Effects of diluted bitumen exposure on Atlantic salmon smolts: Molecular and metabolic responses in relation to swimming performance

Sean R. Aveya, Christopher J. Kennedyb, Anthony P. Farrellc, Todd E. Gillisa, Sarah L. Aldermana,⁎

a Department of Zoology and Faculty of Land and Food Systems, University of British Columbia, Vancouver, British Columbia, Canada
b Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada
c Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada

ABSTRACT

Canada’s oil sands industry continues to expand and the volume of diluted bitumen (dilbit) transported across North America is increasing, adding to spill risk and environmental contamination. Dilbit exposure is known to cause adverse effects in fish, but linking molecular and cellular changes with ecologically-relevant individual performance metrics is needed to better understand the potential consequences of a dilbit spill into the aquatic environment. Therefore, this study examined the effects of dilbit exposure on subcellular responses in cardiac and skeletal muscle in relation to swimming performance in a migratory fish species at risk of exposure, Atlantic salmon. Smolts were exposed subchronically to environmentally relevant concentrations of the water-soluble fraction of dilbit (WSFd) for 24 d, and then a subset of exposed fish underwent a depuration period of 7 or 14 d, for a total of 3 experimental time points. At each time point, repeat swimming performance was assessed using sequential critical swimming speed tests (Ucrit) separated by a 24 h rest period, and then several tissues were collected to determine biotransformation enzyme activation, energetic responses, and gene expression changes. Ucrit was unaffected in fish exposed to 67.9 μg/L total initial polycyclic aromatic compounds (PAC), but fish showed a decreased reliance on lipid metabolism for adenosine triphosphate (ATP) in the heart that was maintained through 7 d depuration. In contrast, Ucrit increased in fish exposed to 9.65 μg/L PAC, corresponding to an increased reliance on anaerobic metabolic pathways in cardiac and red skeletal muscle, with partial recovery after 7 d depuration. As expected, at both concentrations WSFd hepatic cyp 1A-mediated biotransformation reactions increased, as measured by EROD activity, which remained elevated for 7 d but not after 14 d depuration. Transcript abundance of cyp1a was also increased in muscle tissue and recovered by 14 d depuration. The expression of other stress-related genes increased in white muscle of dilbit-exposed fish, but were largely unchanged in cardiac and red muscle. The transcriptional profile of cardiac tissue was compared to that of sockeye salmon similarly exposed to WSFd in a previous experiment, and is provided in supplemental text. Combined, these results demonstrate that dilbit exposure alters gene expression and enzyme activities related to xenobiotic exposure, cellular stress, and muscle energetics in juvenile Atlantic salmon without impairing swimming performance, and that most of these changes are recoverable within 14 d depuration.

ARTICLE INFO

Keywords:
Crude oil
PAH
Fish
Heart
Muscle
Aerobic metabolism

1. Introduction

The oil sands region of western Canada contains one of the world’s largest reserves of bitumen, a heavy crude oil (Crosby et al., 2013). Current bitumen extraction rates exceed 460 million L/d, and the majority of the viscous crude is diluted with lighter condensates to form dilbit for transportation and export (Canadian Association of Petroleum Producers, 2016; Crosby et al., 2013). Dilbit transport to refineries and marine ports across North America involves railcar and pipeline. In Eastern Canada, pipelines carrying diluted bitumen extend as far as the province of Quebec, and the previously proposed TransCanada Energy East Pipeline would extend this route to the Atlantic coast in New Brunswick. As transport volume and infrastructure increases, the risk of accidental release and environmental contamination along transport routes, including into natural waterways, also increases (Dupuis and Ucan-Marin, 2015). The fate of spilled dilbit in aquatic environments is poorly understood. A large spill that contaminated the Kalamazoo River (Michigan, USA) in 2010 revealed that dilbit can sink and become entrained in sediments (Dew et al., 2015), ultimately prolonging exposure times of aquatic biota. In addition, certain fish species, such as Atlantic...
salmon (Salmo salar), may be unable to avoid contaminated waterways if a spill were to occur along their requisite migration routes.

Atlantic salmon are a socioeconomically important species on the East coast of North America, and their anadromous life history links marine and freshwater, as well as aquatic and terrestrial food webs. Many sub-populations of these species are in decline, and the outward migration of salmon smolts from nursery lakes to the ocean is a critical period during which salmon are particularly sensitive to anthropogenic stressors (Thorstad et al., 2012). A dilbit spill into salmon-bearing coastal watersheds could influence the long-term viability of local populations of these fish. Therefore, it is crucial to understand how dilbit exposure impacts physiological endpoints relevant to migration, such as swimming performance and energy metabolism that supports skeletal muscle.

There is a growing body of evidence showing that the fish heart is sensitive to conventional crude oil exposure (Incardona, 2017; Incardona et al., 2011), with a broad range of effects that include reduced contractility and cardiac output (Edmunds et al., 2015; Heuer et al., 2019; Incardona et al., 2013; Nelson et al., 2017, 2016), as well as impaired β-adrenergic stimulation (Cox et al., 2017) and excitation-contraction coupling (Brette et al., 2014). These adverse effects on the heart are likely a major contributor to the reduced cardio-respiratory capacity previously and commonly observed in fish exposed experimentally to crude oil, and may contribute to impaired swimming performance (Claireaux and Davoodi, 2010; Kennedy and Farrell, 2006; Mauduit et al., 2016). Swimming also relies on skeletal muscles, which are similar to cardiac muscle in many ways including phenotypic plasticity in response to physiological and environmental challenges (Alderman et al., 2012; Dindia et al., 2017; Gamperi and Farrell, 2004; Johnston et al., 2013). Yet there is a paucity of information on how contaminants in crude oil impact the physiology of skeletal muscle. Fish exposed to crude oil can experience altered activities of cytochrome c oxidase (COX) and lactate dehydrogenase (LDH) in skeletal muscle (Cohen et al., 2005), which could in turn affect exercise capacity as these are key enzymes in aerobic and anaerobic metabolism, respectively (McClelland, 2012). In addition, pathophysiological changes to muscle tissue can arise from crude oil exposure (Ayandiran and Dahunsi, 2016), and dilbit exposure may leave fish more susceptible to exercise-induced muscle damage (Alderman et al., 2017a). Thus as with the heart, skeletal muscle may also be sensitive to contaminants in crude oil, which could compound the effects of dilbit exposure on the capacity for salmon to complete migratory endurance swims.

One of the main chemicals involved in crude oil toxicity are polycyclic aromatic compounds (PAC), a common constituent of all crude oil types that often exert biological effects through the aryl-hydrocarbon receptor (AhR) signalling pathway (Collier et al., 2013; Kennedy, 2015). AhR is a ligand-activated transcription factor with many molecular targets, making transcriptional responses to crude oil exposure a common endpoint measure. Some genes of interest for dilbit toxicity are those involved in Phase I biotransformation (e.g. cytochrome P450 type 1, cyp1a), oxidative stress defense (e.g. catalase, cat), glutathione peroxidase, gpx; glutathione reductase, gsr; superoxide dismutase, sod), the tumorigenic response (e.g. tumour suppressing protein, p53), cellular stress (e.g. heat shock protein 70 kDa, hsp70), proteasomal degradation (e.g. ubiquitin, ub), and muscle damage (e.g. skeletal muscle creatine kinase, ckm3). Dilbit exposure alters whole-body expression of these genes in fish embryos (Alsaadi et al., 2018; Madison et al., 2017, 2015). Similarly, dilbit exposure exerts a concentration-specific increase in cyp1a expression in the head region of sockeye alevins (Alderman et al., 2018), and in the heart, skeletal muscle, and kidney of juvenile sockeye salmon (Alderman et al., 2017b, 2017a); but tissue-specific induction of other target genes is unknown. Characterizing these molecular responses in juvenile fish at risk of dilbit exposure may reveal new potential biomarkers and help to establish an integrated perspective on the biological response to dilbit exposure.

The purpose of the current study was to determine if dilbit exposure impacts the swimming performance of Atlantic salmon smolts, and then to relate any alterations to molecular and metabolic targets in the muscles that support this activity, namely cardiac and skeletal muscle. In addition, the potential recovery of toxicity endpoints to pre-exposure levels was monitored through a defined depuration period in an effort to understand if, and to what extent, the sub-lethal effects of dilbit exposure are reversible.

2. Materials and methods

2.1. Fish

Juvenile Atlantic salmon (age 8 months) were purchased from Marine Harvest Canada (Campbell River, BC) and transported to Simon Fraser University (Burnaby, BC, Canada). Each fish was implanted with a passive integrated transponder (PIT) tag in the dorsal muscle, and individual mass and fork length were recorded (average mass of 61.29 ± 1.15 g and fork length of 168.79 ± 1.05 mm; N = 272). Fish were randomly distributed among nine 200-L fiberglass tanks supplied with aerated flow-through dechlorinated municipal water (10 ºC). Fish were maintained in constant light during a 4 wk acclimation and throughout the exposure period in order to induce smolting (Stefansson et al., 1991). Fish were fed daily ad libitum. Care and use of animals were approved by the Simon Fraser University Animal Care Committee (animal care protocol 1151B-14), according to the guidelines of the Canadian Council for Animal Care.

2.2. Dilbit exposure

The water-soluble fraction of dilbit (WSFd) was generated exactly as previously described (Alderman et al., 2017b), using Cold Lake Summer Blend dilbit. Briefly, Siporax® 15 mm ceramic beads (Aquatique Eco-Systems Inc., Apopka, FL) were pre-soaked to saturation in dilbit for 2 d, and then the beads were added to PVC generator columns. Water passed up through the generator columns and collected into 1 of 6 2000-L header tanks, and the WSFd exposure water, free of emulsion, was pumped from the bottom of the header tanks into replicate experimental tanks. A range of concentrations was achieved by varying the quantity of dilbit-soaked ceramic beads in the generator columns or by omitting the dilbit (control). Target initial total PAC concentrations sought were 0, 10, and 100 μg/L, reflecting values within reported ranges at shoreline sites in the Gulf of Mexico after the Deepwater Horizon oil spill in 2010 (Allan et al., 2012) and known to be sublethal in juvenile salmonids (Alderman et al., 2017b). Flow-through exposures (7.5 L/min) in triplicate experimental tanks were maintained for 24 d without replenishing the dilbit in the generator columns. Water samples from each experimental tank were collected ~12 h following the initiation of exposures (0 d), and again at 10 d and 21 d. Water samples were pooled by replicate experimental tanks (n = 6 per treatment) and then the beads were added to PVC generator columns. Water passed up through the generator columns and collected into 1 of 6 2000-L header tanks, and the WSFd exposure water, free of emulsion, was pumped from the bottom of the header tanks into replicate experimental tanks. A range of concentrations was achieved by varying the quantity of dilbit-soaked ceramic beads in the generator columns or by omitting the dilbit (control). Target initial total PAC concentrations sought were 0, 10, and 100 μg/L, reflecting values within reported ranges at shoreline sites in the Gulf of Mexico after the Deepwater Horizon oil spill in 2010 (Allan et al., 2012) and known to be sublethal in juvenile salmonids (Alderman et al., 2017b). Flow-through exposures (7.5 L/min) in triplicate experimental tanks were maintained for 24 d without replenishing the dilbit in the generator columns. Water samples from each experimental tank were collected ~12 h following the initiation of exposures (0 d), and again at 10 d and 21 d. Water samples were pooled by replicate experimental tanks fed from the same header tank prior to analysis, resulting in 2 samples per concentration per time point. The abundances of 75 individual PAC were measured by SGS AXYS Analytical Services as previously described (Alderman et al., 2017b). At the end of the 24-d exposure period, the water supply to experimental tanks was switched to uncontaminated, dechlorinated municipal water for a de

2.3. Swimming performance

Critical swimming speed (Ucrit) was assessed following the methods of Jain et al. (1997) in a custom-built swim tunnel filled with
contaminant-free aerated water as previously described (Alderman et al., 2017b). On the day of the swim trial, 4 fish from an experimental tank (n = 8 fish per treatment, from 2 of 3 replicate tanks) were carefully transferred to the swim tunnel and acclimated for 45 min at a water velocity of ~0.7 body lengths per second (BL/s). Water velocity was then steadily increased over a 5-min interval to approximately 60% U_crit (actual ramp was 69% of measured U_crit), after which velocity increments of 12 cm/s (~0.7 BL/s) occurred at 20-min intervals until exhaustion. Exhaustion time was recorded when a fish no longer responded to gentle prodding whenever it rested on the screen at the back of the swim tunnel (Kennedy and Farrell, 2006), and the fish was removed, and the PIT-tag number was recorded. The exhausted fish was then returned to a holding tank of contaminant-free aerated water and allowed to recover for 24 h, and all 4 fish from a swim trial recovered together. A second U_crit test (U_crit2) was performed the following day exactly as described above, and upon exhaustion the fish was removed and placed in a lethal dose (0.5 mg/L) of buffered tricaine methanesulfonate (Syndel, Qualicum Beach, BC) prior to tissue sampling. A value for each U_crit was calculated (Jain et al., 1997), and individual performances in the two U_crit tests were used to calculate a recovery ratio (RR), where RR = U_crit2/U_crit. Due to a technical difficulty, U_crit tests were not performed for the high exposure group at the last 2.6. Tissue metabolite and enzyme assays

The activity and potential induction of liver cytochrome P450 (Cyp1A) was quantified in frozen liver tissue of unexercised fish from all WSFd concentrations at each experimental time point (n = 6 fish per treatment) using an ethoxyresorufin O-deethylase (EROD) assay as previously described (Kennedy and Farrell, 2006).

Ventricle and skeletal muscle samples (10–60 mg) from exercised fish (n = 8 per concentration and time point) were homogenized in 400 μL of ice-cold homogenization buffer (50 mM HEPES, 1 mM EDTA, and 0.1% Triton X-100, pH 7.0) with protease inhibitors (1 mM each of phenylmethylsulfonyl fluoride and benzamidine) using a Precellys Evolution tissue homogenizer (Bertin Technologies). The tissue homogenates were briefly sonicated on ice before centrifugation (500 x g for 10 min at 4°C). For lactate, glucose, and glycerol quantitation, a portion of the tissue homogenate was precipitated with PCA before centrifuging the sample at 10,000 x g for 5 min at 4°C. The supernatant was then neutralized with 3 M K2CO3, centrifuged again at 10,000 x g for 5 min at 4°C, and a portion of the resulting supernatant was used in metabolite assays as described in Section 2.5. Glycogen was digested from the remaining supernatant by re-acidifying with 2 M acetate buffer (pH 7.0), adding 30 μL of amyloglucosidase, incubating overnight at 37°C, and then stopping the reaction with PCA precipitation before centrifuging the sample at 10,000 x g for 5 min at 4°C. The final supernatant was neutralized, centrifuged, and glucose concentration was quantified in the supernatant as described in Section 2.5. Glycogen content was calculated by subtracting the initial concentration of glucose in the homogenate from the glucose concentration following glycogen digestion. Immediately following glyceral quantification, triglyceride levels were quantified by measuring the change in absorbance (340 nm) after 10 min incubation with lipoprotein triglyceride lipase reagent (warmed to 30°C) added to each well.

The remaining tissue homogenate was used to measure the activities of COX and LDH by spectrophotometry in duplicate 200 μL reactions at RT, following previously described methods (Gillis and Ballantyne, 1999; Zhang et al., 2016). Optimal assay conditions for COX were 50 μM reduced cytochrome c in 50 mM imidazole (pH 8.0). The cytochrome c solution was reduced with the addition of sodium hydrosulfite, with excess reducing agent removed by slowly bubbling air into the solution for 5 min. COX activity was calculated using maximal reaction velocity over 90 s at 550 nm (molar extinction coefficient = 19.1). Optimal assay conditions for LDH were 10 mM pyruvate, 80 mM imidazole and 0.2 mM NADH (pH 7.6). Following 10 min incubation at RT, pyruvate was added and LDH activity was calculated over 5 min at 340 nm (molar extinction coefficient = 6.22). All values are expressed relative to total soluble protein concentration of the homogenate (quantified using a bicinchoninic acid assay). All chemicals were purchased from Sigma-Aldrich (Oakville, Canada).

2.7. Reverse-transcription quantitative polymerase chain reaction

Total RNA was extracted from frozen liver, skeletal muscle, and ventricle pieces from fish sampled directly from experimental tanks (n = 6 per concentration and time point) as previously described (Alderman et al., 2017b). Then, 1 μg of total RNA was treated with DNase I and reverse-transcribed to complementary DNA (cDNA) in 20 μL reactions using the High Capacity cDNA Synthesis Kit according to the manufacturer’s instructions (Life Technologies). Reactions that omitted Multi-scribe reverse-transcriptase (10 % of samples, randomly chosen) served as non-reverse-transcribed controls. All cDNA and non-reverse-transcribed reactions were diluted 10-fold with molecular-grade water, and stored temporarily at ~20°C.

Transcript abundances of cyp1a, cat, gpx3, gsr, p53, ub, ckm3, hsp70, and the reference genes elongation factor 1 alpha (ef1α) and β-actin (β-
Table 1
Reverse-transcription quantitative polymerase chain reaction assay information for Atlantic salmon, including source and sequence information for primer sequences. efla, elongation factor 1 alpha; b-actin, β-actin; cyp1a, cytochrome P450 type 1a; ub, ubiquitin; gpx3, glutathione peroxidase 3; cat, catalase; gr, glutathione reductase; ps3, tumour suppressing protein; hp, heat shock protein 70; ckm3, creatine kinase 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Primer sequences (5′-3′)</th>
<th>Amplicon</th>
<th>Efficiency</th>
<th>R²</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>efla</td>
<td>AF321836</td>
<td>gacaagcttggaacgctggcag</td>
<td>71</td>
<td>95</td>
<td>0.99</td>
<td>(Engelund and Madsen, 2014)</td>
</tr>
<tr>
<td>b-actin</td>
<td>BG933897</td>
<td>ccaagagcctggtgctcag</td>
<td>92</td>
<td>95</td>
<td>1.00</td>
<td>(Olsvik et al., 2010)</td>
</tr>
<tr>
<td>cyp1a</td>
<td>AF364076</td>
<td>tgagactgccgctgctggcag</td>
<td>101</td>
<td>99</td>
<td>0.98</td>
<td>(Olsvik et al., 2007)</td>
</tr>
<tr>
<td>ub</td>
<td>BG936428</td>
<td>cgggagcctggtgctcag</td>
<td>93</td>
<td>95</td>
<td>1.00</td>
<td>(Olsvik et al., 2010)</td>
</tr>
<tr>
<td>gpx3</td>
<td>BE518588</td>
<td>gacttgctgaactggcctcag</td>
<td>140</td>
<td>95</td>
<td>0.99</td>
<td>(Olsvik et al., 2010)</td>
</tr>
<tr>
<td>cat</td>
<td>NM001146302</td>
<td>cactcatgctggtgctcag</td>
<td>129</td>
<td>89</td>
<td>1.00</td>
<td>(Aukse and Mortensen, 2011)</td>
</tr>
<tr>
<td>gr</td>
<td>BG934480</td>
<td>cgggagcctggtgctcag</td>
<td>61</td>
<td>110</td>
<td>1.00</td>
<td>(Olsvik et al., 2010)</td>
</tr>
<tr>
<td>ps3</td>
<td>BT058777</td>
<td>gatctgctgctgaactggcctcag</td>
<td>196</td>
<td>101</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>ckm3</td>
<td>BG933934</td>
<td>eactgtggctggtgctcag</td>
<td>121</td>
<td>94</td>
<td>1.00</td>
<td>(Olsvik et al., 2011)</td>
</tr>
<tr>
<td>β-actin</td>
<td>BT043724</td>
<td>gacagctggtgctcag</td>
<td>192</td>
<td>95</td>
<td>1.00</td>
<td>(Rojas et al., 2018)</td>
</tr>
</tbody>
</table>

Fig. 1. Mean total polycyclic aromatic compounds (PAC) in triplicate experimental tanks during the 24 d exposure to dibit, for each of three concentrations: Control (no dibit, circles), Low (squares), and High (triangles) concentrations.

2.8. Statistical analysis
One-way analysis of variance (ANOVA) was used to confirm that initial fish morphometrics (mass, fork length, condition factor) were similar across all nine experimental tanks at the start of the acclimation period, and that the few instances of fish mortality were not WSF exposure related. The effects of WSF concentration on swimming performance (Ucrit, Ucrit2, RR), serum glycerol level, white muscle tissue metabolite levels (lactate, glucose, glycogen, triglycerides), white muscle LDH activity, and muscle gene expression were determined by one-way ANOVA and Holm Sidak multiple comparisons tests where differences were detected. The effects of WSF concentration and depuration time on fish morphometrics, hematocrit, plasma glucose and lactate, liver EROD and cyp1a expression, cardiac and red muscle metabolite levels and enzyme activities, were determined by two-way ANOVA and Holm Sidak multiple comparisons tests where differences were detected. Any data that did not meet the assumptions of normality or equal variances was log or square root transformed prior to analysis. All analyses were performed using SigmaPlot 12.5 (Systat Software Inc.) with α = 0.05. Data is presented as mean ± standard error of the mean (S.E.M.).

3. Results
3.1. WSF exposure
Water samples collected at 0 d, 10 d, and 21 d confirmed the presence of PAC in experimental tanks supplied with WSF, with initial total dissolved PAC concentrations of 9.65 μg/L (low) and 67.9 μg/L (high), which were similar to two of the concentrations used earlier with sockeye salmon, where the same 75 PAC were quantified (Alderman et al., 2017b). Total PAC concentration decreased by 56 % between 0 d and 10 d, and by 83 % between 0 d and 21 d. In contrast, total PAC in the control tanks remained extremely low and stable at 0.018 ± 0.003 μg/L for the duration of the experiment (Fig. 1). As in our previous study (Alderman et al., 2017b), component breakdown for total PAC showed lower molecular weight hydrocarbons (i.e. naphthalenes) dominating initially, with higher molecular weight hydrocarbons (i.e. phenanthrenes) becoming proportionally more abundant with time (Supplemental Data Table S1).

3.2. Effects of WSF exposure on fish biometrics and swimming performance
Initial mass, fork length, and body condition of fish were similar for all experimental tanks (N = 30 per tank). WSF exposure had no significant effect on fish mortality (Control: 7.23 %, Low: 8.29 %, High: 3.23 %). As is expected during smoltification (Hoar, 1988) and irrespective of WSF concentration, all fish demonstrated a similar growth pattern of reduced mass and increased fork length over the course of the experimental period, which resulted in a net decrease in condition factor of approximately 18 % (p < 0.001; Table 2).

Following the 24 d exposure to WSF, the Ucrit for the low exposure group was 19 % higher (P = 0.003) than that of the control and high exposure groups, which were not significantly different (Fig. 2A). Similarly, after a 24 h recovery from the first swim trial, Ucrit2 for the low exposure group was 15 % higher than the high exposure group (P = 0.038) but there were no significant differences in Ucrit2 between the control and high exposure groups, or the control and low exposure groups. The recovery ratio (Ucrit2/Ucrit) was similar between all experimental exposure groups, ranging from 1.05 to 1.10, which meant that fish performed just as well on the second swim test. Following the 7 d and 14 d depuration periods, there were no significant differences in Ucrit, Ucrit2, or recovery ratio between experimental exposure groups (Fig. 2B, C).

3.3. Effects of WSF exposure on blood parameters of Atlantic salmon
Lactate, glucose, and glycerol levels of plasma were similar among WSF exposure groups (Table 3). A significant interaction between time
and concentration existed for hematocrit in fish sampled at rest, however the multiple comparisons test revealed a similar trend across all concentrations, with hematocrit decreasing over time (P_interaction = 0.025; Fig. 3A). In fish sampled after the second swim trial, hematocrit was significantly reduced in fish exposed to the highest concentration of dilbit relative to control fish (P_concentration = 0.016; Fig. 3B).

### 3.4. Effects of WSFd exposure on Phase I biotransformation in Atlantic salmon liver

Following the 24 d exposure to the WSFd, liver EROD activity was significantly elevated 6.5- and 12.6-fold in the low and high exposure groups, respectively, relative to controls (P_interaction < 0.001; Fig. 4A). Following the 7 d depuration period, EROD activity remained elevated in WSFd-exposed fish relative to controls (P_interaction < 0.001),
returning to control levels following the 14 d depuration period. Transcript abundance of cyp1a in the liver was significantly elevated relative to controls in both the low and high exposure group (Pconcentration < 0.001; Fig. 4B).

### 3.5. Effects of WSFd exposure on the metabolic pathways in Atlantic salmon striated muscle during exercise

Following the 24 d exposure to dilbit, a concentration-specific effect on COX and LDH activity existed within the heart, where COX was reduced and LDH was increased in the low exposure group compared to the control group (Fig. 5A, B; Pconcentration = 0.033 and 0.021, respectively). There were no significant differences in cardiac lactate or glycogen levels; however, there was a significant effect of concentration on triglyceride levels, which were elevated in the high exposure group compared to both the control and low exposure groups (Table 3; Pconcentration = 0.002). Red muscle COX activity decreased by ~40 % in the low and high exposure groups, but returned to control levels

Table 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Exp Control</th>
<th>Exp Low</th>
<th>Exp High</th>
<th>Dep 1 Control</th>
<th>Dep 1 Low</th>
<th>Dep 1 High</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmatic (mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td>9.6 ± 0.9</td>
<td>8.3 ± 0.4</td>
<td>7.4 ± 1.2</td>
<td>9.3 ± 0.7</td>
<td>7.1 ± 0.5</td>
<td>8.5 ± 0.9</td>
<td>pconcentration = 0.06</td>
</tr>
<tr>
<td>glucose</td>
<td>10.8 ± 2.1</td>
<td>7.6 ± 0.5</td>
<td>10.9 ± 2.4</td>
<td>11.6 ± 2.6</td>
<td>8.8 ± 1.9</td>
<td>10.3 ± 1.7</td>
<td>nd</td>
</tr>
<tr>
<td>glycerol</td>
<td>0.24 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>nd</td>
</tr>
<tr>
<td>Heart (μmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td>105.5 ± 10.3</td>
<td>76.3 ± 11.7</td>
<td>98.5 ± 15.7</td>
<td>96.8 ± 12.0</td>
<td>97.0 ± 10.4</td>
<td>100.4 ± 12.9</td>
<td>nd</td>
</tr>
<tr>
<td>glucose</td>
<td>12.1 ± 2.5</td>
<td>5.8 ± 2.7</td>
<td>12.7 ± 4.2</td>
<td>17.2 ± 2.8</td>
<td>9.4 ± 1.7</td>
<td>14.5 ± 4.6</td>
<td>nd</td>
</tr>
<tr>
<td>triglycerides</td>
<td>35.0 ± 5.8</td>
<td>24.2 ± 7.0</td>
<td>65.0 ± 13.9</td>
<td>38.0 ± 10.0</td>
<td>36.2 ± 10.9</td>
<td>98.5 ± 24.4</td>
<td>(C, L) &lt; H</td>
</tr>
<tr>
<td>Red muscle (μmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td>121.8 ± 30.2</td>
<td>65.0 ± 8.3</td>
<td>64.8 ± 10.9</td>
<td>118.9 ± 17.3</td>
<td>127.1 ± 14.6</td>
<td>122.1 ± 15.7</td>
<td>Dep 1 &gt; Exp</td>
</tr>
<tr>
<td>White muscle (μmol/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td>126.9 ± 15.3</td>
<td>118.0 ± 14.6</td>
<td>125.2 ± 18.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>nd</td>
</tr>
<tr>
<td>glucose</td>
<td>12.8 ± 3.7</td>
<td>9.6 ± 3.4</td>
<td>15.1 ± 2.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>nd</td>
</tr>
<tr>
<td>glycogen</td>
<td>22.5 ± 10.1</td>
<td>18.2 ± 7.7</td>
<td>18.0 ± 6.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>nd</td>
</tr>
<tr>
<td>triglycerides</td>
<td>21.5 ± 7.1</td>
<td>43.3 ± 10.1</td>
<td>48.8 ± 16.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>nd</td>
</tr>
</tbody>
</table>

Fig. 3. Hematocrit in Atlantic salmon smolts sampled at rest (A) or following a successive critical swimming speed test (B). Fish were exposed to uncontaminated water (Control) or the water-soluble fraction of dilbit (Low: 9.65 μg/L, High: 67.9 μg/L) for 24 d (Exp), and a subset of fish were sampled after a 7 d or 14 d depuration period in uncontaminated water (Dep 1 or Dep 2, respectively). Differences were determined by two-way ANOVA and Holm-Sidak multiple comparisons test (N = 6–8), and are indicated with text in each panel. Data are mean ± S.E.M.

Fig. 4. Indicators of Phase 1 biotransformation in Atlantic salmon smolts exposed to various concentrations (Control: 0 μg/L, Low: 9.65 μg/L, High: 67.9 μg/L total PAC) of WSFd for 24 d (Exp), with a subset of fish given a 7 d or 14 d depuration period in clean water prior to sampling (Dep 1 or Dep 2, respectively). (A) Liver ethoxyresorufin-O-deethylase (EROD) activity was quantified as a measure of cytochrome P450 1 (Cyp1) activation. (B) Relative transcript abundance of cyp1a in the liver. To facilitate comparisons, data are shown standardized to the expression in Exp Control fish. A two-way ANOVA and Holm-Sidak method were used to test for effects of concentration and time, or their interaction (N = 4–6; p < 0.05). Differences are indicated with text for each panel, and asterisks indicate differences between concentrations within a time point. Data are mean ± S.E.M.
following the 7 d depuration period (Fig. 5C; \( P_{interaction} = 0.035 \)). While no significant differences in LDH activity existed in red muscle (Fig. 5D), muscle lactate levels were nearly 2-fold lower in WSFd-exposed fish relative to unexposed controls at the end of the exposure period but recovered by 7 d depuration (Table 3). The potential interaction between concentration and time in this variable did not reach statistical significance, however the significant main effect of time was clearly driven by red muscle lactate values in the low and high exposure groups (\( P_{time} = 0.019 \)). In contrast, dilbit exposure did not significantly change white muscle LDH activity (Control 14.1 ± 0.8 kU/g; Low 13.6 ± 1.0 kU/g; High 13.0 ± 1.6 kU/g), nor were tissue lactate, glucose, glycogen or triglyceride levels altered in white muscle (Table 3).

### 3.6. Effects of WSFd exposure on gene expression in Atlantic salmon striated muscle

Transcript abundance of \( cyp1a \) in the heart was significantly elevated within the low and high exposure groups relative to controls (\( P_{concentration} < 0.001 \); Fig. 6A), but all other genes of interest were unchanged in WSFd exposed fish relative to controls (Table 4). Within the red muscle of the low and high exposure groups, \( cyp1a \) was 3.7-fold and 6.0-fold higher than the control exposure group following the 24 d exposure (\( P_{interaction} = 0.006 \); Fig. 6B), returning to control levels following the 7 d depuration period. The abundance of \( cat \) decreased in red muscle of fish in the low exposure group after 24 d WSFd (\( P = 0.05 \); Table 4). A significant interaction between the main variables of concentration and time existed for \( cyp1a \) expression in the white muscle (\( P_{interaction} = 0.019 \)), with ∼6- to 8-fold higher expression in the low and high exposure groups relative to the control group following the 24 d exposure and 7 d depuration, but this returned to baseline after 14 d depuration (Fig. 6C). Following the 24 d exposure to WSFd, the expression of \( ub \), \( cat \), and \( ckm3 \) were significantly up-regulated ∼2-fold in the white muscle of the high exposure group relative to controls (\( P = 0.01, 0.04, \) and 0.02, respectively), while \( p53 \) and \( ckm3 \) were 2-fold to 3-fold higher in the low exposure group relative to controls (\( P = 0.03 \) and 0.02, respectively; Table 4).

### 4. Discussion

The present study is the first to describe the effects of sublethal, environmentally relevant concentrations of dilbit on the swimming performance and tissue-level biochemical and molecular responses in Atlantic salmon smolts, and to track these parameters through a 14 d depuration period. Subchronic exposure of the Atlantic salmon smolts to WSFd at 9.65 μg/L PAC appeared to increase the reliance of the cardiac muscle on anaerobic metabolism, as evidenced by changes in LDH and COX activities that persisted through 7 d depuration, and this shift was concurrent with a modest improvement in swimming performance. At a higher WSFd concentration (67.9 μg/L), there appeared to be a decrease in the reliance of cardiac muscle on lipid metabolism for ATP production as evidenced by elevated triglyceride levels after the 24 d WSFd exposure and for at least 7 d depuration. The results of this study suggest that crude oil exposure stimulates changes in cellular metabolism and activates cellular biotransformation pathways in all types of striated muscle but that these changes are muscle-type specific. Importantly, the majority of the parameters examined returned to pre-exposure levels following a 14 d depuration period, indicating that the responses observed in striated muscles are reversible after sufficient time in contaminant-free water.

### 4.1. Effects of WSFd exposure on swimming performance and recovery

Multiple studies have demonstrated that cardiorespiratory performance is compromised in fish exposed to increasing concentrations of conventional crude oil, including reduced hypoxia tolerance, thermal tolerance, and \( U_{crit} \) (Claireaux et al., 2013; Incardona et al., 2015; Mager et al., 2014; Mauduit et al., 2016; Nelson et al., 2017). In some cases (Hicken et al., 2011; Incardona et al., 2015; Johansen and Esbaugh, 2017; Mauduit et al., 2016), but not all (Mauduit et al., 2019; Pan et al., 2018), these adverse effects persisted for many months after the exposure, emphasizing the potential for latent impacts of crude oil exposure on fish populations. In the present study with dilbit rather than conventional crude oil, Atlantic salmon smolts exposed to the lowest concentration of WSFd (9.65 μg/L PAC) experienced a modestly
improved $U_{crit}$ that disappeared with 7 d depuration, and fish exposed to the highest concentration of WSFd (67.9 μg/L PAC) attained $U_{crit}$ values comparable to those of unexposed control fish. These results are in partial agreement with our previous study that reported improved $U_{crit}$ in juvenile sockeye salmon exposed to WSFd at 3.5 μg/L PAC but reduced $U_{crit}$ at 66.7 μg/L PAC (Alderman et al., 2017b). Similarly, others report no impact on oxygen consumption or $U_{crit}$ of juvenile golden grey mullets (Lista aurata) exposed acutely to Arabian crude oil (Milinkovitch et al., 2012). While these discrepancies may simply result from differences in experimental design (e.g., oil constituents, concentration, exposure time), the potential for species-specific sensitivities to crude oil exposure should not be ignored. For example, sockeye salmon from our previous study (Alderman et al., 2017b) displayed a notably different molecular response in the heart following WSFd exposure compared to that of Atlantic salmon from the present study, despite comparable exposure durations and concentrations between studies (Supplemental Table S3). These different subcellular responses to WSFd exposure may help explain why sockeye swimming performance declined at high WSFd concentrations while that of the Atlantic did not.

A recovery ratio describes the ability of a fish to repeat its performance in a $U_{crit}$ test after a defined rest period and is a useful proxy for an individual’s return to pre-exercise homeostasis after exhaustive exercise (Farrell, 2008). Surprisingly, despite the frequent use of $U_{crit}$ tests in crude oil exposure studies, most experiments are terminated after a fish reaches exhaustion, and therefore the opportunity to also consider exercise recovery as a meaningful endpoint of oil exposure is lost. Control and dillbit-exposed Atlantic salmon smolts maintained recovery ratios slightly > 1.0 indicating full recovery from fatigue. Likewise, Zhang et al. (2017) reported no difference in excess post-exercise oxygen consumption in European sea bass (Dicentrarchus labrax) that were chased to exhaustion after an acute exposure to crude oil. It is worth noting, however, that some fish such as rainbow trout (O. mykiss) require as little as 45 min to fully recover $U_{crit}$ between tests (Farrell, 2008), and recent work in Atlantic salmon has shown that full recovery of routine oxygen consumption from exhaustion can occur in 12 h (Zhang et al., 2018). It is possible that a shorter rest period in the present study would have produced different results, particularly in light of the differences in anaerobic metabolism described below. A rest period of 24 h was chosen for two reasons. First, a quantitative assessment of changes in the serum proteomes of sockeye salmon exposed to 66.7 μg/L PAC from WSFd for 28 d suggested these fish experience increased exercise-induced muscle damage relative to unexposed fish (Alderman et al., 2017a); similar results in Atlantic salmon were anticipated. Second, previous studies have shown that exposure to crude oil can cause post-swim mortalities, and the design of this study was done to prevent such losses. Specifically, juvenile Pacific herring (Clupea pallasi) exposed to 40 and 100 μg/L PAC from North Slope crude oil for up to 8 wk had higher post-swim mortalities than controls during the first 24 h of recovery (Kennedy and Farrell, 2006), and adult mahi-mahi (Coryphaena hippurus) exposed to 8.4 μg/L PAC from the Deepwater Horizon oil spill for 24 h had an increased rate of pre-test handling-induced mortality (Stieglitz et al., 2016).

### 4.2. Effects of WSFd exposure on Phase I biotransformation in the liver and muscle

Cyp1A-mediated Phase 1 reactions are involved in cellular biotransformation of chemical compounds, including xenobiotics that bind to AhR (Zhou et al., 2010). The AhR-mediated transcription of cyp1a and induction of liver EROD activity, which is a catalytic measurement of cytochrome P4501A, have served as biomarkers for PAC exposure in fish for nearly two decades (Whyte et al., 2000). As expected, there was an increase in liver EROD activity after the 24 d exposure to both concentrations of WSFd; this response required 14 d of depuration to return to pre-exposure levels. Similarly, liver cyp1a mRNA abundance increased in a concentration-specific manner with WSFd exposure. This result aligns with a previous study showing that Polar cod (Boreogadus saida) exposed to crude oil for 4 wk and then given a 2 wk depuration retained elevated liver EROD activity while cyp1a gene expression had returned to baseline (Nahrgang et al., 2010). While a sustained increase in enzymatic activity could reflect a tissue burden of PAC (Cheikyula et al., 2008), it may also reflect differences in the stabilities of mRNA transcripts relative to proteins.

Unlike in liver, striated muscle cyp1a mRNA abundance was elevated in both the low and high exposure groups after 24 d WSFd exposure, with expression returning to pre-exposure levels in red and white muscle during the depuration period (at 7 d and 14 d, respectively). In contrast, cyp1a abundance remained elevated in cardiac muscle after 14 d depuration even at the lowest WSFd concentration. In

---

Fig. 6. Gene expression changes of cytochrome P450 type 1a (cyp1a) in the heart (A), red skeletal muscle (B), and white skeletal muscle (C) of Atlantic salmon smolts exposed to various concentrations (Control: 0 μg/L, Low: 9.65 μg/L, High: 67.9 μg/L total PAC) of WSFd for 24 d (Exp), with a subset of fish given a 7 d or 14 d depuration period in clean water prior to sampling (Dep 1 or Dep 2, respectively). For each tissue, transcript abundance of cyp1a was standardized to expression in Exp Control fish to facilitate comparison. A two-way ANOVA and Holm-Sidak method were used to test for effects of concentration and time, or their interaction (N = 3).

---

*S.R. Avey, et al.* Aquatic Toxicology 221 (2020) 105423
Table 4

Molecular responses to WSF6 exposure in Atlantic salmon muscles. For each gene of interest, expression in the unexposed control fish was normalized to 1 to facilitate comparisons of the magnitude of response between tissues. For each tissue and gene of interest, differences were determined by one-way ANOVA and Holm-Sidak multiple comparisons tests, and values in bold font indicate significant differences from control. Values are mean ± S.E.M. ub, ubiquitin; gpx3, glutathione peroxidase 3; cat, catalase; gsr, glutathione reductase; p53, tumour suppressing protein; hsp70, heat shock protein 70; ckm3, creatine kinase 3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Heart Low 9.65 μg/L</th>
<th>Heart High 67.9 μg/L</th>
<th>Red muscle Low 9.65 μg/L</th>
<th>Red muscle High 67.9 μg/L</th>
<th>White muscle Low 9.65 μg/L</th>
<th>White muscle High 67.9 μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ub</td>
<td>1.06 ± 0.08</td>
<td>1.30 ± 0.15</td>
<td>0.78 ± 0.09</td>
<td>1.09 ± 0.14</td>
<td>1.40 ± 0.15</td>
<td>1.60 ± 0.13</td>
</tr>
<tr>
<td>gpx3</td>
<td>0.94 ± 0.15</td>
<td>1.45 ± 0.17</td>
<td>0.83 ± 0.06</td>
<td>1.53 ± 0.25</td>
<td>0.81 ± 0.20</td>
<td>1.87 ± 0.41</td>
</tr>
<tr>
<td>cat</td>
<td>1.36 ± 0.12</td>
<td>1.23 ± 0.15</td>
<td>0.54 ± 0.08</td>
<td>0.96 ± 0.09</td>
<td>1.43 ± 0.23</td>
<td>1.91 ± 0.30</td>
</tr>
<tr>
<td>gr</td>
<td>0.71 ± 0.08</td>
<td>1.07 ± 0.17</td>
<td>0.72 ± 0.08</td>
<td>0.82 ± 0.04</td>
<td>1.10 ± 0.15</td>
<td>1.22 ± 0.18</td>
</tr>
<tr>
<td>p53</td>
<td>0.94 ± 0.11</td>
<td>1.09 ± 0.19</td>
<td>0.55 ± 0.10</td>
<td>0.62 ± 0.06</td>
<td>2.23 ± 0.39</td>
<td>1.46 ± 0.20</td>
</tr>
<tr>
<td>hsp70</td>
<td>1.00 ± 0.05</td>
<td>0.96 ± 0.10</td>
<td>0.71 ± 0.09</td>
<td>1.32 ± 0.11</td>
<td>1.35 ± 0.11</td>
<td>1.43 ± 0.12</td>
</tr>
<tr>
<td>ckm3</td>
<td>1.10 ± 0.14</td>
<td>1.25 ± 0.14</td>
<td>0.51 ± 0.10</td>
<td>1.05 ± 0.31</td>
<td>2.23 ± 0.30</td>
<td>2.11 ± 0.24</td>
</tr>
</tbody>
</table>

4.3. Effects of WSF6 exposure on energy metabolism in striated muscle during exercise

During the assessment of swimming performance, it is likely that both sustained aerobic and anaerobic burst swimming were utilized to power exercise (Lee et al., 2003). Indicators of metabolic capacity are commonly used to differentiate between these methods of exercise, such as COX activity for aerobic metabolism, and LDH for anaerobic metabolism (Pelletier et al., 1994). In the present study, increased Ucrit, the increased energy demand imposed by sustained swim-
This study presents novel data on sublethal effects and recovery in juvenile Atlantic salmon exposed to dilbit. In contrast to many reports of impaired swimming performance in crude oil exposed fish, Atlantic salmon smolts exposed to WSFd achieved $U_{crit}$ values on par with unexposed control fish, and were able to repeat this performance 24 h later. This result underlines the importance of understanding species and life stage sensitivities to dilbit when considering the potential impacts of a spill into fish habitat. Indeed, Atlantic and sockeye salmon exposed similarly to WSFd displayed differential transcriptional responses in cardiac tissue for genes involved in biotransformation pathways, which may help explain why $U_{crit}$ was impaired in sockeye (Alderman et al., 2017b) but not Atlantic salmon. An important discovery of this study was that subchronic exposure to WSFd increased the capacity for anaerobic metabolism in the cardiac and red skeletal muscle of smolts, and decreased utilization of lipids for aerobic ATP production in the cardiac muscle. This shift towards a less efficient metabolic strategy was maintained for at least 7 d of post-exposure recovery in clean water, and could carry long term consequences if not corrected. In mammals, for example, reduced utilization of lipid fuels in the heart and red muscle affects swimming endurance, as this would no doubt limit migratory success of smolts.

This work was supported by grants from the National Contaminants Advisory group of Fisheries and Oceans Canada to TEG and CJK. APF holds a Tier 1 Canada Research Chair. SRA was supported by an Ontario Graduate Scholarship.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Acknowledgements**

We thank Geoffrey Su and Bruce Leighton (SFU) for experimental support, and Fisheries and Oceans Canada for Cold Lake Summer Blend dilbit.

**Appendix A. Supplementary data**

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105423.

## References


Dupuis, A., Ucan-Marín, F., 2015. A Literature Review on the Aquatic Toxicology of Petroleum Oil: an Overview of Oil Properties and Effects to Aquatic Biota. Aquatic Toxicology 000, 000. https://doi.org/10.1016/j.aquatox.2016.01.0878-0.


