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Developmental and latent effects of diluted bitumen exposure on early life stages of sockeye salmon (*Oncorhynchus nerka*)

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ABSTRACT

The early life stages of Pacific salmon are at risk of environmental exposure to diluted bitumen (dilbit) as Canada's oil sands industry continues to expand. The toxicity and latent effects of dilbit exposure were assessed in sockeye salmon (*Oncorhynchus nerka*) exposed to water-soluble fractions of dilbit (WSFd) from fertilization to the swim-up stage, and then reared in clean water for 8 months. Mortality was significantly higher in WSFd-exposed embryos, with cumulative mortality up to 4.6-fold higher in exposed relative to unexposed embryos. The sublethal effects of WSFd exposure included transcriptional up-regulation of *cyp1a*, a concentration-dependent delay in the onset and progression of hatching, as well as increased prevalence of developmental deformities at total polycyclic aromatic hydrocarbon (TPAH) concentrations $\geq 35\,\mu g\,L^{-1}$. Growth and body composition were negatively affected by WSFd exposure, including a concentration-specific decrease in soluble protein concentration and increases in total body lipid and triglyceride concentrations. Mortality continued during the first 2 months after transferring fish to clean water, reaching 53% in fish exposed to 100 $\mu g\,L^{-1}$ TPAH; but there was no latent impact on swimming performance, heart mass, or heart morphology in surviving fish after 8 months. A latent effect of WSFd exposure on brain morphology was observed, with fish exposed to $4\,\mu g\,L^{-1}$ TPAH having significantly larger brains compared to other treatment groups after 8 months in clean water. This study provides comprehensive data on the acute, sub-chronic, and latent impacts of dilbit exposure in early life stage sockeye, information that is critical for a proper risk analysis of the impact of a dilbit spill on this socioeconomically important fish species.

1. Introduction

Canada holds the third largest crude oil reserves in the world and is the sixth largest producer contributing to the global oil market (National Energy Board of Canada, 2017). The majority of Canadian crude originates as bitumen in the oil sands deposits of western Canada, and extraction rates are projected to increase from 2.4 million barrels of oil per day (Mb/d) to 3.7 Mb/d over the next decade (Canadian Association of Petroleum Producers, 2016). Bitumen is a heavy crude oil with a tar-like consistency, and is diluted with lighter hydrocarbons (3:1 bitumen to diluent, termed dilbit), synthetic oil (1:1 bitumen to diluent, termed synbit), or a combination of the two (termed dilsynbit) to reach a consistency that permits flow and increases buoyancy (Canadian Association of Petroleum Producers, 2016). An expanding network of rails and pipelines carry bitumen products (primarily dilbit) across North America, posing a continual risk of accidental release and environmental contamination. The largest dilbit spill to date occurred in 2010, when 3.2 million liters of dilbit was released into the Kalamzoo River, Michigan. Unrecovered dilbit remained entrained in river sediments at least 3 y following the spill despite extensive clean up and dredging efforts (Dew et al., 2015). Given the known toxicity of petrogenic chemicals to aquatic organisms including fish (Kennedy, 2015), the propensity for spilled dilbit to sink in some aquatic habitats could put biota at risk of long-term exposure (Alsaadi et al., 2018), warranting research directed at understanding the subchronic/chronic and latent effects of exposure.

Recent laboratory studies in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) reported a range of concentration-dependent biological effects in embryonic fish exposed to water accommodated fractions (WAF) of dilbit. These effects included malformations of the heart and skeleton; failure of swim bladder inflation; genomic responses in pathways related to upregulation of phase I and II biotransformation,

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oxidative and cellular stress responses, and tumorigenesis; as well as increased mortality and altered behaviour (Madison et al., 2017, 2015; Philibert et al., 2016). These responses parallel biological effects observed in ELS fathead minnow (*Pimephales promelas*) and white sucker (*Catostomus commersoni*) exposed to natural bitumous sediments (Colavecchia et al., 2006, 2004), and are generally consistent with toxicity studies of conventional crude oils (ex. Dubansky et al., 2013; Incardona et al., 2013, 2004). Polycyclic aromatic hydrocarbons (PAH) are considered major drivers of the teratogenic phenotypes associated with crude oil exposure, owing in part to the affinity of some PAH types for the aryl hydrocarbon receptor (AhR) (Colavecchia et al., 2004; Hodson et al., 2007; Incardona et al., 2006, 2004; Scott and Hodson, 2008; Wu et al., 2012). Indeed, AhR-mediated induction of cytochrome P450-dependent monooxygenases (CYP1A) is an established bioindicator of PAH exposure (Whyte et al., 2000). Importantly, PAH- or crude oil-induced cardiotoxicity has been directly linked to immediate and latent impacts on cardiac function (Brette et al., 2014; Dubansky et al., 2013; Incardona et al., 2015, 2014, 2005; Jung et al., 2013; Khursigara et al., 2017; Nelson et al., 2016) and aerobic swimming performance (Kennedy and Farrell, 2006; Mager et al., 2014; Stieglitz et al., 2016), which are critical traits in migratory salmonids. The nature of crude oil toxicity in other body systems represents a considerable knowledge gap in this field. However, efforts to define the transcriptomic responses to crude oil exposure in developing marine fish species highlight the nervous system as a key target of crude oil toxicity (Xu et al., 2016, 2017a), confirming reports of behavioral alterations in PAH-exposed fish (Brown et al., 2016; Johansen et al., 2017; Philibert et al., 2016).

Pacific salmon hold considerable socioeconomic and environmental value to Canadians (Fisheries and Oceans Canada, 2018), and many populations are at risk of dilbit exposure due to pipeline routes that bring bitumen products to sea terminals on the Pacific coast of North America (Levy, 2009). For example, the expansion of the existing Trans Mountain pipeline will triple the volume of dilbit moving between Edmonton, AB and Burnaby, BC to about 900,000 b/d. This pipeline corridor extends through the lower section of Canada's largest salmon-bearing river system, the Fraser River Watershed (FRW), which supports millions of early life stage (ELS) salmon including sockeye (*Oncorhynchus nerka*). Importantly, these ELS are especially sensitive to anthropogenic pollution (Cohen, 2012) and ELS survival prior to seawater migration is critical to overall population viability (Henderson and Graham, 1991). Some 40% of sockeye populations currently monitored by the Canadian government are listed as stocks of conservation concern (Fisheries and Oceans Canada, 2018), including those that rely on habitat in the lower FRW. Recently, we showed that juvenile sockeye exposed to low, environmentally relevant concentrations of the water soluble fraction of dilbit (WSFd) for 4 wk experience considerable changes in the serum proteome consistent with WSFd-induced tissue damage (Alderman et al., 2017a), as well as altered aerobic swimming performance, cardiac gene expression and cardiac histology (Alderman et al., 2017b). However, the sensitivity of developing sockeye to WSFd remains unknown, as does the nature and persistence of biological effects. Therefore, the present study was conducted to determine the immediate and latent impact of dilbit exposure on ELS sockeye.

2. Materials and methods

2.1. Fish

Fertilized sockeye salmon (*O. nerka*) eggs were supplied by the Inch Creek Hatchery, BC (Fisheries and Oceans Canada) and evenly distributed among 8 Heath trays (MariSource, Fife, WA; n = 470 per tray). Embryos were batch weighed on arrival, and individual mass estimated by assuming homogeneity in size (mean mass = 0.09 g ± 0.02 S.D.). Heath trays were supplied with a continual flow of dechlorinated municipal water at ambient temperature (flow rate 6 L/min; dissolved O_2 > 95% saturation, hardness 6.12 mg/L $CaCO_3$, chlorine undetectable, < 1 mg/L DOC, pH 7.0; mean 11.3 °C). Rearing to the swim-up stage took place in the dark, after which fry were transferred (n = 100 per concentration) to eight 250 L aquaria supplied with dechlorinated municipal water (7.5 L/min) at ambient temperature (13 °C) under a 12 h light:12 h dark photoperiod. Fish were fed twice daily ad libitum with commercial salmonid fry feed (Skretting, Vancouver, BC), and mortality records were maintained. Care and use of fish was approved by Simon Fraser University animal care committee, as per guidelines outlined by the Canadian Council on Animal Care.

2.2. Diluted bitumen exposure

Water-soluble fractions of dilbit (WSFd) were generated as previously described (Alderman et al., 2017b; Kennedy and Farrell, 2005) using Cold Lake Summer Blend dilbit. Four concentrations (in duplicate) of total polycyclic aromatic hydrocarbon (TPAH) were achieved by varying the quantity of dilbit-soaked Siproax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) in PVC generator columns (15 cm diameter, 80 cm length). Ceramic beads were re-coated with dilbit every 14 d, and control columns contained only clean beads. Target TPAH concentrations were chosen to span a nominal range of 3 orders of magnitude, with a maximum concentration reflecting reported values at shoreline sites in the Gulf of Mexico after the Deepwater Horizon oil spill in 2010 (Allan et al., 2012). Dechlorinated municipal water flowed up through the columns and into one of 4 header tanks, and then was piped to Heath trays using submersible pumps. This set-up enabled duplicate exposures, which were applied from 2 d post fertilization (2 dpf) through to swim-up (Fig. 1). Water samples were collected 6 h after initiating the exposure (0 d), and again at 7 d and 14 d for quantification of individual PAH concentrations by GC–MS (SGS Axys Analytical Services Ltd., Sidney, BC) as previously described (Alderman et al., 2017b).

2.3. Development: mortality, deformity, hatching, and growth

Dead embryos were counted and carefully removed daily under red lighting. Hatching was quantified daily from 45 to 73 dpf, when hatch was considered 100% complete (no further eggs hatched for 5 d). Morphometric and deformity analyses were carried out at 76 dpf. A swimming performance were evaluated in juvenile fish after 8 months in clean water.

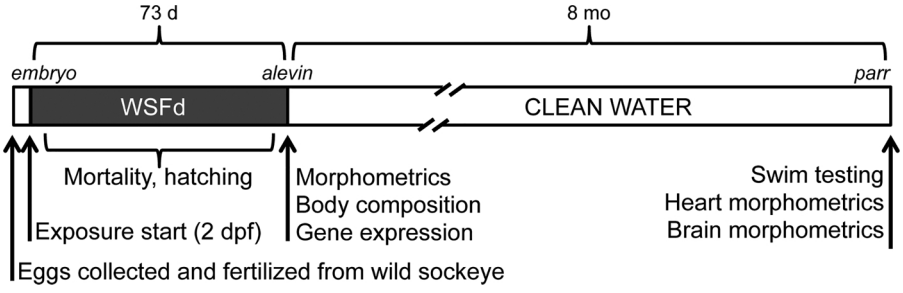

Fig. 1. Schematic of experimental design. Fertilized sockeye eggs were exposed to 1 of 3 concentrations of the water-soluble fraction of diluted bitumen (WSFd) or to clean water (control) from 2 d post fertilization (dpf) to swim-up (76 d total exposure length). A subset of fry were then transferred to clean water and raised for 8 months. Mortality and hatching were monitored throughout the exposure. Morphometrics, including deformity analysis, body composition, and gene expression were evaluated at the end of the exposure in a subset of fish. The latent effects of WSFd exposure on future brain and heart morphology, as well as swim-

random selection of viable alevins (n = 50 per Heath tray) was euthanized with MS-222, blotted dry, then weights and lengths were recorded. Development was scored based on staging series for developing salmonids (Velsen, 1980; Vernier, 1969). Teratogenesis was scored for frequency and severity on a scale of 0 (normal) to 3 (severely deformed) as previously described (Rudolph et al., 2008); deformities included craniofacial, skeletal, and fin deformities, as well as edema of the yolk sac and/or pericardial space. The mean frequency and severity was calculated for each tank and treatment by averaging incidence and scores, respectively.

2.4. Body composition analysis

Following deformity analysis, a subset of randomly selected alevins was weighed, snap frozen whole on dry ice and stored at −80 °C until body composition analysis (n = 60 per treatment). For protein quantification, alevins were thawed on ice (n = 20 per treatment) and homogenized in 1 mL assay buffer (500 mM Tris−HCl, 100 mM DTT, 5.5 mM EDT; pH 7.4) containing 1x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) using a Mixer Mill homogenizer. The crude homogenates were sonicated, centrifuged at 12,000 *g* for 15 min at 4 °C, and then protein content of the supernatant was measured in duplicate using a Bradford Protein Assay Kit and bovine serum albumin standards (Bio-Rad, Mississauga, ON). Whole body lipid content was quantified after drying alevins to constant weight at 65 °C (n = 20 per treatment), using a standard chloroform/methanol extraction protocol exactly as previously described (Johnston et al., 2013). To quantify whole body triglyceride content, alevins were thawed on ice (n = 20 per treatment) and homogenized in 1 mL of assay reagent (Cayman Chemical Company, Ann Arbor, MI) containing 1 mM EDTA as above. The crude homogenates were sonicated, centrifuged at 10,000 *g* for 10 min at 4 °C, and then triglyceride content of the supernatant was quantified in duplicate using a commercial colorimetric assay kit (Cayman). Data are expressed relative to original wet weight of the alevin.

2.5. Gene expression

A subset of randomly selected alevins was bisected into head and tail regions at the rostral boundary of the yolk sac and perpendicular to the body axis. The head regions were snap frozen on dry ice (n = 8 per treatment group) and stored at −80 °C. Total RNA was extracted with Trizol (Life Technologies, Grand Island, NY) following manufacturer's instructions. RNA integrity was verified by gel electrophoresis then purity and quantity were measured using a NanoDrop 2000. After DNase I treatment (Life Technologies), 500 ng total RNA was used to synthesize cDNA (High Capacity cDNA Kit, Life Technologies) according to manufacturer's instructions. Transcript abundances of *cyp1a*, *ahr*, and ribosomal protein L8 (*rpL8*) were quantified in duplicate RT-qPCR reactions, exactly as previously described and including all appropriate control reactions (Alderman et al., 2017b). Data were normalized to the abundance of the stably expressed reference gene, *rpL8*, and are shown as fold-change from control.

2.6. Swimming tests

The latent effect of WSFd exposure on swimming performance was determined in surviving juveniles after 8 months of rearing in clean water using either a critical swimming speed test (U_{crit}) or a constant acceleration test (U_{max}; Farrell, 2008). A total of 52 fish were tested (average mass 48.4 ± 1.2 g; average fork length 16.0 ± 0.1 cm). All tests were performed in a custom-built, temperature-controlled oval raceway equipped with a removable window at the rear gate of the swimming chamber that allowed individual fish to be removed as they fatigued (Alderman et al., 2017b; Kennedy and Farrell, 2006). For each U_{crit} test, 5 fish from the same treatment group were gently transferred to the swim tunnel and acclimated for 45 min at 5.8 cm s^{-1} (0.37 BL s^{-1}). Water velocity was then increased to ~65% U_{crit} (estimated from a preliminary test of unexposed salmon; actual ramp averaged 71 ± 1.6% U_{crit}) over 5 min followed by step increases of 5 cm s^{-1} (0.32 BL s^{-1}) every 20 min until fish were exhausted. A fish was considered fatigued and removed from the swim tunnel when it rested on the rear gate and could not be encouraged to resume swimming with mechanical stimulation. Fish were immediately euthanized in an overdose of 2-phenoxyethanol. The exhaustion time and fork length were noted and used to calculate U_{crit} values (Farrell, 2008). U_{crit} tests were repeated up to 3 times for each treatment (n = 5–15 fish). The U_{max} swim tests were performed on individual fish from the control and high exposure groups only (n = 5). After a fish was acclimated to the tunnel as above, the water velocity was incrementally increased (5.6 cm s^{-1} every 5 min; acceleration 0.027 cm s^{-2}) until the fish reached exhaustion. U_{max} was calculated as per U_{crit} (Farrell, 2008).

2.7. Brain morphometrics

Brain morphometric analyses were conducted in surviving juveniles after rearing for 8 months in clean water (average mass 47.9 ± 0.9 g; average fork length 16.0 ± 0.1 cm; n = 10–21 per treatment, 73 fish total). Following euthanasia, decapitated fish heads were affixed with plastic numbered tags and preserved in a large volume of neutral buffered formalin for long-term storage. Brains were removed, blotted dry and weighed, then digitally photographed using a Nikon D90 equipped with a Tamron 90 mm Macro lens. To orient the brains and ensure symmetrical imaging, brains were gently placed in depressions made in agarose-filled petri dishes. A ruler was included in each photo for calibration, and each brain was imaged in dorsal, ventral, and left lateral views. Regional volumes (*V*) were estimated using previously described methods (Edmunds et al., 2016a, 2016b; Gonzalez-Voyer and Kolm, 2010). Briefly, maximum length (*l*), width (*w*), and height (*h*) to the nearest tenth mm were measured in ImageJ for each of 5 discrete brain regions (olfactory bulbs, telencephalon, optic tectum, cerebellum, hypothalamus) and used in the formula for ellipsoid volume:

$$V = \tfrac{1}{6} (l \times w \times h)\, \pi \quad (1)$$

Bilateral symmetry was assumed for paired regions (olfactory bulbs, telencephalon, optic tectum), so one lobe was measured and the volume doubled. Regional volumes were standardized to individual fork length, which was similar across treatments.

2.8. Heart morphometrics

Excised hearts from exhausted fish were passively cleared of blood in physiological saline, and digitally photographed as described above (Section 2.7). Hearts were then blotted dry and weighed whole and with the ventricle isolated to determine relative ventricular mass (RVM). Digital images were analyzed in ImageJ to determine maximal length and width (Incardona et al., 2015), and normalized to individual fish fork length. Aspect ratios for each heart were calculated as length/width.

2.9. Statistics

Statistical analysis began by interrogating data for tank effects using a one-way ANOVA and including tank as a random factor, or with a Chi-Square test (for frequency data). Without exception, no significant tank effects were detected (median P = 0.51); therefore data were combined within concentration for subsequent analyses. Sigmoidal regressions of percent hatch *v.* exposure day were plotted for each concentration in SigmaPlot 10 according to the equation

$$f = a \,/\, (1 + \exp(-(x - x0)/b)) \quad (2)$$

and used to estimate time to 50%, 75%, or 90% hatch in each treatment

by solving for x (day), where f is percent hatch, a is the maximum asymptote, $x0$ is the inflection point, and b is the slope. The fit of all regressions was high ($R^2 > 99\%$). A two-way analysis of variance (ANOVA) and Holm-Sidak multiple comparison test were then used to detect differences in the progression of hatching among treatments, using the main effects of hatching level (50%, 75%, 90%) and WSFd concentration (Control, Low, Medium, High). All other data sets were analyzed using a one-way ANOVA followed by a Holm-Sidak post-hoc test where differences were detected ($\alpha = 0.05$).

3. Results

3.1. Water chemistry

Aqueous total PAH concentrations (sum of 50 PAH) for each concentration at the beginning of the exposures (0 d) were: Control = < 0.1 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, and High = 100 μg L^{-1}. With the exception of Control, TPAH declined with time and at a similar rate across concentrations during the 14 d between column recharges, so distinct treatments were maintained throughout the exposure period (Supplemental Figure S1). The PAH component breakdown is provided (Supplemental Table S1).

3.2. Developmental effects

Mortality of Control fish during embryogenesis was progressive and minor (final cumulative mortality = 1.7 ± 0.1%) during the first 50 days with no spikes in mortality. In contrast, embryos exposed to 100 μg L^{-1} TPAH experienced higher mortality relative to all other TPAH concentrations throughout the exposure period, largely as a result of an early spike in mortality before day 10. Mortality in the Control, Low and Medium exposure groups was similar until day 28. A pronounced spike in mortality (days 28–32) was seen in all WSFd treatment groups and again at days 41–43 for just the 100 μg L^{-1} TPAH group, which were associated with refreshing the generator columns. Final cumulative mortality was 7.8 ± 0.3% for 100 μg L^{-1} TPAH, 3.2 ± 0.6% and 3.3 ± 0.5% for the 4 μg L^{-1} and 35 μg L^{-1} groups, respectively (Fig. 2A).

Hatching was initiated at 47 dpf in controls, but was delayed 1 to 3 d in Heath trays supplied with water containing WSFd (Fig. 2B). Although hatching progressed in all treatments (significant main effect *hatching level*, $p < 0.001$; Fig. 2C), the concentration-dependent effect of WSFd on hatching time persisted throughout the hatching period (significant main effect *concentration*, $p < 0.001$; Fig. 2C). For example, time to 50% hatch (54 dpf in controls) was 3 to 4 d later in WSFd-exposed embryos. By 27 d after hatching began, 97–98% of eggs had hatched in the 0, 4, and 35 μg L^{-1} treatment groups, whereas only 92% of eggs had hatched in the 100 μg L^{-1} TPAH treatment (Fig. 2B); unhatched eggs at this time were deemed non-viable. Thus the combined influences of mortality and failed hatching increased overall embryo loss from 4.7% in Control (0 μg L^{-1} TPAH) to 15.2% in the 100 μg L^{-1} TPAH treatment. There was no effect of WSFd concentration on developmental stage of alevins at 76 dpf, with average stage across treatments being 33 (Table 1).

Teratogenesis was quantified as the frequency and severity of deformities in viable alevins at the end of the WSFd exposure, but not in embryos prior to hatch; the contribution of WSFd-induced malformations to observed mortalities was not evaluated. The frequency of developmental deformities was higher in WSFd-exposed alevins compared to controls, reaching approximately 10-fold increased frequency over controls in the highest concentration groups (17–20% incidence in 35 μg L^{-1} and 100 μg L^{-1}; Table 1). The most common deformity observed in all treatments was edema, primarily around the yolk sac, followed by craniofacial defects. Skeletal and finfold deformities were rare, with only one observation with WSFd (Table 1). With few exceptions, observed deformities were scored as 1 (minor), resulting in

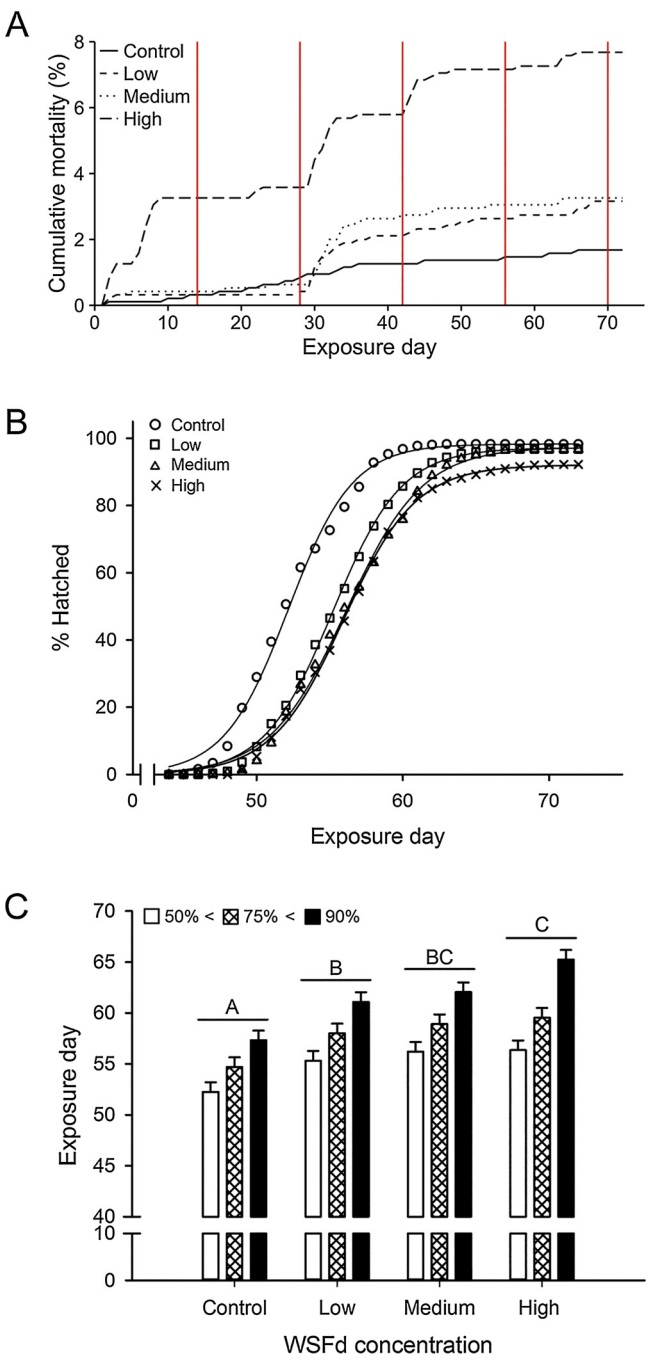

Fig. 2. Mortality and hatching. Sockeye embryos were exposed in duplicate to WSFd (in TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}) from 2 d post fertilization until the end of hatching. (A) Cumulative mortality (%) was monitored daily, and is shown combined for duplicate exposures, with vertical red lines indicating when generator columns were recharged with fresh dilbit. (B) Cumulative hatching success (%) was quantified daily for each duplicate exposure, and is shown combined and fitted with a 3-point sigmoidal regression (Eq. (2)). (C) Time to reach 50%, 75%, and 90% hatch was calculated for each of the duplicate exposures (Eq. (2)), and differences between the main effects of *concentration* and *hatching level* were determined using a two-way ANOVA and Holm-Sidak post-hoc test. Bars that do not share a common letter are significantly different ($p < 0.05$) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Comparison of alevin development across treatments. Alevins from duplicate exposure tanks were analyzed, and data is presented pooled for each concentration. Wet weight, length, and developmental stage are presented as mean ± SEM. Results from the deformity analysis are presented as both frequency of occurrence (%) and severity (mean ± SEM). For each parameter, differences between exposure concentrations were determined by a one-way ANOVA and Bonferroni test for multiple comparisons. For each metric, values that do not share a common letter across concentrations are statistically different (n = 100; $p < 0.05$).

	Control	Low	Medium	High
Weight (mg)	160.0 ± 1.8^{a}	156.0 ± 1.6^{a}	166.0 ± 1.8^{b}	157.0 ± 1.6^{a}
Length (mm)	22.1 ± 0.1^{a}	21.8 ± 0.1^{ab}	21.9 ± 0.1^{ab}	21.6 ± 0.1^{b}
Developmental Stage	33.4 ± 0.1	33.6 ± 0.1	33.6 ± 0.1	33.5 ± 0.1
Frequency of Deformity (%)				
Yolk sac edema	2	6	15	12
Cardiac edema	1	0	4	1
Craniofacial	1	3	3	5
Skeletal	0	1	1	1
Finfold	0	1	0	0
Total	2	8	20	17
Severity of Deformity (score)				
Yolk sac edema	0.02 ± 0.01	0.10 ± 0.05	0.20 ± 0.05	0.13 ± 0.03
Cardiac edema	0.01 ± 0.01	0	0.05 ± 0.03	0.01 ± 0.01
Craniofacial	0.03 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.08 ± 0.04
Skeletal	0	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02
Finfold	0	0.01 ± 0.01	0	0
Total	0.06 ± 0.03	0.15 ± 0.07	0.29 ± 0.07	0.24 ± 0.06

average severity scores of < 1 across all treatments (Table 1).

3.3. Biochemical effects

At the end of the hatching period, differences in alevin mass and length between WSFd concentrations were observed (Table 1). Alevins exposed to 35 μg L^{-1} TPAH during embryogenesis were significantly heavier than all other treatments, and alevins exposed to 100 μg L^{-1} TPAH were significantly shorter in length than controls. There was a concentration-dependent effect of WSFd on the body composition of alevins measured at the end of the exposure period (76 dpf). Soluble body protein content was negatively impacted by WSFd exposure, with the two highest exposure groups having 1.4-fold lower protein concentrations than control fish (Fig. 3A; n = 20; $p < 0.05$). The effect of WSFd on total body lipid was positively correlated to concentration, with 1.5-, 1.9-, and 2.5-fold higher lipid contents in fish from the 4 μg L^{-1}, 35 μg L^{-1}, and 100 μg L^{-1} treatments, respectively, relative to controls (Fig. 3B; n = 20; $p < 0.05$). The threshold concentration for an effect of WSFd on whole body triglyceride content of alevins was 35 μg L^{-1}, but there was no increase in the effect at 100 μg L^{-1}. Relative to controls, average alevin triglyceride contents were 1.6- and 2.1-fold higher in the 35 μg L^{-1} and 100 μg L^{-1} exposure groups, respectively (Fig. 3C; n = 20; $p < 0.05$).

3.4. Molecular effects

The expression of *cyp1a* in alevin head regions was significantly elevated above controls in all exposure groups in a concentration-dependent manner, ranging from 17- to 20-fold higher in fish exposed to 4 μg L^{-1} and 35 μg L^{-1}, respectively, and 37-fold higher in the 100 μg L^{-1} exposure group (Fig. 4; n = 8, $p < 0.001$). The expression of *ahr* did not significantly change with WSFd exposure (Fig. 4; n = 8, P = 0.07).

3.5. Latent effects

Mortality in all treatment groups was followed daily for 8 months after the exposures, during which fish were held in clean water. Final cumulative mortality during this period was similar for Control, Low and Medium treatments (11–15% mortality). In contrast, on-going mortality was appreciably higher in fish exposed to 100 μg L^{-1} TPAH, especially during the first 60 days (40% mortality), with final mortality of 53% (Fig. 5).

There was a concentration-dependent impact of early life stage exposure to WSFd on brain morphology across all brain regions (Fig. 6; n = 10–21; $p < 0.05$) with the exception of the olfactory bulbs

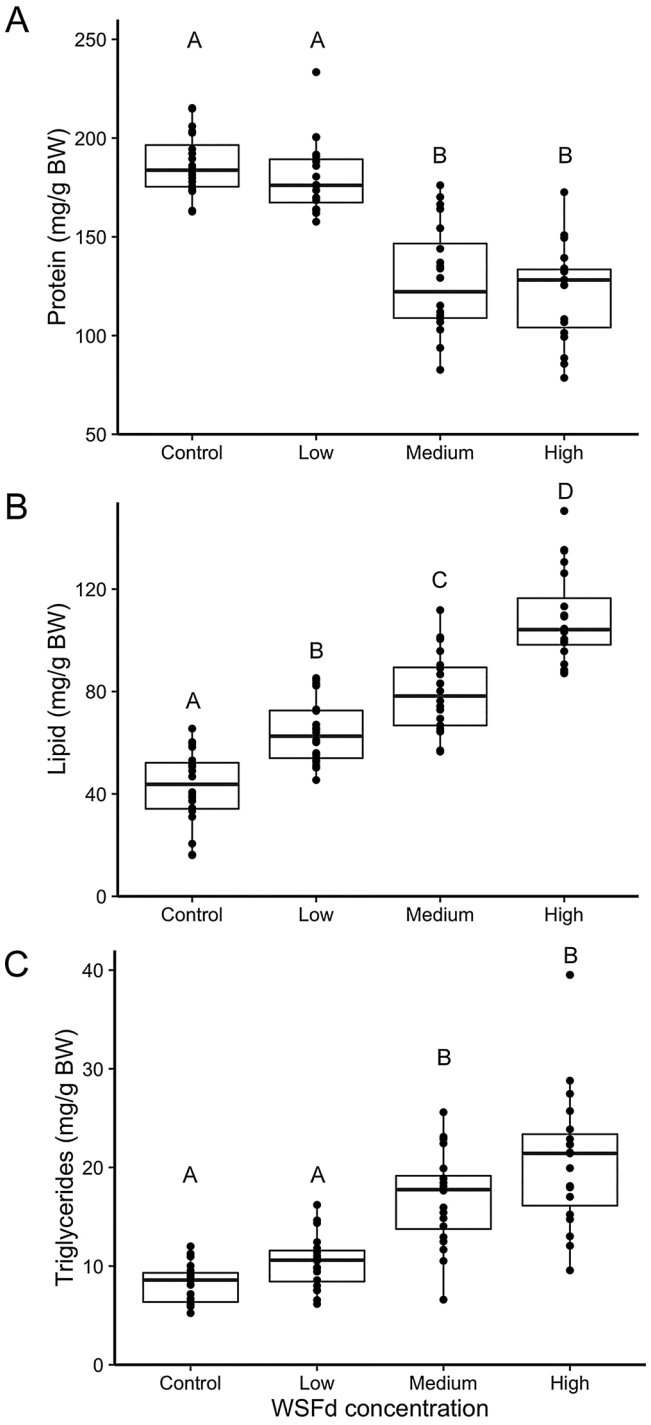

Fig. 3. Body composition. Soluble protein (A), total lipid (B), and total triglyceride (C) content was quantified in alevins following 76 d exposure to WSFd (in TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}). Data were standardized to individual wet weight (BW). Within each plot, boxes that do not share a common letter are significantly different (one-way ANOVA and Holm-Sidak post-hoc test; n = 20; $p < 0.001$).

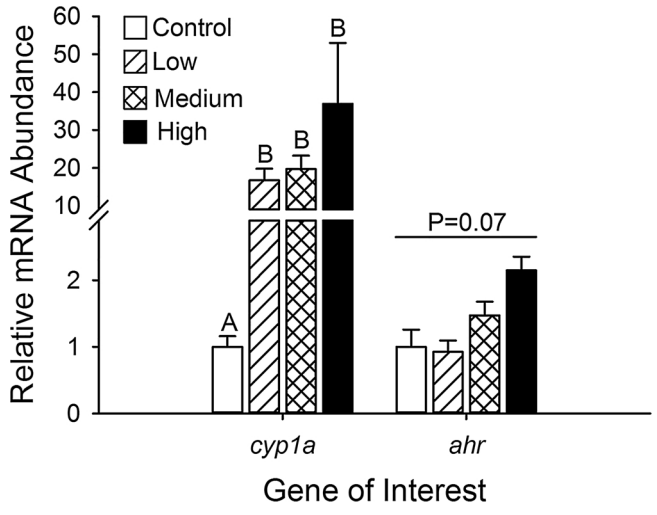

Fig. 4. Gene expression. Relative mRNA abundance of cytochrome P450 1a (*cyp1a*) and aryl hydrocarbon receptor (*ahr*) in alevin heads following chronic exposure to WSFd during early development. Gene expression was quantified by RT-qPCR and standardized to a stable housekeeping gene (*rpL8*). Data are mean ± SEM and is presented as fold-change from the normalized control fish values. Significant differences for each gene were determined with a one-way ANOVA and Holm-Sidak test. Bars that do not share a common letter are statistically different (n = 8; $p < 0.05$).

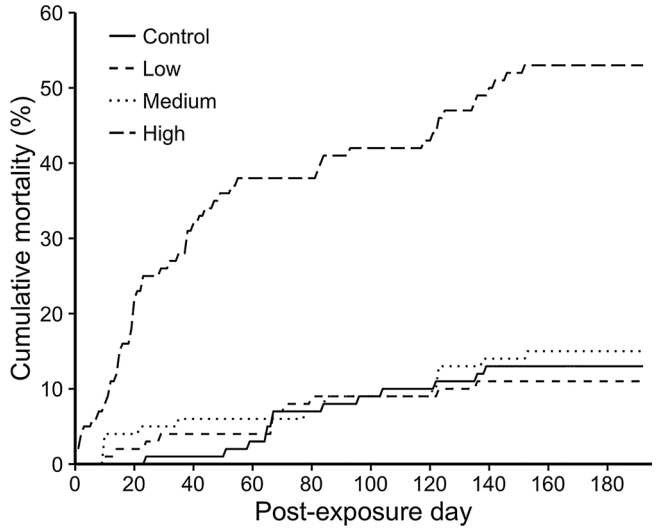

Fig. 5. Post-exposure mortality. Cumulative mortality of sockeye salmon during an 8-month period in clean water following embryonic exposure to WSFd (in TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}).

(Fig. 6B). There was a 9–16% increase in relative volume of the telencephalon (Fig. 6C), hypothalamus (Fig. 6D), optic tectum (Fig. 6E), and cerebellum (Fig. 6F) of fish exposed to 4 μg L^{-1} TPAH during development compared to control fish. The effect at other concentrations was regionally specific. There was a 15% increase in telencephalon volume in fish exposed as embryos to 35 μg L^{-1} TPAH relative to controls (Fig. 6C), while fish exposed as embryos to 100 μg L^{-1} TPAH showed increases in the volumes of the hypothalamus (Fig. 6D) and optic tectum (Fig. 6E), relative to controls.

There were no differences in relative ventricular mass between any WSFd-exposed and control fish, regardless of whether ventricle mass was expressed relative to whole heart mass or body mass (n = 24–61, $p > 0.05$; Table 2). Heart shape was assessed from digital images of isolated hearts taken from a subset of fish in each treatment. There were no significant differences in ventricle length or width, normalized to fish length, among the treatment groups (n = 4–9, $p > 0.05$; Table 2). The aspect ratio was significantly lower only in fish that were exposed to 35 μg L^{-1} WSFd during development relative to controls ($p < 0.05$; Table 2).

There were no differences in absolute (cm s^{-1}) or standardized (BL s^{-1}) critical swimming speed (U_{crit}) of juvenile fish exposed as embryos to WSFd (Table 3; n = 5–15). Similarly, absolute and standardized maximum swimming speeds (U_{max}) did not differ in juvenile sockeye exposed as embryos to control or 100 μg L^{-1} TPAH (Table 3; n = 5).

4. Discussion

This study provides the first data on the biological effects of dilbit exposure in sockeye embryos, a native fish species at risk of exposure. We describe immediate effects of dilbit exposure that are consistent with petrochemical toxicity, including mortality, deformities, and delays in hatching, as well as novel findings of altered body composition and delayed effects on mortality and brain morphology. Considering that the typical egg-to-fry survival rate of wild Pacific salmon is only 7–8% (Bradford, 1995), the adverse effects of dilbit exposure on the survival and development of ELS sockeye in a natural environment is likely to carry a substantial effect on population dynamics.

The immediate effects of WSFd exposure on sockeye ELS were examined by measuring mortality and sub-lethal effects on development. A concentration-dependent increase in mortality occurred with increasing concentrations of WSFd, and mortality was higher in all WSFd-exposed groups relative to unexposed controls. This lethal response is comparable to results from a study in pink salmon (*O. gorbuscha*) where embryos were reared on gravel coated in *Exxon Valdez* oil, and the lowest dose to significantly induce mortality (20% over controls) had an initial aqueous TPAH concentration of 18 μg L^{-1} (Heintz et al., 1999). In the present study, spikes in mortality events tended to coincide with recharging of the WSFd generating columns, suggesting that much of this embryonic mortality is driven by high initial concentrations of WSFd components that deplete or disperse relatively quickly.

The development of sockeye was significantly impaired by dilbit exposure, as evidenced by a concentration-dependent delay in the onset and progression of hatching, increased prevalence of developmental deformities including yolk sac edema, induction of *cyp1a* gene expression, changes to major energy stores, and reduced growth. The sub-lethal effects on embryo development observed in the present study are generally consistent with other crude oil toxicity assessments in developing fish. For example, hatching was comparably delayed in pink salmon exposed to unreplenished WSF Alaskan North Slope Crude Oil (ANSCO; initial [TPAH] 22 μg L^{-1}), and yolk sac resorption was slower (Carls and Thedinga, 2010). Mahi mahi exposed to high energy WAF (HEWAF) of Deepwater Horizon oil during embryogenesis also experienced a hatching delay when exposures were performed in conjunction with UV light (Pasparakis et al., 2017; Sweet et al., 2017). Cardiac and/or yolk sac edema is a common phenotype observed in multiple fish species exposed during development to various conventional crude oils (Edmunds et al., 2015; Incardona et al., 2013; Jung et al., 2013; Pasparakis et al., 2016; Wu et al., 2012), dilbit (present study; Madison et al., 2017, 2015; Philibert et al., 2016), and natural bitumous sediments from Canada's oil sands region (Colavecchia et al., 2006, 2004). One mechanism ascribed to the development of this phenotype is AhR activation by PAHs and subsequent CYP1A induction (Barron et al., 2004; Billiard et al., 2006; Fallahtafti et al., 2012; Hodson et al., 2007; Scott and Hodson, 2008). For example, Hodson et al. (2007) suggest that retene toxicity (a model PAH) is principally driven by its hydroxylated metabolites, since inhibition of CYP1A induction and activity by the antagonist, α-naphthoflavone, significantly reduced mortality and teratogenesis in rainbow trout larvae (Hodson et al., 2007). In addition, CYP1A catalytic measurement (EROD activity) is considered a sensitive biomarker for xenobiotic exposure (Whyte et al., 2000), and

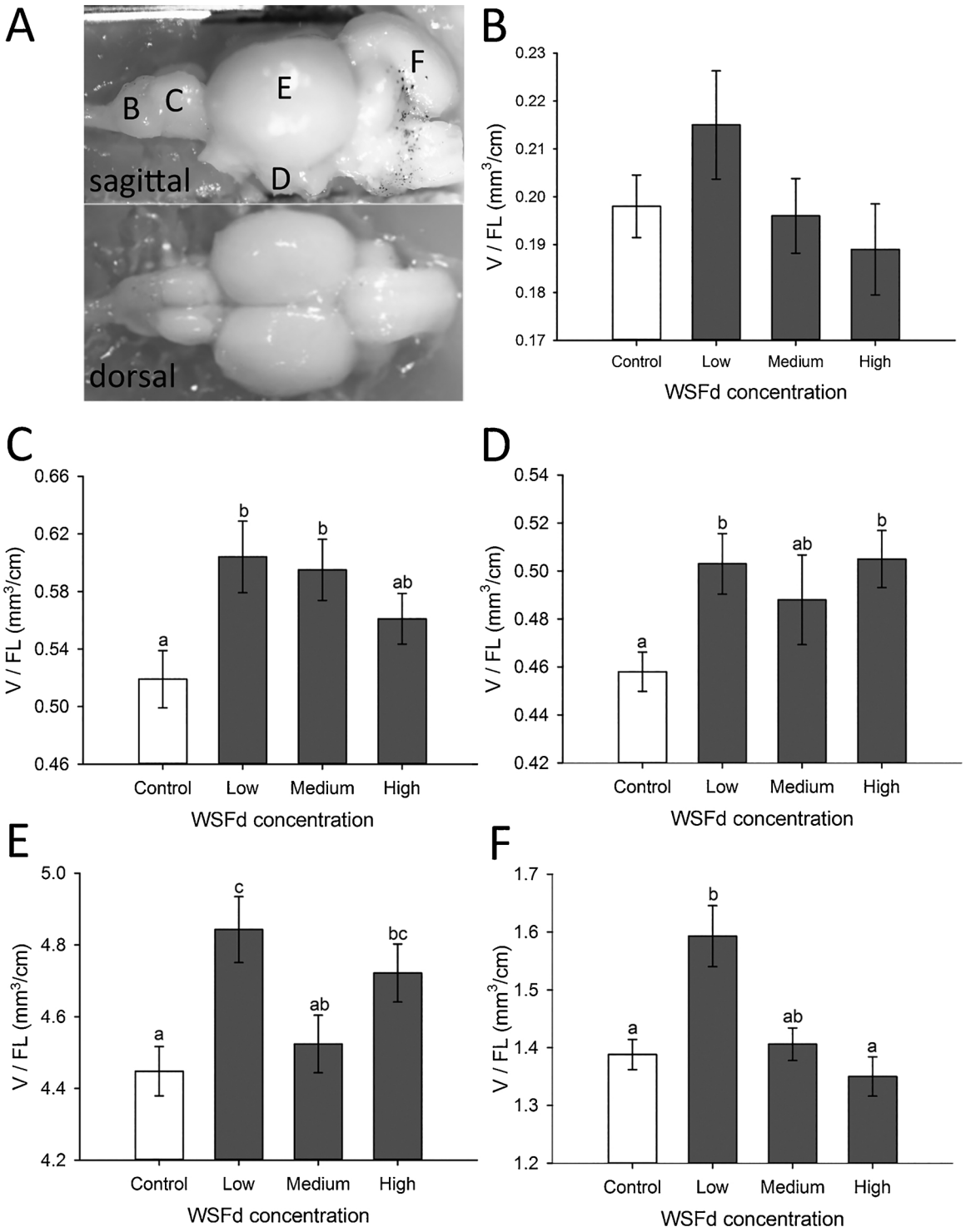

Fig. 6. Brain morphometrics. Comparisons of brain region volumes (V) in sockeye salmon that were exposed as embryos to WSFd (in TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}) and then raised in clean water for 8 months. (A) Representative brain, photographed in sagittal and dorsal planes, with 5 quantified regions indicated: (B) olfactory bulbs, (C) telencephalon, (D) hypothalamus, (E) optic tectum, and (F) cerebellum. The maximum length, width, and height of each region were used to calculate volume (Eq. (1)), doubled if the region contained paired lobes (olfactory bulbs, telencephalon, optic tectum), and standardized to individual fork length (FL). Data are shown as mean ± SEM, and differences were determined for each region using one-way ANOVA and Holm-Sidak tests (n = 10–21; $p < 0.05$).

EROD activity is reliably increased in fish gill (e.g. Blanc et al., 2010) and liver (e.g. Kennedy and Farrell, 2005) tissues when exposed to low levels of petrogenic chemicals. Here we show that *cyp1a* is increased as much as 35-fold in the head region of exposed ELS sockeye and at concentrations as low as 4 μg L^{-1} TPAH, supporting the sensitivity of this parameter for confirming contaminant uptake and biological response in ELS sockeye. By contrast, we did not detect a significant difference in *ahr* abundance, supporting our previous finding of a modest and transient transcriptional response in this receptor to dilbit exposure (Alderman et al., 2017b).

WSFd exposure altered growth and body composition of sockeye alevins, indicating a potential direct effect of ELS exposure on energy

Table 2
Heart biometrics of juvenile sockeye exposed during early development to WSFd (in total polycyclic aromatic hydrocarbons, TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}), and then raised in clean water for 8 months. Relative ventricular mass (RVM) is standardized to both whole heart mass and body mass (n = 24–61 per treatment). Ventricular dimensions (length, width) were determined from digital images of the excised hearts of a subset of fish that also underwent a swimming trial (n = 4–9), and are normalized to individual fork length. The aspect ratio of the ventricle was calculated as length:width for each heart. All data is expressed as mean ± SEM. Significance was determined using a one-way ANOVA and Holm-Sidak test.

	Control	Low	Medium	High	Statistics
RVM (whole heart; %)	70.2 ± 0.85	71.1 ± 0.52	69.8 ± 0.93	69.9 ± 0.94	n.s.d.
RVM (body mass; %)	0.096 ± 0.002	0.098 ± 0.001	0.096 ± 0.003	0.094 ± 0.002	n.s.d.
Ventricle length	0.339 ± 0.005	0.308 ± 0.034	0.316 ± 0.005	0.322 ± 0.010	n.s.d.
Ventricle width	0.360 ± 0.009	0.340 ± 0.025	0.369 ± 0.006	0.372 ± 0.003	n.s.d.
Aspect ratio	0.946 ± 0.020[a]	0.899 ± 0.037[ab]	0.858 ± 0.016[b]	0.899 ± 0.020[ab]	$p < 0.05$

Table 3
Swimming performances of juvenile sockeye exposed during early development to WSFd (in total polycyclic aromatic hydrocarbons, TPAH: Control = 0 μg L^{-1}, Low = 4 μg L^{-1}, Medium = 35 μg L^{-1}, High = 100 μg L^{-1}), and then raised in clean water for 8 months. A ramp-critical swimming speed test (U_{crit}) and a maximal swimming speed test (U_{max}) were performed on separate fish (n = 5–15 per test per treatment). Data is expressed as absolute swimming speed (cm s^{-1}) and standardized to body length (BL s^{-1}). U_{max} was not determined (N.D.) for the 2 intermediate concentrations. No differences in swimming performance were detected among the treatments (one-way ANOVA, $p > 0.05$).

	U_{crit}		U_{max}	
	cm s^{-1}	BL s^{-1}	cm s^{-1}	BL s^{-1}
Control	57.3 ± 1.38	3.61 ± 0.11	62.1 ± 3.12	4.15 ± 0.22
Low	60.4 ± 1.86	3.66 ± 0.10	N.D.	N.D.
Medium	58.4 ± 1.60	3.63 ± 0.14	N.D.	N.D.
High	59.7 ± 0.63	3.80 ± 0.06	64.7 ± 4.22	4.07 ± 0.28

storage and utilization. After emergence and prior to the onset of endogenous feeding, alevins resorb a large external yolk sac to support somatic growth; therefore, the higher lipid content and shorter length of alevins exposed to WSFd, in the absence of any developmental delay, may indicate a reduced yolk sac conversion capacity which could be driven by direct and/or indirect mechanisms. An example of a direct toxic effect of WSFd on energy pathways would be reduced macromolecule metabolism, as is seen with fish exposed to selenium. Here, fish show altered triglyceride storage (Bennett and Janz, 2007; Thomas and Janz, 2011) concomitant with changes in transcriptional networks involved in fatty acid metabolism and synthesis (Knight et al., 2016). Alternately, indirect mechanisms of WSFd-induced changes to energy utilization could result from a shift in basal metabolic rate, as with rainbow trout exposed to developmental hypoxia (Johnston et al., 2013; Miller et al., 2008), and/or as a consequence of PAH-induced cardiotoxicity (Incardona et al., 2004).

The latent effects of WSFd exposure were examined by measuring mortality, brain and cardiac morphology, and swimming performance of juvenile fish that were held in uncontaminated water for 8 months after the ELS exposure. Immediately following transfer to clean water, fry that had been exposed to 100 μg L^{-1} TPAH experienced considerable mortality compared to unexposed control fry, with 40% mortality occurring during the first 60 d and final mortality surpassing 50%. Similarly, Johansen and colleagues report increased latent mortality in several species of coral reef fish exposed to weathered HEWAF of Deepwater Horizon crude oil during development (Johansen et al., 2017), and Brown and colleagues found a higher post-exposure incidence of mortality in Atlantic killifish (*Fundulus heteroclitus*) exposed during development to a complex PAH mixture (Brown et al., 2016). This suggests that delayed mortality is a common outcome of ELS crude oil exposure, and when combined with acute mortality and the numerous early sub-lethal effects of crude oil on fish development, warns of exacerbated population-level impacts of an oil spill in the aquatic environment.

The impacts of crude oils on developing fish hearts are well-studied, including molecular and morphological changes (Edmunds et al., 2015; Hicken et al., 2011; Jung et al., 2013; Madison et al., 2015) and functional impairment (Incardona et al., 2014, 2005; Pasparakis et al., 2016; Xu et al., 2016; Khursigara et al., 2017). Crude oil exposure at older life stages of fish can also impact the heart, including molecular and histological changes (Alderman et al., 2017b) and functional impairments (Brette et al., 2014; Nelson et al., 2016). Similarly, at the organismal level the effects of crude oil on the cardiorespiratory system can manifest as impaired swimming performance (Alderman et al., 2017b; Kennedy and Farrell, 2006; Mager et al., 2014; Mauduit et al., 2016; Stieglitz et al., 2016) and hypoxia tolerance (Maudit et al., 2016; Zhang et al., 2017). Fewer studies have investigated the latent effects of ELS oil exposure on the cardiovascular system. Hicken et al. (2011) showed that changes in heart shape and impaired swimming capacity were still evident 1 y after embryonic exposure of zebrafish to low concentrations of ANSCO (Hicken et al., 2011). Similarly, pink salmon and Pacific herring exposed during cardiogenesis to ANSCO crude oil retained significant differences in cardiac morphology 7–9 months later that coincided with reduced aerobic swimming performance (Incardona et al., 2015). In contrast, with the exception of a moderate decrease in aspect ratio in sockeye exposed as embryos to 35 μg L^{-1} TPAH, the present study detected no lasting changes in gross heart morphology (RVM, normalized ventricle length and width) or swimming performance (U_{crit} or U_{max}) following 8 months in clean water. However, considering that these metrics were only quantified in fish surviving the full 8 months, it is reasonable to speculate that much of the high mortality observed during and following WSFd exposure occurred in individuals with oil-induced cardiac defects, precluding any apparent latent impact on cardiac form and function.

The brains of sockeye juveniles that were exposed during development to WSFd were significantly larger than unexposed fish when corrected for individual fish length, and this effect was most pronounced and consistent across brain regions in fish exposed to 4 μg L^{-1} TPAH during development. To the best of our knowledge, this is the first study to assess the lasting effects of ELS crude oil exposure on brain morphology; however previous studies have shown that regional (Irie et al., 2011; Kawaguchi et al., 2012) or total brain area (Xu et al., 2017a) is reduced in fish larvae exposed to crude oil, and this can impact larval swimming behavior (Kawaguchi et al., 2012). The mechanisms responsible for crude oil-induced changes to the central nervous system are not known, but ELS mahi-mahi and red drum (*Sciaenops ocellatus*) exposed to Deepwater Horizon HEWAF undergo pronounced changes in the expression of genes associated with neurodegeneration, brain development and neuronal function, supporting the CNS as a major target of crude oil induced toxicity (Xu et al., 2017a, 2017b). Furthermore, latent behavioral impacts of ELS exposure suggest that the consequences of this neurotoxicity can persist to later life stages. For example, killifish exposed as ELS to a complex PAH mixture experience impaired mobility, activity patterns, and tank positioning as adults (Brown et al., 2016), and coral reef fishes exposed as ELS to

Deepwater Horizon HEWAF show impaired shoaling behavior and habitat use that result in reduced survival in the presence of a natural predator (Johansen et al., 2017). While more research is needed to fully appreciate the mechanisms and consequences of crude oil induced neurotoxicity, it is clear from these recent studies that ELS exposure to crude oil affects brain development, which can cause significant behavioral and cognitive deficits that impact survival.

5. Conclusions

This study presents data on adverse effects of WSFd exposure to developing sockeye embryos, including increased mortality, delayed hatching, teratogenesis, decreased utilization of energy stores, and reduced growth. In addition, high post-exposure mortality of fry and lasting changes in brain morphology could extend the impact of ELS dilbit exposure by further reducing population size and impairing the physiology and behavior of surviving fish. Continued efforts to understand the nature and consequences of latent effects will deepen our understanding of the impacts of petrochemical exposure.

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