a useful biomarker of dilbit exposure. Importantly, biochemical assays to quantify CK activity in blood samples are already widely available and do not rely on species-specific protein identification (ex. antibody-based detection methods). CK activity is routinely used in both human and veterinary clinical practice to diagnose myocardial infarction and other myopathies, and the extension of this measurement to ecotoxicological studies offers a simple and cost-effective tool for biomonitoring.

There is, of course, significant work involved in moving from identifying candidate biomarkers of dilbit exposure to using them as bio-monitoring tools in post-spill scenarios. Key steps in this validation process will be to establish a pre-exposure population baseline that considers variation across biological variables (ex. age, gender) and environmental heterogeneity (ex. season, degradation from human

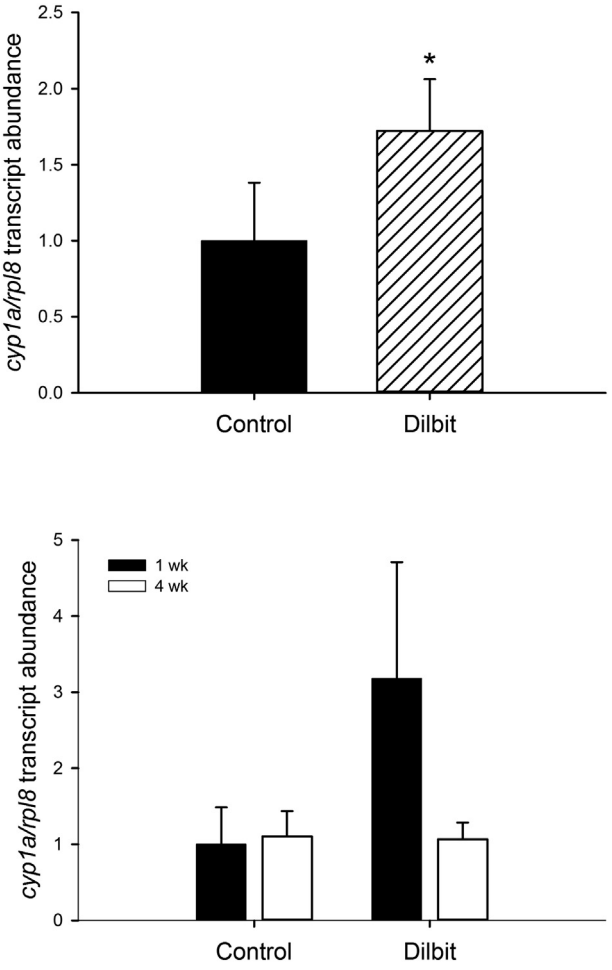

Fig. 4. Effect of dilbit exposure on *cyp1a* transcript abundance in peripheral tissues. The transcript abundance of *cyp1a* was quantified by RT-qPCR in (A) skeletal muscle and (B) head kidney of fish exposed for 1 wk or 4 wk to either clean water (control) or the water-soluble fraction of dilbit. Gene expression in each sample was standardized to the housekeeping gene, *rpl8*. Data is mean ± S.E.M. and is shown normalized to *cyp1a* expression at 1 wk for each tissue. (A) Muscle *cyp1a* abundance was significantly higher following 1 wk of dilbit exposure (hashed bar) relative to controls (black bar), as determined by a one-tailed *t*-test ($n = 8$, $p < 0.05$), but was not quantified at 4 wk due to loss of samples. (B) Head kidney *cyp1a* abundance was not statistically different between dilbit and control exposed fish at either 1 wk (black bars) or 4 wk (white bars), as determined by a two-way ANOVA ($n = 6$, $p > 0.05$).

activities), as well as to confirm dose and specificity in the responses of these proteins to crude oil exposure (Simmons et al., 2015).

4.3. Proteomic insights on swim performance impairment

Sublethal crude oil exposure imposes an array of physiological effects in fish (Kennedy, 2015), including altered swimming behaviors (Kawaguchi et al., 2012; Lari et al., 2016; Philibert et al., 2016) and reduced critical swimming speed (Alderman et al., 2017; Kennedy and Farrell, 2006; Mager et al., 2014; Stieglitz et al., 2016). Contributing to these effects on swim performance are cardiotoxicity (Incardona et al., 2011; Incardona and Scholz, 2016) and general impairments in cardiorespiratory function (Lari et al., 2016; Nelson et al., 2016; Stieglitz et al., 2016), as well as osmoregulatory compromise (Kennedy and Farrell, 2005, 2006) and neurotoxicity (Almeida et al., 2012; Irie et al., 2011; Kawaguchi et al., 2012). Moreover, increased post-exercise mortality was observed in herring exposed for as little as 96 h to environmentally relevant concentrations of crude oil (Kennedy and Farrell, 2006). Importantly, in migratory fish like sockeye, any impairment in swimming performance and/or recovery can have fitness repercussions because it can limit the successful transition between life history stages. Therefore, in addition to identifying candidate biomarkers of dilbit exposure, the proteomic data acquired in this study was further analyzed to uncover changes in the serum proteome unique to the combined treatment effects of dilbit-exposure and exercise at 4 wk, when a reduction in critical swimming speed was observed in these fish (Alderman et al., 2017).

The most pronounced effect on the serum proteome of exercised and dilbit-exposed fish was an increase in non-constituent serum proteins, accounting for nearly 80% of the DA proteins in this treatment group (compared to 69% in exercised control fish). For example, proteins associated with cell membranes (ex. HPHL1, hemicentin, fibronectin, SCO-spondin), cytoplasmic proteins (ex. sacsin-like isoform, myosin-7, DNAH8), and organelle-specific proteins (ex. small subunit processome component 20, vacuolar protein sorting-associated proteins 13B and 13C) were all elevated. Considering that (i) over 50% of fish mass is skeletal muscle (Mommsen and Moon, 2001), (ii) intense exercise induces muscle damage, and (iii) muscle-specific proteins are among the proteins increased by exercise in dilbit-exposed sockeye (ex. myosin-7, myosin heavy chain fast skeletal muscle, creatine kinase), it is proposed that skeletal muscle is either more susceptible to exercise-induced damage following dilbit exposure, or simply contributes more to the serum proteins by virtue of its proportional mass in a salmonid. As confirmation of a cellular response to dilbit exposure in muscle, *cyp1a* transcript abundance was significantly elevated at 1 wk of dilbit exposure relative to controls, indicating PAH-induced AhR activation. Similarly, *cyp1a* transcription in the ventricle was induced at both 1 wk and 4 wk of dilbit exposure, and this coincided with a maximal induction of liver EROD activity (Alderman et al., 2017). Conversely, in the head kidney, *cyp1a* induction was variable in fish exposed to dilbit for 1 wk, and no difference in *cyp1a* abundance was evident at 4 wk. These tissue specific responses in *cyp1a* induction are likely a reflection of the differential expression patterns of AhRs in fish (Abnet et al., 1999; Doering et al., 2012; Hansson and Hahn, 2008), and certainly there are alternative mechanisms of crude oil toxicity in tissues that are independent of AhR (Incardona et al., 2005) and/or are driven by other chemical classes of crude oil mixtures (Kennedy, 2015). Irrespective of mechanism, the observed changes in the serum proteome suggest that skeletal muscle, and likely the myocardium (Alderman et al., 2017), are target tissues for damage by dilbit. This observation could help explain the previously observed impairment of swimming performance in these fish (Alderman et al., 2017). This finding is of great concern from an ecological perspective, as increased cellular damage from strenuous activity will impede recovery, which could have a significant effect on salmon escapement and survival during the oceanic transition.

The three proteins with the highest fold-changes in the combined dilbit/exercise treatment group offer strong insight into dilbit-induced performance impairment. These were: protein piccolo (PCLO), hephaestin-like protein 1 (HEPHL1), and dynein heavy chain 8 axonemal (DNAH8). PCLO is a component of the synaptic active zone involved in vesicle trafficking (Fenster et al., 2000), and is implicated in several neuropathologies including major depressive disorder (Sullivan et al., 2009). Outside of the central nervous system, PCLO has been localized to neuromuscular junctions in rodents (Juranek et al., 2006; Tokoro et al., 2007). To the best of our knowledge only one study has examined PCLO in fish, confirming the presence of two piccolo genes (*pcloa*, *pclob*) in several teleost species and expression in the zebrafish brain (Nonet, 2012). If the peripheral expression of PCLO is conserved in fish, then the large increase in serum PCLO following the swimming test after 4 wk of dilbit exposure supports the hypothesis of increased neuromuscular junction sensitivity to dilbit-induced damage. This is a novel discovery of crude oil toxicity that warrants further investigation. In humans, HEPHL1 is a multi-copper oxidase primarily expressed in the small intestine epithelium, where it regulates iron and copper absorption (Vashchenko and MacGillivray, 2013). The tissue distribu-

tion of HEPHL1 in fish is not known, but zebrafish larvae increase whole-body transcript levels during cold-acclimation and hypoxia exposure (Long et al., 2015). Axonemal dynein proteins, such as DNAH8, are integral components of the microtubules found in motile cilia. These motile cilia are prominent in the ciliated epithelia of fish renal tubules (Hellman et al., 2010; Wessely and Obara, 2013), and suggests that dilbit exposure impacts kidney morphology and function. Histological evidence of renal cell damage induced by crude oil exposure was previously shown in common carp (*Cyprinus carpio*) fed a diet containing heavy oil for 2 wk (Pal et al., 2011).

5. Conclusions

This study provides for the first time a detailed analysis of the quantitative changes to the serum proteome incurred by dilbit exposure in fish. Numerous proteins were identified as candidate biomarkers for dilbit exposure, including proteins involved in immune and inflammatory responses, iron homeostasis, and coagulation. The fact that these changes were consistently detected across 3 wk and with 2 very different physiological states makes these proteins particularly amenable to field applications, where exact exposure time and exercise status are not known. The data also indicate that dilbit exposure caused increased cellular damage in exercised fish, including muscle and kidney injury. Specifically, the substantial increases in serum concentrations of protein piccolo and dynein heavy chain 8 axonemal are potentially novel discoveries of crude oil toxicity to neuromuscular junctions and renal tubules, respectively.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbd.2017.04.003.

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