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Diluted Bitumen Affects Multiple Physiological Systems in Sockeye Salmon (*Oncorhynchus nerka*) Embryo to Juvenile Life Stages

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Abstract: An understanding of the risks associated with diluted bitumen (dilbit) transport through Pacific salmon habitat necessitates the identification and quantification of hazards posed to early life stages. Sockeye from the embryo to juvenile stage (8 months old) were exposed to four concentrations of the water-soluble fraction of Cold Lake dilbit (summer blend; concentrations of 0, 13.7, 34.7, and 124.5 µg/L total polycyclic aromatic compounds). Significant mortality (up to 18% over controls) only occurred in the embryo to swim-up fry stage. Impaired growth was seen in the alevin, swim-up, and juvenile stages (maximum reduction 15% in mass but not fork length). Reductions in both critical (maximum 24% reductions) and burst (maximum 47% reductions) swimming speed in swim-up fry and juveniles were seen. Alterations in energy substrate reserves (reductions in soluble protein and glycogen content, elevations in whole-body lipid and triglyceride levels) at all stages may underlie the effects seen in swimming and growth. Dilbit exposure induced a preexercise physiological stress response that affected the recovery of postexercise biochemistry (cortisol, glycogen, lactate, triglyceride concentrations). The transcript abundance of the cytochrome P450 1A gene (*cyp1a*) was quantified in alevin head regions (containing the heart) and in the hearts of swim-up fry and juveniles and showed a concentration-dependent increase in the expression of *cyp1a* at all life stages. *Environ Toxicol Chem* 2022;41:1937–1949. © 2022 SETAC

Keywords: Diluted bitumen; Sockeye salmon; Early life stages; Swimming; Gene expression; Biochemistry

INTRODUCTION

Canada has the world's third largest crude oil reserves, which are estimated at 1.67 trillion barrels, with 96% of proven reserves contained in oil sands deposits located in the Western Canada Sedimentary Basin (Natural Resources Canada, 2020). The extraction of bitumen from the oil sands has increased exponentially over the past decade, with extraction rates projected to increase from the current 2.8 to 4.5 million barrels/day by 2040 (Canada Energy Regulator, 2019). Raw bitumen naturally has high viscosity and density; extracted bitumen is processed and diluted with other, lighter petroleum products (e.g., natural gas condensate or synthetic oil) to facilitate transportation via pipeline (Dew et al., 2015). Diluted bitumen (dilbit; 20%–30% natural gas condensate, 70%–80% bitumen) is the most frequently transported bitumen product in currently employed pipeline networks across North America (Crosby et al., 2013; Environment and Climate Change Canada et al., 2013). To cope with increasing global demand for petroleum products, multiple pipeline projects have been proposed in recent years, aiming to increase the exports of Canadian oil sands products (Levy, 2009; National Energy Board [NEB], 2019). The construction of new pipeline and the expansion of existing infrastructure are expected to provide convenient and cost-efficient means for transporting dilbit from remote production sites to coastal regions for refining and eventual overseas shipping (NEB, 2019). The anticipated increase in dilbit transportation (e.g., pipeline, tanker, and rail) raises concerns regarding the potential for a spill event following a pipeline failure or a tanker accident.

Different petroleum products will exhibit unique environmental fate/behaviors and environmental impacts if spilled; limited evidence with dilbit indicates that releases will result in challenging postspill cleanup and habitat recovery (Alsaadi, Hodson, & Langlois, 2018; Dew et al., 2015). For example, it is estimated that nearly 1 million L (up to 30%) of the residual oils from the Kalamazoo River dilbit spill (2010) are associated with sediments and have remained in the river system for years

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following cleanup efforts (US Environmental Protection Agency, 2013); this suggests that investigations utilizing sublethal and chronic exposure scenarios are needed (Alderman et al., 2018).

Dilbit is a mixture of various petrogenic hydrocarbons (e.g., benzene, toluene, ethylbenzene, xylenes [BTEX], polycyclic aromatic compounds [PACs], and naphthenic acids, among others) with demonstrated toxicity to fish. Acute and sublethal effects following exposure to other crudes or their constituents include developmental defects at early life stages (ELS), impaired growth, reductions in reproductive capacity, changes in behavior, alterations in biochemistry and gene expression, suppressed immune function, genetic damage, and endocrine disruption (Dupuis & Ucan-Marin, 2015; Kennedy, 2014; National Academies of Sciences, Engineering, and Medicine [NASEM], 2016).

Limited studies exist on the toxicity of dilbit to fish species (Dupuis & Ucan-Marin, 2015; NASEM, 2016); therefore, uncertainties regarding the hazards associated with this complex mixture exist. Constituent profiles can vary greatly between products and blends, leading to reservations in predicting dilbit toxicity from data that exist for other crude oils (NASEM, 2016). Early studies on dilbit toxicity focused on embryonic and larval life stages, where effects tend to be greatest in fish exposed to conventional crude oils (see Alsaadi, Madison et al., 2018; Madison, Hodson, & Langlois, 2015; Madison et al., 2017; McDonnell et al., 2019; Philibert et al., 2016). More recent studies investigating effects in older life stages at risk of dilbit exposure support the need for a deeper appreciation of life-specific responses (e.g., fry and juveniles; Alderman et al., 2018, 2020; Alderman, Dindia et al., 2017; Alderman, Lin et al., 2017; Avey et al., 2020; Lin et al., 2020, 2021).

Freshwater and estuarine salmon habitats are at risk because of proposed and existing pipeline and rail routes, as well as the use of marine tanker terminals in the Canadian Pacific Northwest (Levy, 2009; Raincoast Conservation Foundation, 2018). As one of the most productive salmon migration routes in the world, the Fraser River watershed and its estuary serve as vital spawning and nursery habitat for all five species of Pacific salmon (Henderson & Graham, 1998; Labelle, 2009). There is an increasing body of evidence suggesting that dilbit exposure can negatively affect the survival, early development, and critical physiological systems of Pacific salmon at extremely low environmental concentrations. These effects include delayed hatching time, mortality during embryonic development, deformities, impairment of growth, and alteration of body composition (Alderman et al., 2018). Older ELS are also affected by dilbit exposure. For example, exposed 1+-year-old sockeye exhibit altered gene expression, decreased swimming ability, as well as alterations in cardiac tissues and the plasma proteome (Alderman, Dindia et al., 2017; Alderman, Lin et al., 2017). The potential for longer-term exposures to dilbit following a spill in salmon habitat and the growing evidence of pronounced effects in salmon were the impetus for the present study. In the present study, the effects of a chronic dilbit exposure on developing sockeye through several life stages from the embryo to the juvenile stage were investigated using a suite of endpoints known to have direct relevance to salmon survival and performance (survival, growth, biochemistry, gene expression, swim performance, and exercise recovery).

MATERIALS AND METHODS

Fish

Sockeye gametes were obtained from the Upper Pitt River Hatchery (Fisheries and Oceans Canada) and fertilized according to standard procedures (Ontario Ministry of Natural Resources, 2009). Embryos were incubated in heath trays (MariSource; 372 embryos per tray; mean mass 0.24 ± 0.11 g [mean ± standard deviation]) supplied with dechlorinated municipal water (flow rate 6 L/min; dissolved O_2 > 95% saturation, hardness 6.12 mg/L $CaCO_3$, dissolved organic carbon < 1 mg/L, pH 7.0) at 11.3 °C in the dark until the swim-up fry stage (no visible external yolk sac). Mortality was recorded daily under red light, and dead embryos were immediately removed from trays. Swim-up fry were collected from rearing trays and transferred to 250-L fiberglass tanks supplied with dechlorinated water at 13 °C (flow rate 7.5 L/min and 12:12-h light:dark photoperiod). Fry were fed 5% body weight/day commercial salmonid feed (Skretting Canada), which was increased weekly according to a growth equation that included a feed conversion efficiency of 20% (Meador et al., 2006) until fish were 8 months of age (juveniles). The care and use of all fish were approved by the University Animal Care Committee at Simon Fraser University following Canadian Council on Animal Care guidelines (1315B-20).

Exposure

The water-soluble fraction of dilbit was generated as previously described (Alderman, Dindia et al., 2017; Kennedy & Farrell, 2005). In brief, Siproax® ceramic beads (Aquatic Eco-Systems) were soaked (except controls) in unweathered Cold Lake Blend summer dilbit (COOGER, DFO) for 24 h and then placed into polyvinyl chloride columns (16 cm diameter × 80 cm length) supplied with an upward-directed continuous flow of dechlorinated municipal water (6 L/min). Varying the number of beads/column provided four different water-soluble fraction concentrations (in duplicate); columns were "recharged" every 14 days with newly soaked beads. Water containing the water-soluble fraction of dilbit flowed into 500-L fiberglass header tanks and was then distributed into heath stacks. Embryos were exposed to dilbit in duplicate heath stacks immediately postfertilization and continued until fish reached the swim-up stage. At this stage, fish were transferred into 200-L fiberglass tanks (n = 200 fish in each duplicate tank) supplied with dilbit-water-soluble fraction water and exposed for a further 90 days until fish were 8 months of age (8-month total exposure). The detailed experimental design is depicted in Figure 1.

Water samples were collected from duplicate header tanks at 0, 7, and 14 days after initiation of water-soluble fraction generation and analyzed for individual PACs using gas chromatography–mass spectrometry, as previously described

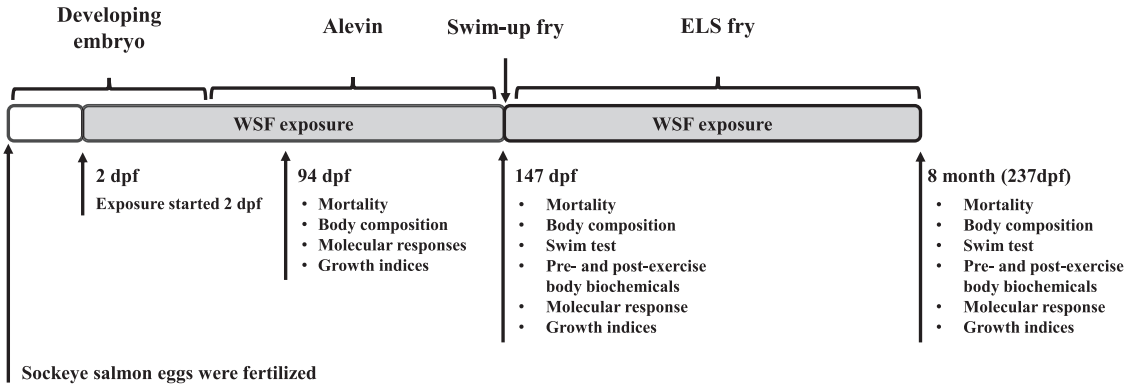

FIGURE 1: Schematic of the experimental design. ELS = early life stage; WSF = water-soluble fraction; dpf = days postfertilization.

(Alderman, Dindia et al., 2017). Individual PAC concentrations were measured by Axys Analytical Services following standard procedures. All samples were spiked with deuterated surrogate standards prior to dichloromethane extraction and cleanup with silica column chromatography. Low-resolution mass spectrometry using an RTX-5 capillary gas chromatography column operated in the electron impact ionization mode using multiple ion detection was used, acquiring at least one characteristic ion for each target analyte and surrogate standard. Reporting limits for individual compounds ranged from 0.13 to 1.13 ng/L (mean 0.42 ng/L), and the mean percentage recovery was 100.3%. Concentrations of target PACs were calculated using the isotope dilution method of quantification and expressed as percentage of total PAH (Alderman, Dindia et al., 2017).

Tissue collection

Developing alevins ($n = 35$–94 days postfertilization [dpf]), swim-up fry ($n = 44$–147 dpf), and juveniles ($n = 44$–237 dpf) were randomly sampled from each exposure group (and replicate tanks), euthanized with buffered tricaine mesylate (MS-222; 1 g/L), and weighed and measured for length. For biochemical analysis, a subset of these fish ($n = 16$–20/treatment) were frozen in liquid N_2 and then transferred to −80 °C until analysis for whole-body lipid and triglyceride content. For gene transcript analysis, the head region of alevins (bisected at the rostral boundary of the yolk sac and perpendicular to the body axis) or the isolated hearts (fry and juveniles) were individually frozen in liquid N_2 and stored at −80 °C for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis ($n = 8$/developmental stage, $n = 8$/treatment group, $n = 4$/replicate tank).

Swim tests

Critical swimming speed (U_{crit}) and burst swimming speed (U_{burst}) tests (Farrell, 2008; Osachoff et al., 2014) were performed for swim-up fry and juveniles ($n = 10$ from each treatment) using an isolated/blacked-out mini–swim tunnel system (Loligo® Systems). The swim tunnel system (a 1.5-L cylindrical glass chamber submerged inside a reservoir) was temperature-regulated by a custom chilled bath circulator, and dissolved oxygen was maintained at >95% by constant aeration. Fish were first acclimated for 20 min at a water velocity of 1.5 body lengths per second (BL/s) before the swim tests. Water velocity was then increased by 1.5 BL/s every 20 min until fish were exhausted (Farrell, 2008). The U_{burst} test was initiated at a water velocity of 1.0 BL/s (Farrell, 2008; Nendick et al., 2009), rapidly increased to 2 BL/s over a 1-min interval, and subsequently increased by 0.5 BL/s every 1 min. Both tests were complete when exhausted fish were inactive on the rear baffle for over 2 s and would not resume swimming after a brief decrease in water velocity. Fish were immediately removed from the tunnel and euthanized in buffered MS-222, and wet weight (grams) and fork length (centimeters) were recorded. Values for U_{crit} and U_{burst} were calculated according to Farrell (2008). The cross-sectional area of all swim-tested fish was found to be <10% of swim tunnel cross-sectional area, and fish density was <0.2 g/L; therefore, U_{crit} and U_{burst} values were not corrected for solid blocking effects (Bell & Terhune, 1970). Euthanized U_{crit}-tested fish were frozen in liquid nitrogen and immediately transferred to −80 °C for postexercise body composition analysis, as described below (see *Body composition*).

Biochemical measurements

Whole-body lipid content was measured using a standard protocol (Folch et al., 1957). Preweighed fish were thawed on ice and minced into pieces in envelopes (Whatman filter paper) that were then sealed and saturated in a chloroform:methanol (2:1) mixture (solvent:tissue 20:1), incubated for 20 min in a glass container, and shaken at 30 rpm. Samples were then washed with chloroform and dried in an oven (60 °C) for 24 h. Total-body lipid content of each individual fish was calculated by subtracting the sample's original wet weight by the net weight of the dried sample.

Total soluble protein content was quantified in preweighed whole fish that were thawed on ice and homogenized in nine volumes of lysis buffer (0.5 M Tris-HCl and 0.1 mM ethylenediaminetetraacetic acid at pH 8) using a Mixer Mill homogenizer (model MM 300; Qiagen; Cassidy et al., 2016). Crude homogenates were centrifuged at 13,000*g* at 4 °C for 60 min, and the soluble protein content in supernatants was

measured using a Bradford protein assay kit with bovine serum albumin as the standard (catalog no. 5000002; Bio-Rad).

For whole-body pre- and postexercise cortisol, glycogen, triglyceride, and lactate concentrations, preweighed alevin or fry were thawed on ice and homogenized in 0.2 M sodium citrate buffer at pH 5 (catalog no. 200-675-3; EMD Chemicals) using a Tissue Tearor (Fisher Scientific). Each crude homogenate sample was aliquoted into separate microcentrifuge tubes and stored at −80 °C until subsequent analysis. The glycogen concentration was determined following a standard protocol (Weber et al., 2008) using Type IX bovine liver glycogen as a standard (C940M53; Sigma-Aldrich). The whole-body triglyceride concentration was determined following the microplate spectrophotometric assay protocol as described in Weber et al. (2003). Whole-body lactate content of each fish was determined using a commercial colorimetric assay kit (catalog no. 120001400P; Eton Bioscience) performed according to the manufacturer's protocol. Total cortisol concentration was quantified using a commercial enzyme-linked immunosorbent assay kit (catalog no. EA65; Oxford Biomedical Research; McPhee & Janz, 2014). All colorimetric assays were performed in duplicate using an Epoch™ 2 microplate spectrophotometer (Bio-Tek) and a Corning® 96-well microplate (Greiner Bio-One International).

RT-qPCR analysis

Transcript abundances of cytochrome P450 1a (*cyp1a*; Phase I biotransformation) and ribosomal protein L8 (*rpl8*; housekeeping gene) were quantified in alevin head regions (containing the heart) and in the hearts of swim-up fry and juveniles following standard quality control guidelines (Bustin et al., 2009) and using total RNA extraction, complementary DNA synthesis, and RT-qPCR methods exactly as previously described (Alderman et al., 2018). Primer sequences were *cyp1a* (F: tcatcaacgacggcaaga, R: gttcaccaagcccaacag, 110% efficiency) and *rpl8* (F: ttggtaatgttctgcctgtg, R: gggttgtgggagatgactg, 103% efficiency). Data were normalized to the abundance of the stably expressed reference gene, *rpl8*.

Statistical analysis

No statistically significant tank effect was found (using either one-factor or two-factor analysis of variance [ANOVA]) in which *tank* was included as a random factor); therefore, data from replicate tanks were pooled for all analyses. Mortality, body composition measures (including condition factor [mass/length^3]), and swim test data were analyzed using one-factor ANOVA and Tukey's multiple comparison test ($\alpha = 0.05$). The preexercise and postexercise biochemical data from different treatment groups were combined and compared using two-factor ANOVA followed by Tukey's multiple comparison test ($\alpha = 0.05$). Differences in transcript abundances were determined using one-factor ANOVA and Tukey's multiple comparisons test, and the relative abundance of *cyp1a* in the hearts of swim-up fry and 8-month-old juveniles was compared with a two-factor ANOVA and Sidak's multiple comparisons test ($n = 8$; $\alpha = 0.05$).

RESULTS

Water chemistry

Water samples collected (two composite samples per concentration) at 0 days confirm the presence of PAC in experimental tanks supplied with water-soluble fraction of dilbit, with initial total PAC (TPAC) values ranging from 0.2 µg/L (control) to 13.7 µg/L (low) to 34.7 µg/L (medium) to 124.5 µg/L (high). The initial TPAC concentrations in water-soluble fraction of dilbit exposures are used hereafter to

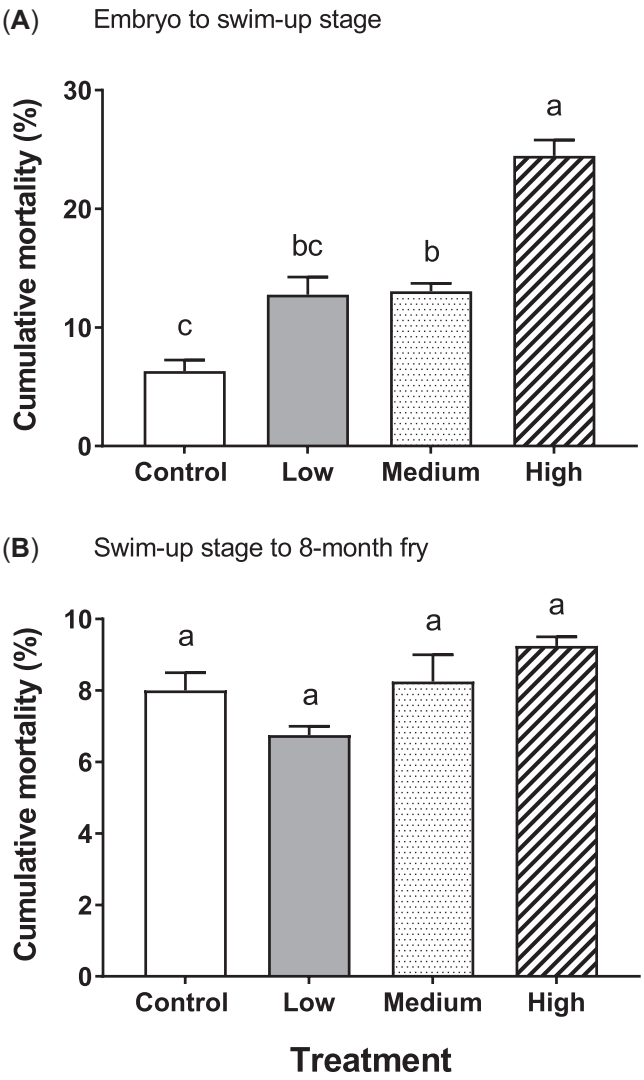

FIGURE 2: Cumulative mortality of embryos exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) µg/L. Time of exposure was (**A**) 147 days for embryo to swim-up fry and (**B**) 90 days for swim-up stage to 8-month-old juveniles. One-factor analysis of variance and Tukey's multiple comparison test were used to test for significant differences between water-soluble fraction treatment groups. Bars that do not share a common letter are statistically different ($p < 0.05$).

TABLE 1: Wet weight, fork length, and Fulton's condition factor of fish exposed to water-soluble fraction of Cold Lake Blend dilbit

Life stage	Treatment	Body mass (mg)	Fork length (mm)	Condition factor	*n*
Alevin	Control	182.9 ± 2.0A	22.6 ± 0.2A	1.61 ± 0.03A,B	70
	Low	184.1 ± 2.1A	22.2 ± 0.1A	1.70 ± 0.03A	70
	Medium	178.9 ± 1.9A,B	22.4 ± 0.1A	1.60 ± 0.02B	70
	High	173.3 ± 2.4B	22.3 ± 0.2A	1.58 ± 0.03B	70
Swim-up fry	Control	161.9 ± 0.9A	28.8 ± 0.1A	0.68 ± 0.003B	227
	Low	163.7 ± 0.7A	28.4 ± 0.1B	0.72 ± 0.003A	179
	Medium	140.2 ± 0.9B	27.0 ± 0.1C	0.72 ± 0.004A	177
	High	137.4 ± 1.6B	26.9 ± 0.1C	0.71 ± 0.006A	92
Juveniles	Control	673.8 ± 6.9A	39.7 ± 0.2A	1.08 ± 0.008A	368
	Low	651.3 ± 6.9A	39.3 ± 0.2A	1.08 ± 0.008A	373
	Medium	561.5 ± 6.8B	39.4 ± 0.2A	0.92 ± 0.009B	367
	High	583.8 ± 7.1B	39.6 ± 0.2A	0.94 ± 0.008B	363

The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) µg/L. Time of exposure was 147 days for embryo to swim-up fry and 90 days for swim-up stage to 8-month-old juveniles. Data are means ± standard error for $n = 20$ fish. Treatments that do not share a common letter are statistically different ($p < 0.05$).

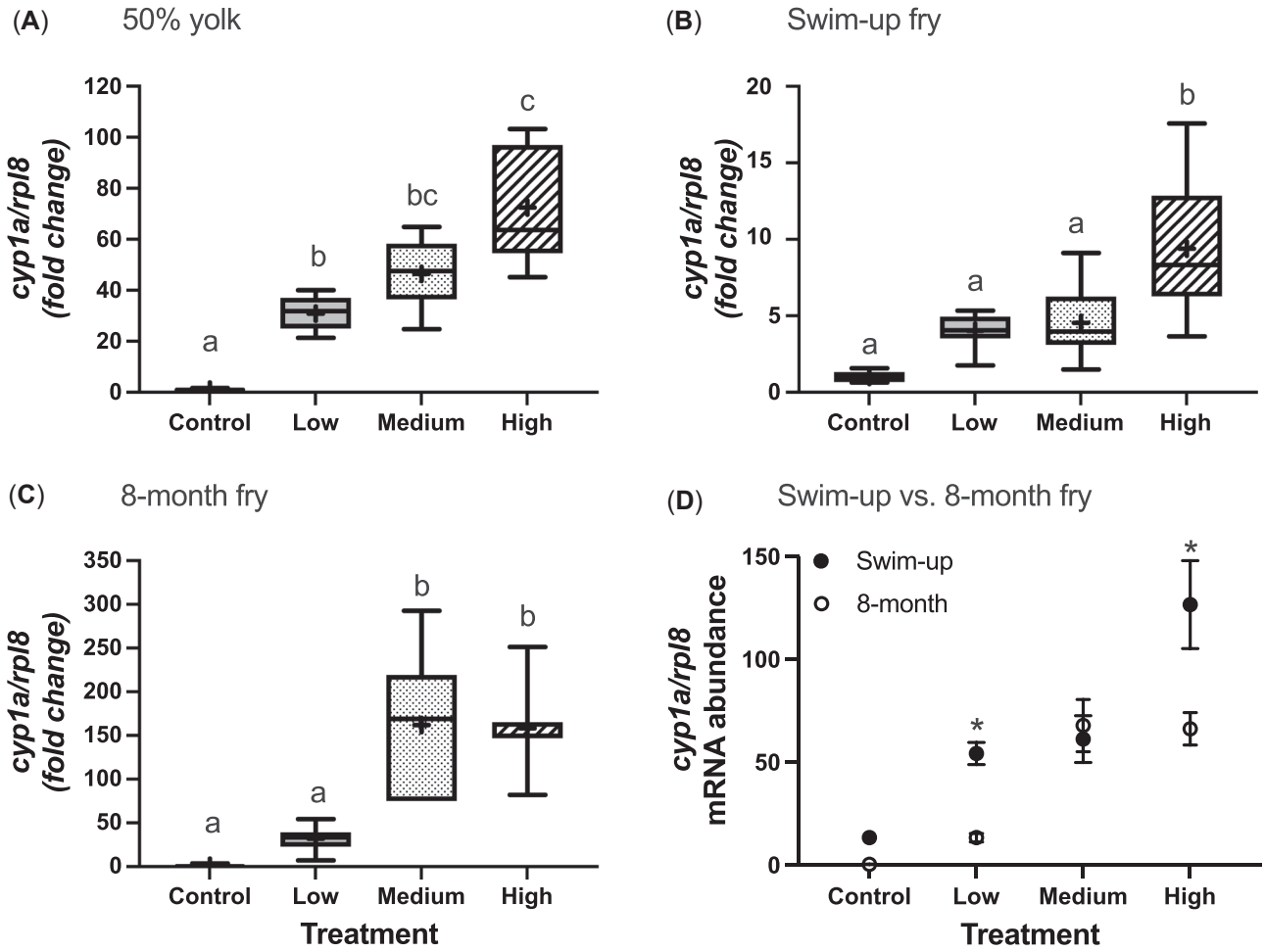

FIGURE 3: Cytochrome P450 1a (*cyp1a*) expression in (**A**) alevin head regions (50% yolk) and isolated whole hearts of (**B**) swim-up fry and (**C**) 8-month-old juveniles exposed to various concentrations of dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) µg/L. Expression was normalized to the housekeeping gene *rpl8*. Within each life stage, data are expressed as fold-change from unexposed controls, and boxes that do not share a common letter are significantly different (one-way analysis of variance [ANOVA] and Tukey's test, $n = 8$; $p < 0.001$). The mean abundance of *cyp1a* in swim-up fry versus juveniles at each concentration is shown in (**D**), with significant differences between life stages indicated with an asterisk (two-way ANOVA and Sidak's post hoc test, $n = 8$; $p_{interaction} = 0.013$). *rpl8* = ribosomal protein L8.

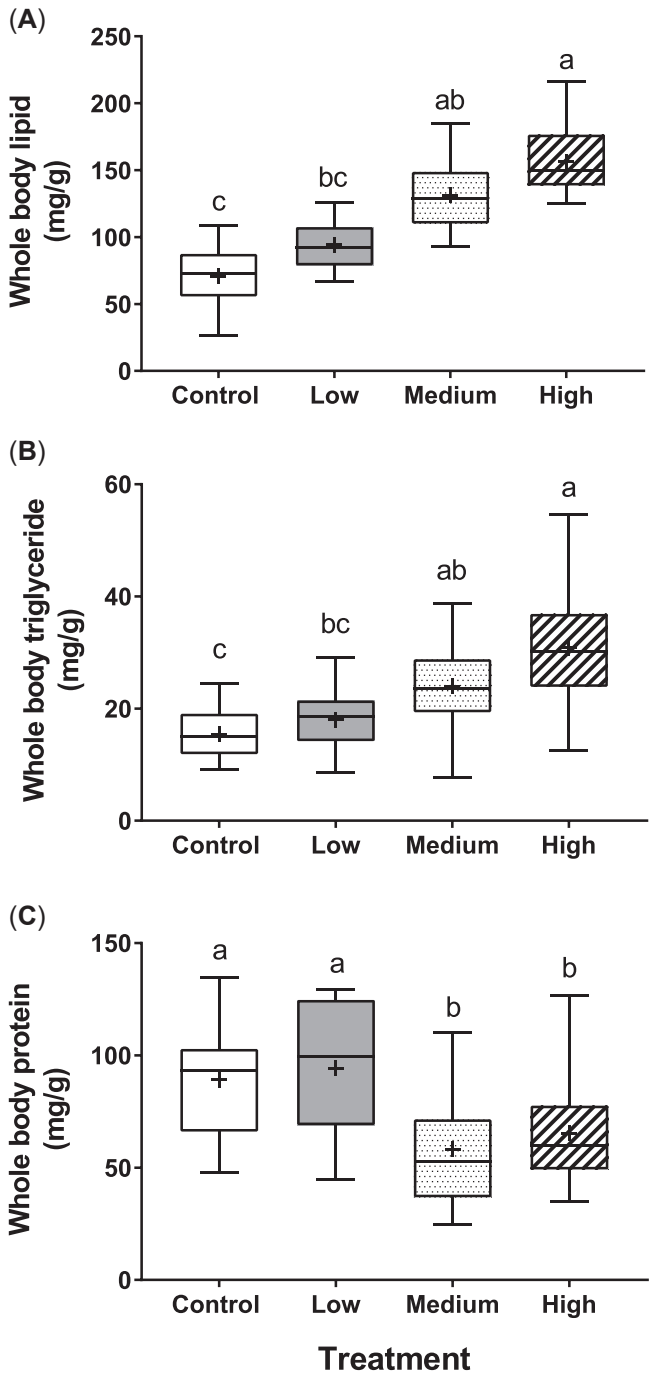

FIGURE 4: Whole-body total (**A**) lipid, (**B**) triglyceride, and (**C**) protein content in alevins exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) µg/L. Within each plot, ✚ indicates mean for $n = 20$ fish; boxes that do not share a common letter are statistically different ($p < 0.05$).

designate treatment groups. In this system, TPAC concentrations decreased by 50%–70% between 0 and 12 days and then changed very little between 12 and 25 days (Alderman, Dindia et al., 2017; Lin et al., 2020). Component breakdown for TPAC shows that smaller and more volatile hydrocarbons (e.g., naphthalenes) predominate initially, with larger PACs (e.g., phenanthrenes) becoming relatively more abundant with time (Lin et al., 2020).

Mortality and growth

Cumulative mortalities in embryos and swim-up fry in the medium and high water-soluble fraction treatments were significantly higher (medium vs. control, $p = 0.0482$; high vs. control, $p < 0.01$) than in controls; however, no significant mortality occurred in older fish from exposure ($p > 0.05$; Figure 2). Body mass was significantly lower in alevins exposed to the highest concentration compared with controls, and at the swim-up stage significantly lower body mass was seen in both the medium and high treatment groups (medium and high both $p < 0.01$), a trend which continued to the juvenile stage (Table 1). Significantly reduced length was only seen in water-soluble fraction-exposed swim-up fry (Table 1). Condition factor was marginally but significantly higher in swim-up fry and lower in juveniles at the higher two concentrations compared with controls (Table 1).

Molecular responses

There was a concentration-dependent increase in transcript abundance of *cyp1a* at all stages examined, with maximal responses of 72-fold (50% yolk sac; Figure 3A), 9-fold (swim-up fry; Figure 3B), and 162-fold (8-month-old juveniles; Figure 3C) relative to unexposed controls. The apparent low response in swim-up fry, however, is relative to an already high background expression of *cyp1a*. Specifically, expression of *cyp1a* in the hearts of unexposed control swim-up fry was 32-fold greater than in the hearts of unexposed control juveniles, and the peak response observed in fish exposed to the highest concentration of dilbit was also relatively higher in swim-up fry hearts (Figure 3D).

Body composition

Developing alevins exposed to dilbit exhibited increased total-body lipid and triglyceride concentrations and a reduced total soluble protein content compared with controls (Figure 4); whole-body lipid contents in the medium and high treatment groups were 1.9-fold and 2.2-fold higher than in controls ($p = 0.048$, $p = 0.015$), and triglyceride levels in these two groups were elevated by 1.6-fold and 2.0-fold, respectively ($p = 0.034$, $p < 0.01$). Lower protein content (35%, high vs. control, $p = 0.025$; 27%, medium vs. control, $p < 0.01$, respectively) was also seen. Swim-up fry in the two higher treatment groups exhibited an increased total lipid content, 2.0-fold and 2.2-fold higher than controls (Figure 5; medium vs. control, $p < 0.01$; high vs. control, $p < 0.01$) as well as lower whole-body soluble protein levels (medium vs. control, by 33.6%, $p = 0.0241$; high vs. control, 42.1%, $p < 0.01$; Figure 5). Juveniles in the high exposure group had a higher lipid content (1.7-fold, $p < 0.01$) and lower whole-body soluble protein levels

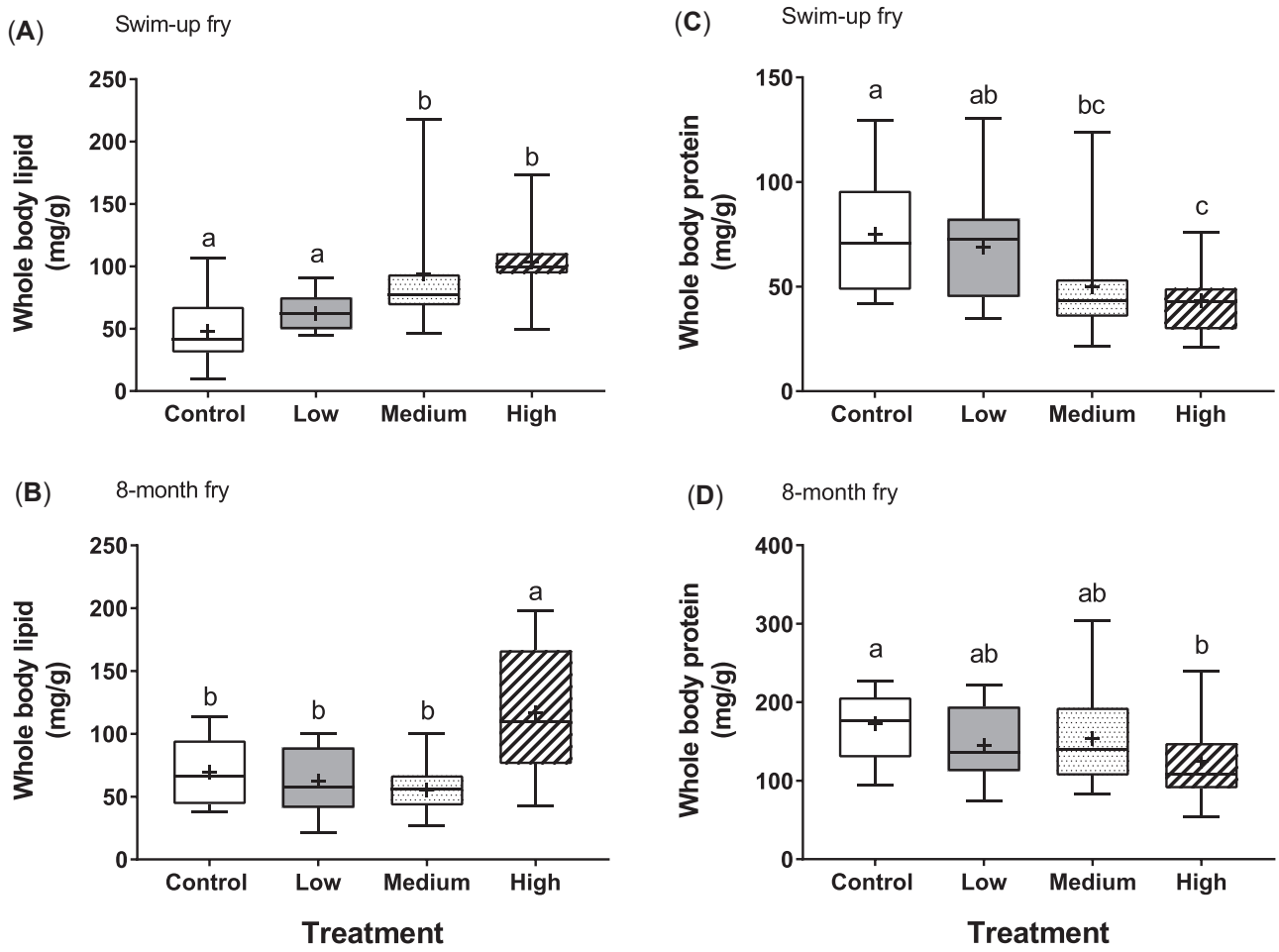

FIGURE 5: Total lipid content (**A,B**) and total protein content in (**C,D**) swim-up fry and juveniles exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) μg/L. Within each plot, ✚ indicates mean for $n = 16$ fish; boxes that do not share a common letter are statistically different ($p < 0.05$).

(by 27%) in the high treatment group ($p = 0.048$; Figure 5) compared with controls.

Swim performance

Values of U_{crit} and U_{burst} were affected by exposure in both swim-ups and juveniles. Values of U_{crit} for swim-up fry in the medium and high treatments were 20% lower than those of controls (Figure 6; medium vs. control, $p < 0.01$; high vs. control, $p < 0.01$); for juveniles, U_{crit} was reduced by 22.8% compared with controls in the high treatment group ($p < 0.01$). Values of U_{burst} were decreased in swim-up fry in the medium and high treatments (26% and 39%, respectively) compared with controls (Figure 6; medium vs. control, $p < 0.01$; high vs. control, $p < 0.01$). For the juveniles, the medium and high groups exhibited decreased U_{burst} by 16.3% and 22.2%, respectively, compared with controls ($p < 0.01$, $p < 0.01$).

Pre- and postexercise biochemistry

Preexercise (baseline) whole-body cortisol concentrations in swim-ups and juveniles were higher (range 1.7- to 2.7-fold) in fish exposed to the medium and high treatment groups compared with controls ($p < 0.01$, $p = 0.037$; Figure 7). Fish that underwent the U_{crit} test exhibited a significant elevation in whole-body cortisol concentrations in controls (2.1-fold, $p = 0.049$) and the low treatment group (2.2-fold, $p = 0.023$) in swim-up fry. In contrast, postexercise cortisol concentrations in fish from the medium and high treatments were not significantly different from preexercise baseline values. This lack of an exercise-induced cortisol increase was consistently observed in juveniles in all three dilbit treatment groups (Figure 7).

Preexercise body glycogen reserves were lower in fish in the higher treatment groups compared with controls (medium vs. control, $p < 0.01$; high vs. control, $p < 0.01$; Figure 7). The U_{crit} test resulted in significant reductions in total-body glycogen content in both control and water-soluble fraction-exposed swim-ups and juveniles; however, in juveniles, exposure to the highest concentration further reduced glycogen stores compared with controls ($p < 0.01$; Figure 7).

For both swim-ups and juveniles, preexercise lactate levels of exposed fish were not significantly different from those of controls ($p > 0.05$; Figure 8). For both swim-up fry and juveniles, significant increases in whole-body lactate levels were

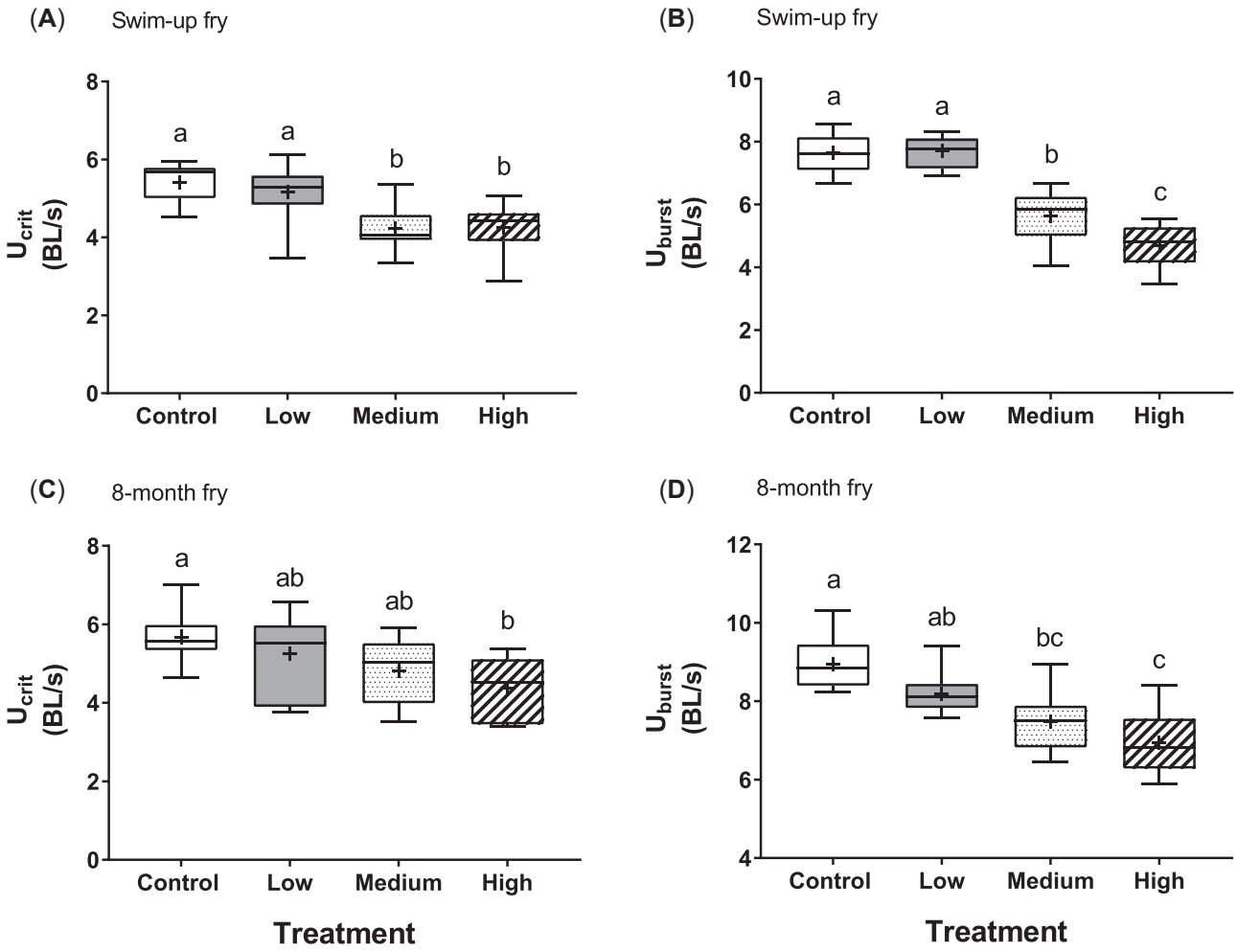

FIGURE 6: Critical and burst swimming speed of (**A,B**) swim-up fry and (**C,D**) juveniles exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) μg/L. Within each plot, ✚ indicates mean for $n = 10$ fish; boxes that do not share a common letter are statistically different ($p < 0.05$). U_{crit} = critical swimming speed; U_{burst} = burst swimming speed; BL = body length.

observed following the U_{crit} trial in both control and exposed fish ($p < 0.01$; Figure 8). For both swim-up fry and juveniles, only fish exposed to the highest water-soluble fraction had higher postexercise lactate when compared with the controls ($p = 0.0225$, $p = 0.0193$).

Whole-body triglyceride levels in preexercised swim-ups were significantly higher in the medium and high treatment groups ($p < 0.01$ and $p < 0.01$, respectively) and in the high treatment group in juveniles compared with controls ($p < 0.01$, Figure 8). Exercise caused significant decreases in control and low treatment swim-ups ($p = 0.0189$, $p = 0.025$) and in juveniles ($p = 0.0484$, $p = 0.0275$). For both swim-up fry and juveniles, fish exposed to the highest concentration of water-soluble fraction did not exhibit a significant decrease in whole-body triglyceride following exhaustive swimming exercise as was seen in other groups ($p > 0.05$; Figure 8).

DISCUSSION

Evidence is growing that Pacific salmon ELS, particularly developing embryos and alevins, are more sensitive to dilbit than other species and that tolerance in salmon increases with age. Dilbit-induced lethality in developing sockeye was life stage–dependent; embryos through the swim-up stage were most sensitive with mortality occurring at TPAC as low as 34.7 μg/L. Alderman et al. (2018) reported higher sensitivity, with mortality in sockeye embryos at TPAC concentrations as low as 4 μg/L; however, at a similar concentration (35 μg/L TPAC), overall mortality was similar (8% vs. 13%). No mortality was seen in dilbit-exposed 1+-year sockeye parr exposed to concentrations between 3.5 and 66.7 μg/L (Alderman, Dindia et al., 2017). Developing pink salmon (*Oncorhynchus gorbuscha*) embryos exhibited mortality at a similar total polycyclic aromatic hydrocarbon (TPAH) range (18–48 μg/L) using a similar dosing method (Heintz et al., 1999). In contrast, larval fathead minnow (*Pimephales promelas*) and inland silverside (*Menidia beryllina*) exhibited no acute lethality at TPAH of 8–40 μg/L (Barron et al., 2018). Low lethal toxicity has also been reported in zebrafish (*Danio rerio*, 28 μg/L), fathead minnow, Japanese medaka (*Oryzias latipes*), and yellow perch (*Perca flavescens*; at TPAH <100 μg/L) during embryonic development (Alsaadi, Madison et al., 2018; Madison, Hodson, & Langlois, 2015;

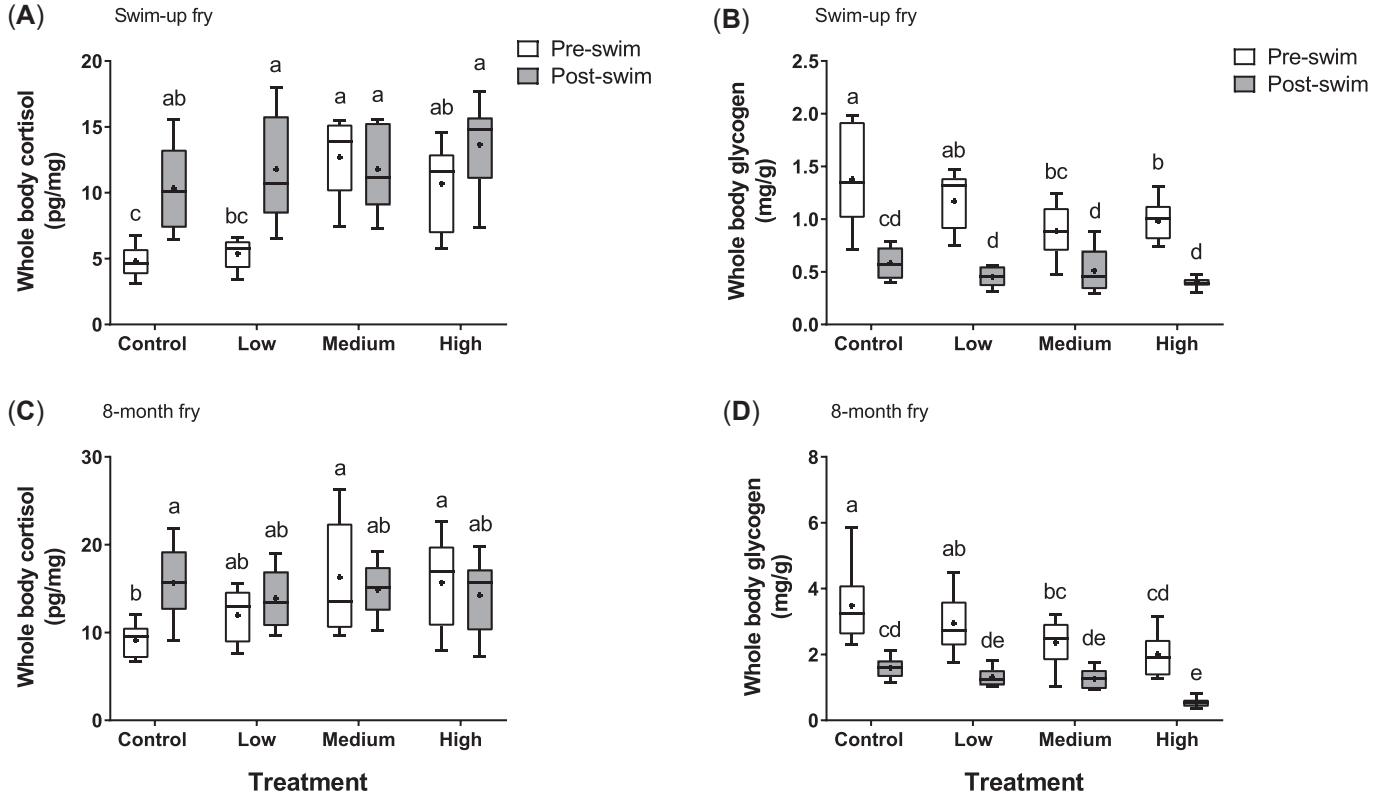

FIGURE 7: Preexercise and postexercise whole-body cortisol content and glycogen content in (**A,B**) swim-up fry and (**C,D**) juveniles exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) μg/L. Within each plot, ✚ indicates mean for $n = 10$ fish; boxes that do not share a common letter are statistically different ($p < 0.05$).

Madison et al., 2017; McDonnell et al., 2019; Philibert et al., 2016).

Body mass, but not overall length, was reduced in sockeye at all life stages when exposed to dilbit; and marginal inconsistent effects were seen in condition factor. Similar results were reported for various petroleum products in other salmonid species (Atlantic salmon, *Salmo salar* [Vignier et al., 1992]; cutthroat trout, *Oncorhynchus clarkii* [Woodward et al., 1983]; rainbow trout, *Oncorhynchus mykiss* [Lockhart et al., 1996]; pink salmon [Wang et al., 1993]; and Chinook salmon, *Oncorhynchus tshawytscha* [Meador et al., 2006]). Multiple mechanisms have been suggested to underlie growth reductions, including the suppression of feeding behavior and decreases in food conversion efficiency (Moles & Rice, 1983; Vignier et al., 1992), physiological stress (Kennedy & Farrell, 2005, 2006; Kochhann et al., 2015), and elevations in metabolic rate (dos Santos et al., 2006; Klinger et al., 2015).

The availability and utilization of critical energy substrates have direct bearing on the success of embryonic development and the growth of fish (Srivastava & Brown, 1991). Dilbit-induced alterations in whole-body lipid, triglyceride, glycogen, and protein concentrations at all stages suggest that disturbances in lipid and protein metabolism or their utilization likely underlie the alterations in mass, length, and calculated condition factor seen at the whole-animal level. Altered lipid stores and reductions in free protein levels have been reported in salmon alevins exposed to dilbit and are suggested to underlie delayed development and shortened body lengths (Alderman et al., 2018). Similarly, the transient exposure of polar cod (*Boreogadus saida*) to North Slope crude oil during embryogenesis caused significant elevations in lipid content (e.g., triacylglycerols, free fatty acids, sterols), reductions in posthatching body size, and poor survival (Laurel et al., 2019). In developing Atlantic haddock (*Melanogrammus aeglefinus*), crude oil exposure disrupted yolk lipid utilization and the biosynthesis of intrinsic cholesterol (Sørhus et al., 2017). Disruptions of lipid utilization may result in the use of protein and carbohydrate as alternative substrates, affecting their concentrations. Increased oxygen consumption in developing mahi-mahi (*Coryphaena hippurus*) exposed to Deep water Horizon crude oil is possibly fueled by enhanced endogenous protein catabolism (Pasparakis et al., 2016). Transcriptomic studies in larval mahi-mahi and red drum (*Sciaenops ocellatus*) have shown that pathways involved in amino acid metabolism and protein digestion are significantly altered following exposure to *Deep water Horizon* crude oil (Xu et al., 2016, 2017).

As in the present study, effects on swimming in various teleosts can occur at concentrations of TPAC as low as 0.23–200 μg/L (Hicken et al., 2011; Johansen & Esbaugh, 2017; Kennedy & Farrell, 2006; Mager et al., 2014). Aberrant molecular responses, functional deficits, and morphological/histopathological alterations during cardiogenesis (Alsaadi, Madison, et al., 2018; Madison, Hodson et al., 2015; Madison et al., 2017; McDonnell et al., 2019; see also Brette et al., 2014; Incardona, 2017); remodeling in cardiac tissues

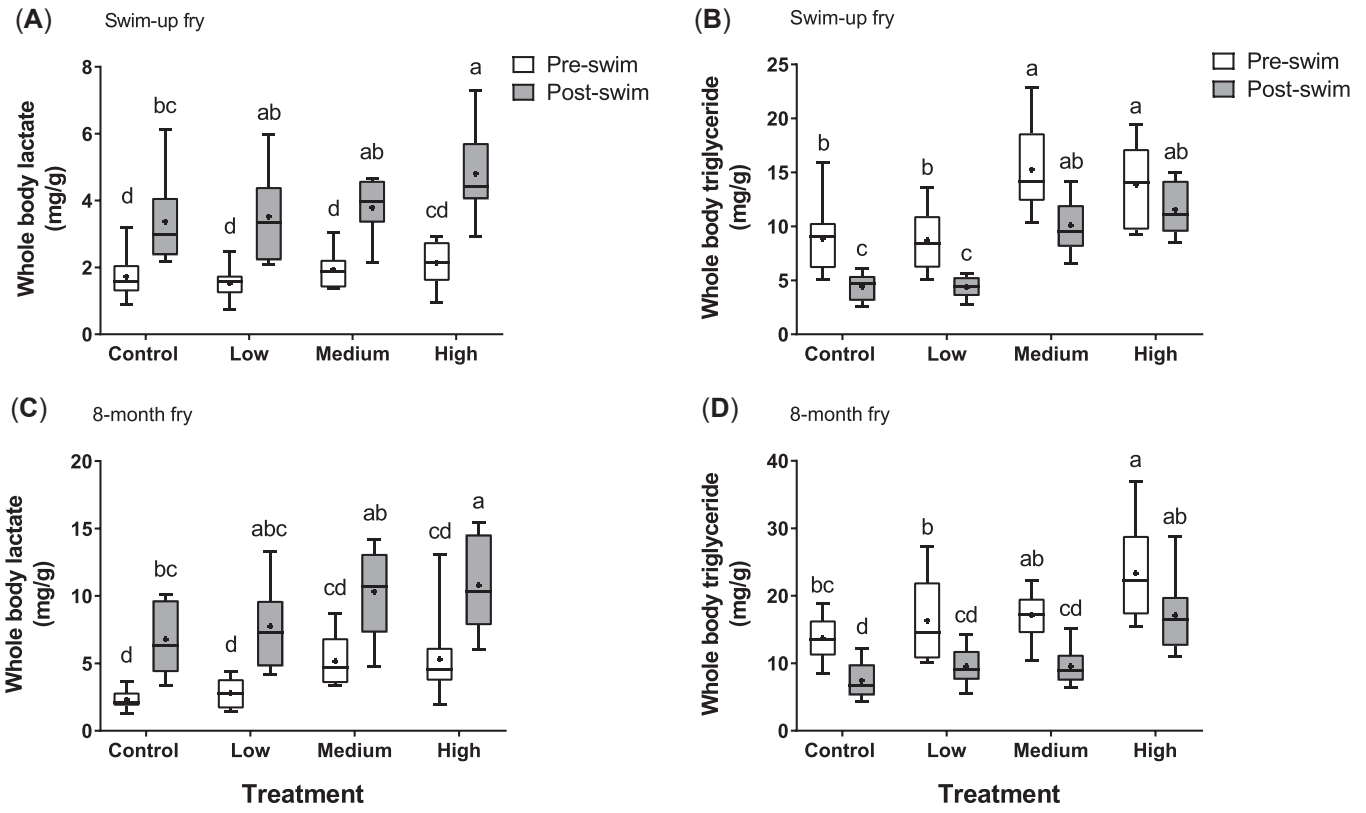

FIGURE 8: Preexercise and postexercise whole-body lactate and triglyceride content in (**A,B**) swim-up fry and (**C,D**) juveniles exposed to dilbit. The initial total polycyclic aromatic compound concentrations for each water-soluble fraction were 0.2 (control), 13.7 (low), 34.7 (medium), and 124.5 (high) μg/L. Within each plot, ✚ indicates mean for $n = 10$ fish; boxes that do not share a common letter are statistically different ($p < 0.05$).

(Alderman, Dindia et al., 2017; Alderman, Lin et al. 2017); and disruptions to cardiovascular capacity (Johansen & Esbaugh, 2017; Nelson et al., 2017; Stieglitz et al., 2016) are often linked to impaired swimming performance in fish exposed to dilbit. In the present study, an induction of *cyp1a* in the heart was observed in dilbit-exposed swim-up fry and juveniles, consistent with cardiotoxicity as a driving mechanism for the observed reductions in swimming performance.

Routine, sustained, and prolonged swimming are primarily fueled by triglyceride oxidative metabolism in slow-twitch red skeletal muscle; and sprint/burst swimming is fueled by glycolytic metabolism in fast-twitch white muscle (Hammer, 1995; Moyes & West, 1995). Elevations in whole-body triglycerides in exposed fish did not provide advantages in swimming performance, and unchanged triglyceride content post-U_{crit} test may reflect a decreased lipolytic capacity; a reduced lipid utilization during the aerobic exercise and diminished carbohydrate availability for anaerobic bursting may underlie impairment. Similarly, Avey et al. (2020) reported that U_{crit} was not affected in Atlantic salmon smolts exposed to water-soluble fraction of dilbit (up to 67.9 μg/L TPAC), but the fish exhibited a reduced reliance on lipid metabolism for adenosine triphosphate in the heart. Exposure to a lower water-soluble fraction concentration at 9.65 μg/L TPAC resulted in an increased reliance on anaerobic metabolism in both cardiac and red skeletal muscle (Avey et al., 2020). Burst swimming is almost exclusively fueled anaerobically through the utilization of muscle glycogen, and reductions in U_{burst} may be directly attributed to the lowered body glycogen stores seen prior to the swim trial. A greater accumulation of lactate and depletion of glycogen postexercise in fish exposed to dilbit suggest a potentially enhanced anaerobic debt during burst swimming.

Dilbit exposure activated a physiological stress response, followed by a short-term hyperglycemic response similar to the response induced by crude oil exposure; this has been attributed to the irritant properties of the lighter, more volatile, and acutely toxic components of oil (e.g., naphthalenes, BTEX, and naphthenic acids; Kennedy & Farrell, 2005, 2006; P. Thomas et al., 1980). Cortisol elevation can reduce feeding and food conversion efficiency (Gregory & Wood, 1999; Madison, Tavakoli, et al., 2015) and may be responsible for the reduced growth seen in the present study. Consistent with cortisol's key role in mediating the peripheral mobilization of energetic substrates during stress, stress may have resulted in the catabolism of body carbohydrate and protein (Milligan, 2003; Mommsen et al., 1999). However, elevated cortisol generally increases peripheral and hepatic lipolysis through increases in lipase activity (Baltzegar et al., 2014), increased glycerol utilization (Vijayan et al., 1991), and reductions in hepatic lipogenic potential (López-Patiño et al., 2014), which is not consistent with the higher lipid and triglyceride content seen in exposed fish that were stressed.

Exhaustive exercise resulted in increased circulating cortisol concentrations, which did not increase over preexercise levels in

response to exercise, data that are contrary to those seen in other species acutely exposed to crude oil (Kennedy & Farrell, 2006; R. E. Thomas & Rice, 1987). Chronic exposure, however, can cause a muted cortisol response following exercise (Kennedy & Farrell, 2005, 2006). The main role of the cortisol-induced stress response is to supply an immediate energy source for fuel-intensive behaviors and physiological processes, and a deemphasized cortisol response can be considered maladaptive. Repeated pulse exposures to petroleum may cause hyperactivity and exhaustion of cortisol-producing cells (Hontela, 1997); act as an endocrine disruptor, targeting pituitary or adrenocortical tissues (Dorval et al., 2003) and affecting multiple sites in the hypothalamic–pituitary–interrenal axis (Kennedy & Farrell, 2005); or result in the necrosis of interrenal tissues (DiMichele & Taylor, 1978).

CONCLUSIONS

Chronic exposure of sockeye to dilbit significantly reduced survival and growth, impaired aerobic and anaerobic swimming performance, and altered body biochemical composition as well as cardiac gene expression, providing evidence that this complex mixture likely has multiple targets, resulting in a complex suite of toxicological outcomes. Dilbit release into the natural habitat of Pacific salmon, under similar exposure scenarios, is likely to produce adverse effects that will affect the viability and sustainability of local salmon populations.

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Author Contributions Statement—**Christopher J. Kennedy:** Funding acquisition; Conceptualization; Project administration; Resources; Writing—review & editing. **Todd E. Gillis:** Funding acquisition; Conceptualization; Project administration; Resources; Writing—review & editing. **Feng Lin:** Investigation; Writing—original draft; Writing—review & editing. **Sarah L. Alderman:** Investigation; Writing—review & editing.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (ckennedy@sfu.ca).

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