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

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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 (U_{crit}) separated by a 24 h rest period, and then several tissues were collected to determine biotransformation enzyme activation, energetic responses, and gene expression changes. U_{crit} 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, U_{crit} 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.

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

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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; Gamperl 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 (ex. cytochrome P450 type 1, *cyp1a*), oxidative stress defense (ex. catalase, *cat*; glutathione peroxidase, *gpx*; glutathione reductase, *gsr*; superoxide dismutase, *sod*), the tumorigenic response (ex. tumour suppressing protein, *p53*), cellular stress (ex. heat shock protein 70 kDa, *hsp70*), proteasomal degradation (ex. ubiquitin, *ub*), and muscle damage (ex. 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 °C). 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 (Aquatic 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 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 to initiate the depuration period. Fish were randomly subsampled from replicate experimental tanks at one of three time points: at the end of the 24 d exposure, or after depuration in clean water for a defined period of 7 d or 14 d. At each time point, fish were either sampled directly from experimental tanks (n = 6 per treatment) or used to assess swimming performance (n = 8 per treatment) and then sampled, as detailed below.

2.3. Swimming performance

Critical swimming speed (U_{crit}) 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 total 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 final 14 d depuration timepoint.

2.4. Tissue collection

Each euthanized fish was blotted dry, PIT-tag recorded, and mass and fork length taken to calculate condition factor (K), where K = [mass (g) x 10^5]/[fork length (mm)^3]. Blood was collected by free-flow from caudal vessels into heparinized microcapillary tubes and into untreated 1.5 mL microcentrifuge tubes. Microcapillary tubes were immediately centrifuged for hematocrit determination, while the remaining blood sample was first clotted for 30 min at room temperature prior to centrifugation, and then the serum was flash frozen in liquid nitrogen. Skeletal muscle was biopsied across the lateral line below the dorsal fin, and the red and white portions were isolated separately and freeze clamped in liquid nitrogen. The liver was removed, minced, and flash frozen in liquid nitrogen. The heart was removed, cleared of blood by passive perfusion in physiological saline, and then freeze clamped in liquid nitrogen. All tissues were stored at −80 °C until used for analysis. During tissue biopsy, all fish were confirmed to be sexually immature by gonad inspection.

2.5. Quantification of blood parameters

Frozen serum was thawed on ice and immediately deproteinated with 5.5 % perchloric acid (PCA) followed by centrifugation at 1500 x *g* for 10 min at 4 °C. Lactate, glucose, and glycerol concentrations in the supernatant were determined spectrophotometrically in duplicate 200 µL reactions by fitting absorbance values against standard curves generated from known dilutions of lactate, glucose, and glycerol, respectively (Alderman et al., 2017b; Gatrell et al., 2019). Briefly, lactate was quantified in duplicate reactions (0.2 M glycine, 0.16 M hydrazine sulphate, 1 mM β-nicotinamide adenine dinucleotide (NAD); pH 9.4) by measuring the change in absorbance (340 nm) after incubating for 30 min at room temperature (RT) in the presence of 5 U LDH. For glucose, optimal reaction conditions were 120 mM Tris, 80 mM Tris−HCl, 5 mM NAD, 2 mM $MgSO_4$, 5 mM adenosine triphosphate (ATP) and 0.08 U glucose-6-phosphate dehydrogenase (pH 7.4), measured as the change in absorbance (340 nm) after incubating for 45 min at RT in the presence of 0.1 U hexokinase. For glycerol, optimal conditions were 0.2 M glycine, 0.16 M hydrazine sulfate, 3.6 mM ATP, 2.2 mM NAD, 1.5 mM dithiothreitol, 0.8 U glyceraldehyde-3-phosphate dehydrogenase (pH 9.4), measured as the change in absorbance (340 nm) after incubating for 30 min at RT in the presence of 1.3 U glycerol kinase. All chemicals were purchased from Sigma-Aldrich (Oakville, Canada).

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 K_2CO_3, 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 U 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 glycerol 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 (*b-*

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

Gene	GenBank	Primer sequences (5′-3′)		Amplicon	Efficiency	R^2	Reference
ef1α	AF321836	F: agaaccattgagaagttcgagaag	R: gcacccaggcatacttgaaag	71	95	0.99	(Engelund and Madsen, 2014)
b-actin	BG933897	F: ccaaagccaacagggagaag	R: agggacaacactgcctggat	92	95	1.00	(Olsvik et al., 2010)
cyp1a	AF364076	F: tggagatcttccggcactct	R: caggtgtccttgggaatgga	101	99	0.98	(Olsvik et al., 2007)
ub	BG936428	F: gatcttcgctggcaaacaact	R: cgaagacgcagcacaagatg	93	95	1.00	(Olsvik et al., 2010)
gpx3	BE518588	F: gattcgttccaaacttcctgcta	R: gctcccagaacagcctgttg	140	95	0.99	(Olsvik et al., 2010)
cat	NM001140302	F: catccagaaacgttgggttc	R: gaggcacctctacgggtgta	129	89	1.00	(Arukwe and Mortensen, 2011)
gsr	BG934480	F: cagtgatggctttttgaactt	R: ccggcccccactatgac	61	110	1.00	(Olsvik et al., 2010)
p53	BT058777	F: tggtcagagttgaggggaac	R: gtctccagggtgatgatggt	196	101	0.99	
hsp70	BG933934	F: ccctgtccctgggtattg	R: caccaggctggttgtctgagt	121	94	1.00	(Olsvik et al., 2011)
ckm3	BT043724	F: gcaagaggaatctggcacc	R: gacatgctcgttccacatgaag	192	95	1.00	(Rojas et al., 2018)

actin) were measured in separate duplicate reactions using a Bio-Rad CFX96 and manufacturer recommended cycling conditions. Each 12 µL reaction contained 1x Power SYBR Green (Life Technologies), 200 nM of each gene-specific primer (Table 1), and 3 µL of diluted cDNA, non-reverse-transcribed control, or molecular-grade water. All cDNA reactions produced single-peak dissociation curves at the predicted melt temperature. Reactions with non-reverse-transcribed controls or water as template failed to amplify, verifying primer specificity. Average threshold cycle values for each sample were used to calculate the transcript abundances from 5-point calibration curves generated for each primer set using serially diluted cDNA (Table 1). Abundance values were then standardized to the mean expression of the 2 reference genes, which did not change across experimental treatments.

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 WSFd related. The effects of WSFd concentration on swimming performance (U_{crit1}, U_{crit2}, 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 WSFd 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 $\alpha = 0.05$. Data is presented as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. WSFd exposure

Water samples collected at 0 d, 10 d, and 21 d confirmed the presence of PAC in experimental tanks supplied with WSFd, 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).

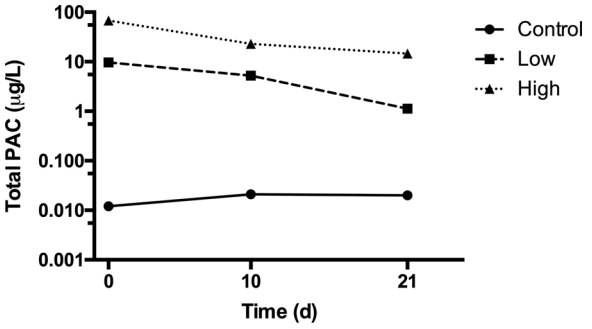

Fig. 1. Mean total polycyclic aromatic compounds (PAC) in triplicate experimental tanks during the 24 d exposure to dilbit, for each of three concentrations: Control (no dilbit, circles), Low (squares), and High (triangles) concentrations.

3.2. Effects of WSFd 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). WSFd 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 WSFd 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 WSFd, the U_{crit} 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, U_{crit2} for the low exposure group was 15 % higher than the high exposure group ($P = 0.038$) but there were no significant differences in U_{crit2} between the control and high exposure groups, or the control and low exposure groups. The recovery ratio (U_{crit2}/U_{crit}) 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 U_{crit}, U_{crit2}, or recovery ratio between experimental exposure groups (Fig. 2B, C).

3.3. Effects of WSFd exposure on blood parameters of Atlantic salmon

Lactate, glucose, and glycerol levels of plasma were similar among WSFd exposure groups (Table 3). A significant interaction between time

Table 2
Change in mass (ΔM), fork length (ΔFL), and condition factor (ΔK) of Atlantic salmon smolts after 24 d exposure (Exp) to uncontaminated water (Control) or one of two concentrations of WSFd (Low, High). A subsample of fish from each exposure group were additionally sampled after a 7 d (Dep 1) or 14 d (Dep 2) depuration period in uncontaminated water. Differences in each parameter were determined by two-way ANOVA and Holm-Sidak multiple comparisons test. Values are mean ± S.E.M. The number of biological replicates is given in brackets. *nsd*, not statistically different.

	ΔM (g)			ΔFL (mm)			ΔK		
	Exp	Dep 1	Dep 2	Exp	Dep 1	Dep 2	Exp	Dep 1	Dep 2
Control	−3.32 ± 0.32 (14)	−4.14 ± 0.39 (14)	−4.54 ± 0.58 (11)	3.64 ± 0.55 (14)	5.21 ± 0.79 (14)	4.67 ± 0.93 (11)	−0.15 ± 0.01 (14)	−0.19 ± 0.01 (14)	−0.19 ± 0.02 (11)
Low	−4.01 ± 0.28 (13)	−4.69 ± 0.29 (14)	−4.74 ± 0.46 (14)	3.77 ± 0.32 (13)	4.50 ± 0.31 (14)	4.79 ± 0.68 (14)	−0.17 ± 0.01 (13)	−0.18 ± 0.01 (14)	−0.20 ± 0.01 (14)
High	−4.26 ± 0.52 (14)	−4.51 ± 0.71 (13)	−5.07 ± 0.7 (6)	4.36 ± 0.67 (14)	4.54 ± 0.73 (13)	4.67 ± 0.42 (6)	−0.18 ± 0.01 (14)	−0.18 ± 0.02 (13)	−0.20 ± 0.02 (6)
Statistics	Concentration: *nsd* Time: P = 0.078 Interaction: *nsd*			Concentration: *nsd* Time: *nsd* Interaction: *nsd*			Concentration: *nsd* Time: P = 0.045 (Dep 2 > Exp) Interaction: *nsd*		

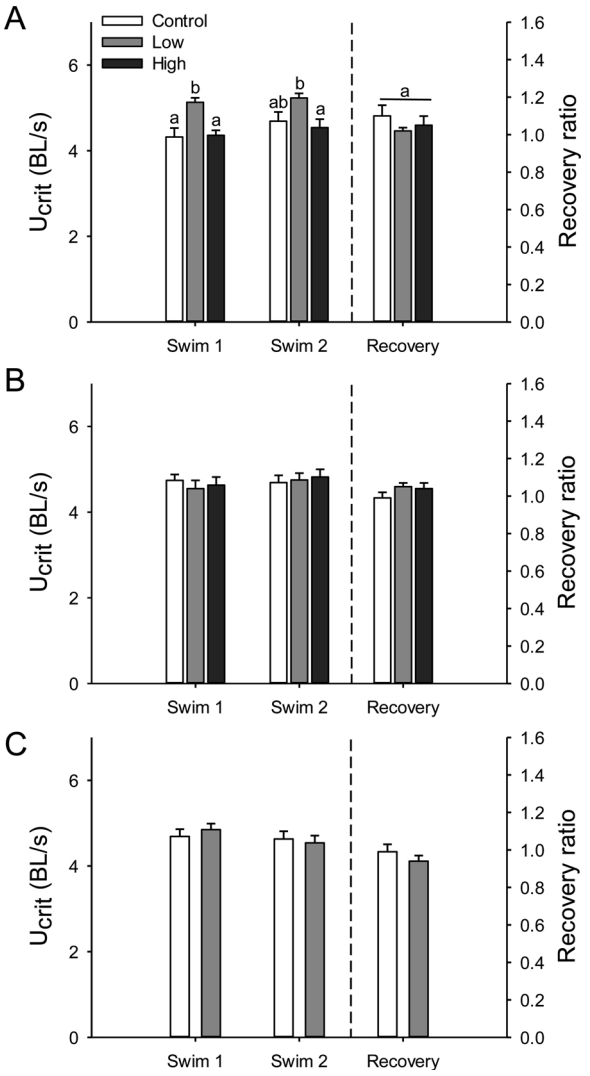

Fig. 2. Swimming performance of Atlantic salmon smolts, measured in successive critical swimming speed tests (Swim 1, Swim 2) separated by a 24 h rest period. Recovery ratio was quantified for each individual as Swim 1/Swim 2, and data are mean ± S.E.M. for each treatment (N = 8). Swimming trials were performed on subsets of fish exposed to the water-soluble fraction of diluted bitumen (WSFd) at one of three concentrations (Control: 0 µg/L, Low: 9.65 µg/L, High: 67.9 µg/L) for 24 d **(A)**, and after a 7 d **(B)** or 14 d **(C)** depuration period in freshwater. No fish from the High exposure group were tested at 14 d depuration. Within each parameter, bars that do not share a letter are significantly different (one-way ANOVA and Holm-Sidak method).

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),

Table 3
Metabolite concentrations in plasma and striated muscles of fish exposed to initial PAC concentrations of 0 µg/L (Control, C), 9.65 µg/L (Low, L), or 67.9 µg/L (High, H) for 24 d (Exp) with or without a subsequent 7 d depuration (Dep 1). Data were analyzed by two-way ANOVA and Holm-Sidak multiple comparisons tests, and differences are presented in bold font. Values are mean ± S.E.M. of n = 6–8 biological replicates; ND, not determined; *nsd*, no statistical differences.

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

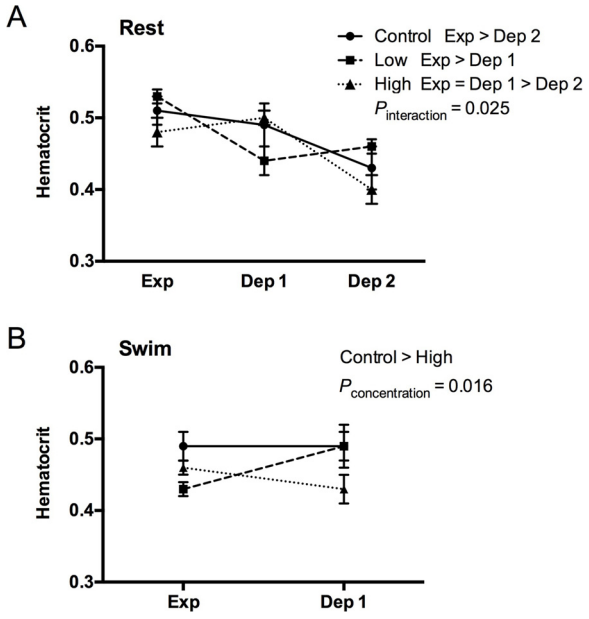

Fig. 3. Hematocrit in Atlantic salmon smolts sampled at rest **(A)** or following a successive critical swimming speed test **(B)**. Fish were exposed to uncontaminated water (Control) or the water-soluble fraction of dilbit (Low: 9.65 µg/L, High: 67.9 µg/L) for 24 d (Exp), and a subset of fish were 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.

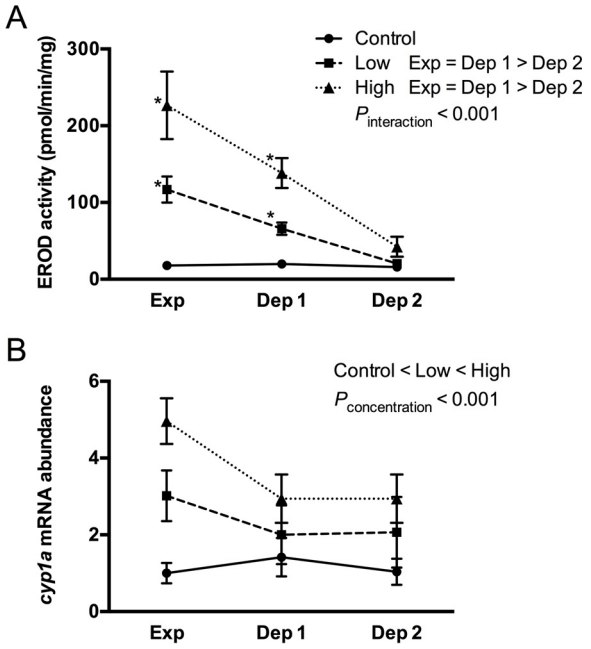

Fig. 4. Indicators of Phase 1 biotransformation in Atlantic salmon smolts exposed to various concentrations (Control: 0 µg/L, Low: 9.65 µg/L, High: 67.9 µg/L total PAC) of WSFd for 24 d (Exp), with a subset of fish given a 7 d or 14 d depuration period in clean water prior to sampling (Dep 1 or Dep 2, 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.

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 ($P_{concentration}$ < 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; $P_{concentration}$ = 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; $P_{concentration}$ = 0.002). Red muscle COX activity decreased by ~40 % in the low and high exposure groups, but returned to control levels

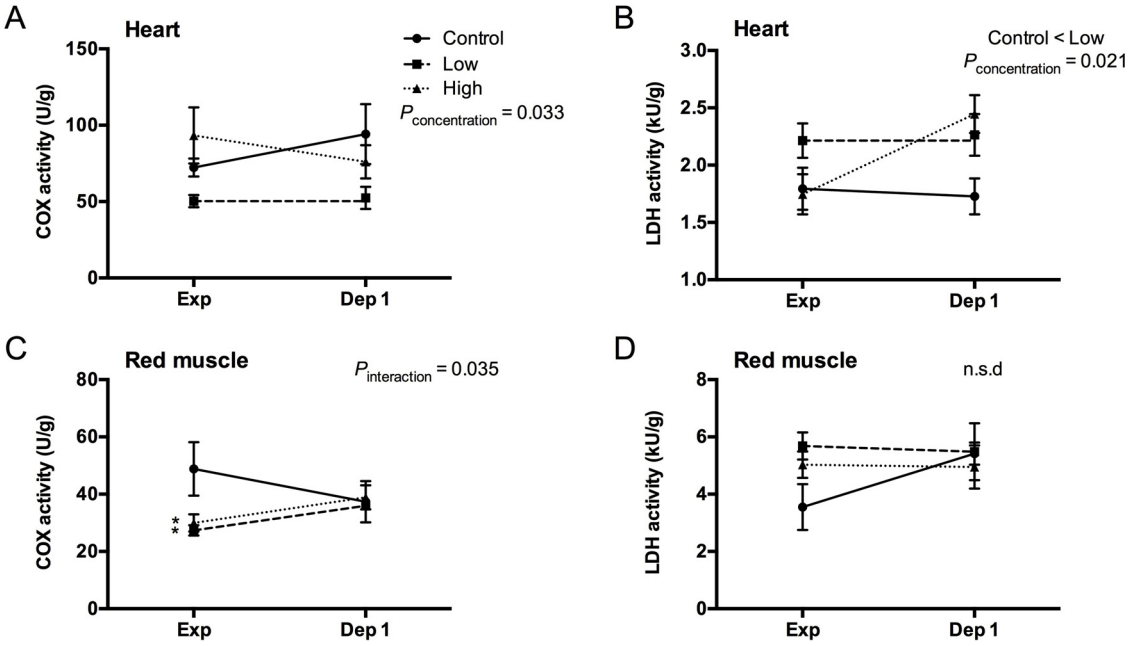

Fig. 5. Cytochrome c oxidase (COX) and lactate dehydrogenase (LDH) activities in heart (**A** and **B**, respectively) and red muscles (**C** and **D**, respectively) of Atlantic salmon smolts exposed to various concentrations (Control: 0 μg/L, Low: 9.65 μg/L, High: 67.9 μg/L initial PAC) of WSFd for 24 d (Exp), with a subset of fish given a 7 d depuration period in clean water prior to sampling (Dep 1). Differences were determined by two-way ANOVA and Holm-Sidak multiple comparisons tests (N = 6–8; $p < 0.05$). Differences are indicated with text for each panel. 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

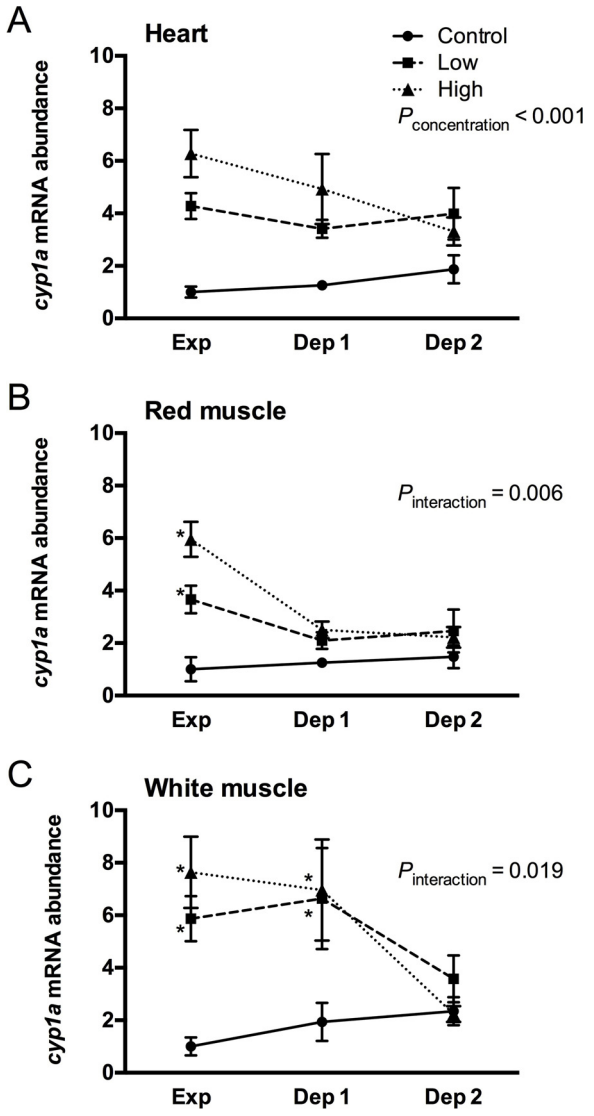

Fig. 6. Gene expression changes of cytochrome P450 type 1a (*cyp1a*) in the heart **(A)**, red skeletal muscle **(B)**, and white skeletal muscle **(C)** of Atlantic salmon smolts exposed to various concentrations (Control: 0 µg/L, Low: 9.65 µg/L, High: 67.9 µg/L total PAC) of WSFd for 24 d (Exp), with 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 = 3–6; $p < 0.05$). Differences are indicated with text for each panel, and asterisks indicate differences between concentrations within a time point. Data are mean ± S.E.M.

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 (*Liza 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 Atlantics 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 dilbit-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 pallasii*) 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

Table 4
Molecular responses to WSFd 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.

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

light of the functional anatomy of the teleost circulatory system, where the heart is exposed to more blood per gram of tissue per unit time than any other tissue, a sustained increase in *cyp1a* may help protect the heart from immediate and future contaminant exposure. While *cyp1a* induction is common in the endocardial vasculature of teleosts (Sarasquete and Segner, 2000), it will be important to consider PAC uptake rates and bioaccumulation in this tissue to fully appreciate the role of cardiac Cyp1A in crude oil exposed fish.

4.3. Effects of WSFd 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 U_{crit} in fish exposed to the lowest concentration of WSFd coincided with reduced COX activity in cardiac and red skeletal muscles as well as increased LDH activity in the heart. While cardiac and red skeletal muscles are highly aerobic and typically rely on mitochondrial respiration for most of their cellular energy (Hickey et al., 2009), these findings suggest that a shift towards anaerobic metabolism may have aided in enhancing the swimming performance in these fish. A shift towards glycolytic metabolism following an acute exposure to crude oil was also demonstrated in European sea bass with a hypoxia-tolerant phenotype, however this response reduced hypoxia resistance in these fish (Zhang et al., 2017).

Despite an apparent increased reliance on anaerobic metabolism within the cardiac and red muscles of exposed Atlantic salmon in the current study, tissue and blood lactate levels did not increase in these fish, nor was the decrease in cardiac glycogen content statistically different from that of unexposed control fish. Previous studies of young adult mahi-mahi acutely exposed to Deepwater Horizon oil revealed reduced mitochondrial affinity for ADP, which would ultimately limit cellular ATP supply within aerobic tissues (Kirby et al., 2019). In contrast, mitochondrial respiration was largely unaffected in cardiac and red muscle fibres of red drum (*Sciaenops ocellatus*) exposed acutely to crude oil (Johansen and Esbaugh, 2019). It is possible that in the current study, the increased energy demand imposed by sustained swimming results in lactate being shunted to the TCA cycle *via* pyruvate decarboxylation to be utilized for ATP, or is being used for the formation of glucose *via* gluconeogenesis (Bembenek-Bailey et al., 2019; Lin et al., 2009; Van Scoy et al., 2010). It is known that rainbow trout hearts readily metabolize lactate (Farrell et al., 1988). Additionally, the elevated levels of triglycerides in the cardiac muscle of Atlantic salmon exposed to 67.9 μg/L PAC, suggests decreased lipid utilization for aerobic ATP production; this effect persists for at least 7 d post-exposure. This is in agreement with previous studies, where sockeye embryos exposed chronically to dilbit show a concentration-dependent increase in total body triglyceride levels (Alderman et al., 2018). Also, fish exposed acutely to unweathered crude oil had an increased prevalence of lipid vacuole accumulation in the cardiac muscle (Hook et al., 2018) although it is not clear whether this was due to the reduced activity of enzymes involved in fatty acid metabolism or an increase in macromolecule synthesis.

4.4. Effects of WSFd exposure on gene expression in striated muscle

Activation of the AhR can increase the production of reactive oxygen species (ROS) during Cyp1A-mediated xenobiotic metabolism, resulting in oxidative stress in cells (Regoli and Giuliani, 2014). A first line of defense against ROS is the antioxidant defense system that includes superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase (Regoli and Giuliani, 2014). The composition of the antioxidant system, as well as the relative activities of the different enzymes, varies between tissues and can be reorganized to establish ROS homeostasis with varying oxidative demand (Pavlović et al., 2004). Thus aerobic tissues such as the heart and red muscle are expected to respond differently to any oxidative stress induced by PAC exposure compared to white muscle, which has comparatively lower mitochondrial content and oxidative metabolic capacity. In the current study, the increased expression of genes associated with oxidative and cellular stress, protein degradation, and the tumorigenic response in the white muscle of Atlantic salmon exposed to WSFd suggests that there may be an active response to reduce cellular damage associated with PAC exposure. Conversely, we found no support for a similar response in cardiac or red muscle tissue based on our gene expression analysis. Previous studies have shown altered expression of *gsr, gpx, cat, p53*, and *hsp70* following exposure to dilbit (Alsaadi et al., 2018; Madison et al., 2017, 2015) and conventional crude oil (Nahrgang et al., 2010). However, this is the first study to examine *ub* and *ckm3* as potential muscle biomarkers of crude oil exposure, both of which were upregulated in the white muscle of exposed Atlantic salmon. Ubiquitin labels proteins for proteasomal degradation, and increased protein ubiquitination has been observed with cellular injury following ROS exposure (Figueiredo-Pereira and Cohen, 1999). The skeletal muscle creatine kinase isoform has been used as a biomarker of muscle damage or inflammation in fish (Rojas et al., 2018), and was indicated as a potential blood biomarker of dilbit exposure in sockeye (Alderman et al., 2017a). Whether or not the gene expression changes described here support the suggestion that dilbit exposure increases muscle damage (Alderman et al., 2017a) is unknown. Regardless, white muscle appears to show a greater and broader molecular sensitivity to dilbit exposure than cardiac and red skeletal muscles, and may therefore be a more useful tissue

for biomarker quantification.

5. Conclusions

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 marks the pathological progression into heart failure (Foster et al., 2016; Tuomainen and Tavi, 2017). A metabolomics based approach would offer a more comprehensive view of dilbit-induced changes to energy production in fish muscles. It will also be important to determine whether an increased reliance on anaerobic metabolism in the heart and red muscle affect swimming endurance, as this would no doubt limit migratory success of smolts.

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Declaration of Competing Interest

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.

CRediT authorship contribution statement

Sean R. Avey: Investigation, Formal analysis, Writing - original draft, Visualization. **Christopher J. Kennedy:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Anthony P. Farrell:** Writing - review & editing, Funding acquisition. **Todd E. Gillis:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Sarah L. Alderman:** Investigation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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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.

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