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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