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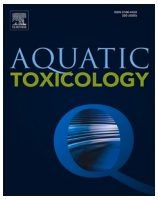

Age matters: Comparing life-stage responses to diluted bitumen exposure in coho salmon (*Oncorhynchus kisutch*)

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ABSTRACT

Millions of liters of diluted bitumen (dilbit), a crude oil product from Canada's oil sands region, is transported through critical Pacific salmon habitat each day. While the toxicity of the water-soluble fraction of dilbit (WSFd) to early life-stages of salmon is known, quantitative data on life-stage differences in sensitivity to WSFd is missing. To fill this knowledge gap, we exposed two juvenile life-stages of coho salmon (*O. kisutch*) in parallel to very low (parts per billion), environmentally-relevant concentrations of WSFd for acute (48 h) and sub-chronic (4 wk) durations. The relative sensitivities of the two life-stages (fry and parr) were assessed by comparing the timing and magnitude of biological responses using common organismal and molecular endpoints of crude oil exposure. A significant reduction in body condition occurred in both fry and parr after 4 wk exposure to WSFd. Both life-stages also experienced a concentration-dependent decrease in time-to-loss-of-equilibrium during a hypoxia challenge test at both 48 h and 4 wk of exposure. Although organismal responses were similar, molecular responses were distinct between life-stages. In general, unexposed fry had higher baseline values of hepatic phase I biotransformation indicators than unexposed parr, but induction of EROD activity and *cyp1a* mRNA expression in response to WSFd exposure was greater in parr than in fry. Neither *gst* nor *hsp70* mRNA expression, markers of phase II biotransformation and cell stress, respectively, were reliably altered by WSFd exposure in either life-stage. Taken together, results of this study do not support differential sensitivities of coho fry and parr to WSFd. All the same, the potential for ontogenic differences in the expression and induction of phase I biotransformation need to be considered because age does matter for these endpoints if they are used as bioindicators of exposure in post-spill impact assessments.

1. Introduction

Canada is home to the third largest proven crude oil reserve in the world, largely found as bituminous oil sands deposits in the Western Canada Sedimentary Basin (National Energy Board of Canada, 2019). Canada is currently the sixth largest contributor to the global oil market, but as armed conflicts and political sanctions disrupt the global energy market, there is added incentive to increase the extraction and export of Canadian crude to satisfy markets in Europe and Asia. A critical component of Canada's crude oil industry is the transport infrastructure that carries extracted bitumen from the oil sands region to coastal seaports and to refineries in the USA. Bitumen, a heavy type of crude oil, is diluted ~3:1 with light hydrocarbon condensate and then shipped thousands of kilometers across North America by pipeline and rail (Canadian Association of Petroleum Producers, 2016). This transport network includes the Trans Mountain Pipeline that spans 1150 km between Strathcona County, Alberta and Burnaby, British Columbia with a daily carrying capacity of over 140 million L of diluted bitumen (dilbit). Importantly, this pipeline corridor transects critical freshwater nursery habitat for Pacific salmon (Levy, 2009). Three of the five species of Pacific salmon in the Fraser River Watershed, Canada's largest salmon-bearing system, are currently listed as Threatened or Endangered by the Committee on the Status of Endangered Wildlife in Canada, including coho (*Oncorhynchus kisutch*; COSEWIC, 2016). Dilbit spilled into freshwater systems can sink (Alsaadi et al., 2018a; Dew et al., 2015; Stoyanovich et al., 2019), which complicates clean-up, recovery, and

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habitat restoration efforts; thus, a pipeline leak or failure leading to contamination of salmon habitat could pose a long-term threat to the viability of these ailing salmon stocks (Levy, 2009).

Petrogenic hydrocarbons, like dilbit, are complex mixtures of chemicals with known toxicity to fish. These chemicals include monocyclic aromatic hydrocarbons (i.e., benzene, toluene, ethylbenzene, and xylenes, or BTEX), polycyclic aromatic compounds (PACs), and naphthenic acids (Ftoon M. Alsaadi et al., 2018a; Kennedy, 2015). While it is this mixture of compounds that drive crude oil toxicity (Meador and Nahrgang, 2019), the summed concentration of soluble PACs is frequently used to define exposure conditions, particularly in laboratory studies, owing to well-described modes of action for specific PACs present in crude oils. For example, PACs with high affinity for the aryl hydrocarbon receptor (AhR) are important contributors to adverse outcomes in fish (Billiard et al., 2006; Collier et al., 2013; Incardona et al., 2006; Scott and Hodson, 2008). The AhR is a ligand-activated transcription factor that interacts with xenobiotic response elements to alter the expression of thousands of target genes, including *cytochrome p450 1a* (*cyp1a*) and *glutathione s transferase* (*gst*) that encode enzymes involved in Phase I and Phase II biotransformation, respectively. Xenobiotic biotransformation in the liver is critical to detoxification and tissue depuration, as sequential enzyme-mediated modifications of parent compounds increase their solubility to facilitate excretion. However, reactive intermediate metabolites formed during biotransformation of PACs underlie their biotoxicity. Therefore, induction of the biotransformation pathway as well as markers of cell stress, such as increased *heat shock protein 70* (*hsp70*) expression, are frequently used as quantitative bioindicators of PAC exposure and early indicators of potential adverse organismal outcomes (Hodson et al., 1991; Madison et al., 2015; Nahrgang et al., 2010; Santana et al., 2018; Whyte and Tillitt, 1995).

Crude oil spills are dynamic, unpredictable events. As such, the environmental impacts of a spill will be influenced by myriad factors including the prevailing abiotic conditions at the time of the spill, the rate and volume of spilled product, and the biotic community within the spill zone. Assessment of environmental impacts is further complicated by the potential for differential sensitivity to the contaminants in crude oil both within and across species. For example, within a species, and across life-stages, ontogenic differences may influence contaminant uptake (surface area to volume ratio decreases with growth) and detoxification (as biotransformation pathways mature) (Petersen and Kristensen, 1998). To date, most studies investigating the toxicity of dilbit to fish have emphasized effects in embryonic and larval life-stages, and demonstrate a range of lethal and sublethal responses including increased mortality, teratogenesis, growth impairment, and altered behaviours (Alderman et al., 2018; Alsaadi et al., 2018b; Bérubé et al., 2021; Lin et al., 2022a; Madison et al., 2020, 2017, 2015; McDonnell et al., 2019; Philibert et al., 2016; Robidoux et al., 2018). In post-larval life-stages, dilbit exposure also induces molecular, tissue, and organismal level changes capable of impacting individual fitness (Alderman et al., 2020, 2017b, 2017a; Avey et al., 2020; Lin et al., 2022c, 2021, 2020). Although life-stage specific sensitivities to crude oil exposure are rarely explicitly tested, current evidence suggests that sensitivity decreases with age. For example, the toxicity of dispersed weathered crude oil to Atlantic herring (*Clupea harengus*) embryos decreased with age of initial exposure, as evidenced by an increase in the 48 h median effective concentration (EC50) and decreased severity of blue sac disease (McIntosh et al., 2010). Similarly, increased mortalities occur in embryonic salmonids exposed to total PAC concentrations as low as 4 μg/L (Alderman et al., 2018; Lin et al., 2022a), whereas exposures as high as 125 μg/L are sublethal in salmon fry and parr (Alderman et al., 2017b; Avey et al., 2020; Lin et al., 2022c).

Despite a growing body of literature on the adverse outcomes of crude oil exposure in fish, our capacity to define life-stage specific sensitivities is restricted by comparing data across studies, where differences in exposure conditions can confound interpretation. Quantitative data that define the relative sensitivity of early life-stages of salmon to dilbit exposure are needed to aid spill managers in estimating population impacts through the integration of data across life-stages. To address this knowledge gap, this study investigated the differential sensitivity of juvenile coho to dilbit exposure by quantifying the temporal induction of Phase I and Phase II biotransformation in the liver alongside organismal effects on cardiorespiratory performance.

2. Materials and methods

2.1. Fish

Coho salmon (*Oncorhynchus kisutch*) fry and parr ($n = 432$ each) were acquired from Capilano Fish Hatchery (North Vancouver, BC) and transported to Simon Fraser University (Burnaby, BC, Canada). Upon arrival, each fish was given one of three unique fin clips (one per exposure concentration), and then acclimated for 2 wk in one of 18 250-L fiberglass tanks ($n = 48$ fish per tank, one life-stage per tank). Each experimental tank was supplied with aerated flow-through dechlorinated municipal water (12 °C) and the photoperiod was 12:12 (light: dark). Fish were fed commercial salmon chow daily ad libitum. Care and use of animals were approved by the Simon Fraser University Animal Care Committee (animal care protocol #1315B-20), according to the guidelines of the Canadian Council for Animal Care.

2.2. Dilbit exposure and water chemistry

For each life-stage, fish were exposed in triplicate tanks to uncontaminated water (control) or to one of two concentrations of the water-soluble fraction of dilbit (WSFd; 1 μg/L and 5 μg/L nominal total PAC) as previously described (Alderman et al., 2017b). Briefly, WSFd exposure was initiated by redirecting water intake through generator columns containing ceramic beads soaked in Cold Lake Summer Blend dilbit and then into header tanks. Water from the middle of the header tanks was pumped into experimental tanks such that fish were exposed only to dissolved contaminants. Target concentrations were achieved by varying the number of ceramic beads or by omitting the dilbit (0 μg/L, control). These concentrations reflect plausible exposure conditions of an upstream spill resulting in contamination of downstream nursery lakes, and are known to be sublethal in other species of juvenile salmonids (Alderman et al., 2017b; Avey et al., 2020). Fish were exposed continuously without recharging the generator columns for 48 h or 4 wk, and then subsampled as described below (Fig. 1). Water samples were collected at 12 h, 36 h, and 4 wk after initiating the exposures to confirm the presence of PAC in experimental tanks. The concentrations of 75 PAC were quantified by GC–MS (SGS Axys Analytical Services Ltd., Sidney, BC) as previously described (Alderman et al., 2017b).

2.3. Hypoxia challenge test (HCT)

The HCT was performed according to Claireaux et al. (2013) with some modification. After 48 h and 4 wk of continuous WSFd exposure, a subset of 12 parr was removed from one replicate experimental tank per concentration and combined into a 250-L fiberglass tank fitted with a transparent lid (i.e., $n = 36$ parr per test representing each concentration). Following a 1 h acclimation period at 100% dissolved oxygen (DO), the DO content in the testing arena was rapidly dropped from full saturation to 20% at a rate of 3.8% per min, and then gradually and steadily (−0.13% per min) to just below 4% when the test was completed. The DO content was controlled by bubbling nitrogen gas into the testing arena at one of two flow rates *via* a ceramic diffuser manually controlled using a standard two-valve gas regulator. The real-time DO content in the testing arena was continuously monitored using a fiber optic oxygen sensor probe and a Witrox 1 minisensor oxygen meter (Loligo® Systems, Tjele, Denmark). When an individual fish could no longer maintain position in the water column and floated to the surface,

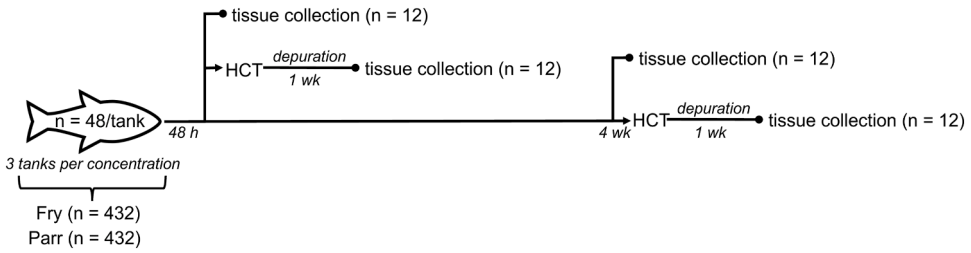

Fig. 1. Schematic of the experimental design indicating exposure and sampling timelines for each experimental tank containing 48 fry or parr (9 tanks total per life-stage). After 48 h or 4 wk of continuous exposure, 12 individual fish were subsampled for tissue collection and an additional 12 fish were subsampled following a hypoxia challenge test (HCT) and 1 wk depuration in clean water.

the time-to-loss-of-equilibrium (TLOE), DO level, and fin-clip pattern (denoting concentration group) were recorded, and the fish was removed to an aerated recovery tank. The HCT was completed when the last fish lost equilibrium (~3 h per test). The HCT was repeated for the two remaining parr tank replicates, and then the tests were performed exactly the same for fry, for a total of 6 tests per time point. Fish were recovered for a 1 wk depuration period in uncontaminated water, and then terminally sampled as described below. No mortalities occurred during or after the HCT.

2.4. Tissue collection

After 48 h and 4 wk of continuous WSFd exposure, a subset of 12 fish was removed from each experimental tank and euthanized in buffered tricaine methanesulfonate (0.5 mg/L; Syndel, Qualicum Beach, BC). Wet body mass and fork length were recorded. Blood was collected (parr only) by free flow from the severed caudal vessels in the tail into heparinized microcapillary tubes, and the plasma separated by centrifugation at 6000 *g* for 5 min. Packed cell volume was measured using digital callipers. The liver was removed, diced, and snap frozen using liquid nitrogen. All samples were stored at −80 °C until analysis. Fish recovered from the HCT were euthanized after a 1 wk depuration period in clean water and the liver was sampled as above.

2.5. Liver ethoxyresorufin O-deethylase assay

The induction of liver cytochrome P450 1 (CYP1) was quantified using an ethoxyresorufin O-deethylase (EROD) assay on a subset of frozen livers ($n = 3$ fish per replicate tank) for a total of 9 fish per treatment (concentration, exposure time, and depuration). Hepatic EROD activity in the microsomal fraction was determined following standard protocols (Kennedy and Farrell, 2006) and is expressed relative to total protein, as determined by a Bradford assay.

2.6. Gene expression and RT-qPCR

Frozen liver tissue was homogenized in TRIzol reagent (Invitrogen, Massachusetts, USA) using a Precellys Evolution (Bertin Technologies, Saint Quentin en Yvelines Cedex, France) and 1.4 mm zirconium oxide beads (Bertin) for two 25 s cycles at 5800 rpm. Cell debris was pelleted by centrifugation (12 000 g for 5 min), and total RNA was extracted following manufacturer's instructions. Purity and yield of total RNA samples were verified using the NanoDrop 2000 (Thermo Scientific), and then 1 µg of total RNA was treated with DNase I and reverse transcribed to cDNA in 20 µL reactions using the High-Capacity cDNA Synthesis Kit, all according to manufacturer's instructions (Life Technologies, Grand Island, NY). To confirm the absence of genomic DNA co-amplification, duplicate reactions that omitted the Multiscribe RT enzyme were included for 10% of randomly chosen samples.

Transcript abundances of*cytochrome p450 1a* (*cyp1a*), *glutathione s transferase* (*gst*), *heat shock protein 70* (*hsp70*), and the reference genes β-*actin*, *ribosomal protein L8* (*rpl8*), and *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) were quantified in separate duplicate RT-qPCR reactions using a Bio-Rad CFX96 and recommended cycling conditions. Each 15 µL reaction contained 1x SSO SYBR Green (Life Technologies), gene-specific primer pair (Table 1), and 5 µL of either diluted cDNA, non-reverse-transcribed sample, or water. Each reaction plate included an internal control sample (intra-assay coefficient of variation was <5%). All cDNA reactions produced a dissociation curve with a single peak at the predicted amplicon melt temperature, while reactions with water as template failed to amplify and non-reverse-transcribed controls either did not amplify or amplified >10 cycles after the average threshold cycle value for that gene. 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. Of the three reference genes, only *β-actin* was stably expressed across experimental groups; therefore, transcript abundance values of target genes were standardized to *β-actin*.

2.7. Statistical analyses

The absence of a tank effect was confirmed for each dataset prior to proceeding with formal statistical analysis. Body mass (g) and fork length (cm) were used to calculate Fulton's condition factor (K) according to the formula:

$$K = (100 \times \text{mass}) \div (\text{fork length})^3$$

A two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test were used to determine life-stage specific effects of WSFd exposure on K. The effect of WSFd exposure on cardiorespiratory performance was assessed by fitting a probability curve to model equilibrium probability against test time using a Kaplan-Meier survival test

Table 1
Forward (F) and reverse (R) primer sequences for genes of interest and three reference genes, indicating final primer concentrations for each assay, as well as the reaction efficiency (E) and R^2 determined from a standard curve of serially diluted cDNA. *cytochrome P450 1a, cyp1a; glutathione S-transferase, gst; heat shock protein 70, hsp70; beta actin, β-actin; ribosomal protein L8, rpl8; glyceraldehyde 3-phosphate dehydrogenase, gapdh.*

Gene	Sequence (5′ – 3′)	Conc. (nM)	E (%)	R^2	Refs.
cyp1a	F: agtgctgatggcacagaactcaa R: agctgacagcgcttgtgctt	400	110	0.98	Matsuo et al. (2008)
gst	F: ctctgctccagttgcctggat R: gttgccattaatgggcagtttct	300	107	0.97	Espinoza et al. (2012)
hsp70	F: ctgctgctgctggatgtg R: gctggttgtcggagtaagtg	400	104	0.96	Yar Ahmadi et al. (2014)
β-actin	F: gacccacacagtgcccatct R: gtgcccatctcctgctcaaa	300	109	0.99	Matsuo et al. (2008)
rpl8	F: ttggtaatgttctgcctgtg R: gggttgtgggagatgactg	200	93	0.96	Alderman et al. (2017b)
gapdh	F: tctgtgttggaatcaacgga R: tgaagaagactccggtggac	400	104	0.98	Espinoza et al. (2012)

followed by a Mantel-Cox test (Claireaux et al., 2013). No differences between replicate tanks were observed, therefore data were pooled for final analysis (n = 36 fish per treatment). To facilitate quantitative interpretation of results, differences in the median TLOE for each treatment (n = 3 replicate tanks per concentration per life-stage) were compared using a two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. A two-way ANOVA and Tukey's post-hoc test determined the effects of WSFd concentration and exposure time on packed cell volume of parr. A two-way ANOVA and Tukey's post-hoc test determined the effects of WSFd concentration and life-stage on liver EROD activity and gene expression. T-tests determined whether life-stage differences in liver gene expression and EROD activity were present in unexposed control fish (n = 16 and 36, respectively). Where data were not normally distributed (Shapiro-Wilk), a conservative outlier detection method, ROUT (Motulsky and Brown, 2006), was used to identify and remove definitive outliers (Q = 0.1%) prior to analysis (48 h *cyp1a* = 4 outliers; 4 wk *cyp1a* = 3 outliers; 48 h *gst* = 5 outliers; 4 wk *gst* = 1 outlier; 48 h *hsp70* = 5 outliers; 4 wk *hsp70* = 2 outliers). All analyses were performed in GraphPad Prism (v9.4) at $\alpha = 0.05$. Data is presented as mean ± standard error of the mean (S.E.M).

3. Results

3.1. WSFd exposure

Water samples collected at 12 h, 36 h, and 4 wk confirmed the presence of PAC in experimental tanks supplied with WSFd, with initial total dissolved PAC concentrations (TPAC) of 2.9 µg/L and 6.3 µg/L (used hereafter to define the two concentration groups; Fig. 2). TPAC concentration decreased throughout the 4 wk, as expected (Alderman et al., 2017b), with the abundances of lower molecular weight hydrocarbons (i.e., naphthalenes) dominating the early exposure and heavier hydrocarbons (i.e., pyrenes) becoming relatively more abundant by 4 wk (Supplementary Data Table S1). TPAC concentration of the control tanks was effectively zero throughout the experiment (geometric mean 0.023 ± 0.0009 µg/L; Fig. 2).

3.2. Organismal responses to WSFd exposure

Fry and parr (measured at 48 h exposure) had similar initial body mass and fork length across treatments (in fry: mass = 1.3 g, length = 4.8 cm; in parr: mass = 26.4 g, length = 13.6 cm). There were no differences in Fulton's condition factor (K) among treatment groups at 48 h (Table 2). Fish growth over the course of the 4 wk experimental period varied with WSFd concentration. While unexposed control fry doubled their body mass and increased fork length by 24%, exposure to WSFd significantly reduced growth in fry, with a maximal effect for the 6.3 µg/L initial TPAC (57% mass gain, 17% length gain). Growth of parr was similarly impaired, but not in a concentration-dependent manner. While unexposed control parr gained 18% in mass and 7% in length over the course of the experiment, parr exposed continuously to WSFd for 4 wk lost weight (−7% and −3% in 2.9 and 6.3 µg/L initial TPAC, respectively) and experienced negligible length gain which resulted in decreased K relative to unexposed control parr ($P_{concentration} < 0.0001$; Table 2).

Inter-individual variation in performance during the HCT, as indicated by oxygen saturation at TLOE, was ~5% within each experimental group (n = 36 per life-stage per concentration per exposure time). Nevertheless, TLOE was significantly faster for fry and parr exposed to WSFd at 48 h and 4 wk compared with unexposed control fish (Fig. 3A and B, respectively). The effect of WSFd exposure on TLOE was concentration and life-stage dependent after 48 h of exposure ($P_{interaction}$ = 0.003), driven by a significantly longer TLOE in control fry relative to control parr (Fig. 3C). After 4 wk of WSFd exposure, differences in TLOE were concentration-dependent ($P_{concentration} < 0.0001$) and independent of life-stage (Fig. 3D).

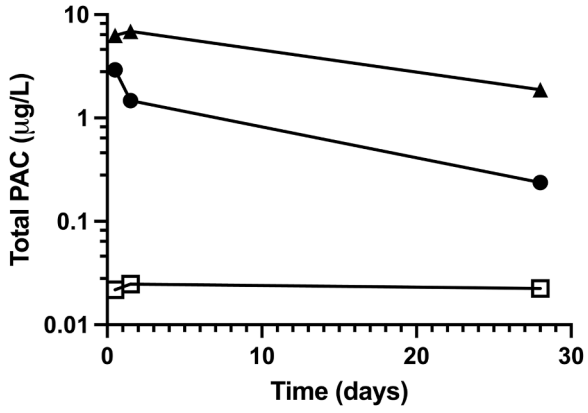

Fig. 2. Mean total polycyclic aromatic compounds (TPAC) in triplicate experimental tanks during the 4 wk exposure to dilbit, for each of three concentrations: 0 µg/L initial TPAC (control, no dilbit, open squares), 2.9 µg/L initial TPAC (filled circles), and 6.3 µg/L initial TPAC (filled triangles).

3.3. Tissue responses to WSFd exposure

Packed cell volume remained constant across WSFd concentrations and exposure durations in parr (Table 2). A sufficient blood volume could not be acquired from fry for similar measurements.

Exposure to WSFd for 48 h did not alter liver EROD activity in either fry or parr (fry = 22.54 ± 1.00 *vs* parr = 14.84 ± 0.83; $P_{lifestage} < 0.0001$; Fig. 4A). The same pattern was observed after 1 wk depuration from the 48 h exposure ($P_{lifestage} < 0.0001$; Fig. 4B). After 4 wk of exposure, liver EROD activity increased in fry only at 6.3 µg/L TPAC; whereas a concentration-dependent increase in liver EROD activity occurred in parr and reached a maximal response greater than that of fry ($P_{interaction}$ = 0.0007; Fig. 4C). After 1 wk depuration from 4 wk exposure, liver EROD activity in parr exposed to 2.9 µg/L TPAC was recovered, but EROD activity remained elevated in both fry and parr exposed to the 6.3 µg/L TPAC ($P_{interaction} < 0.0001$; Fig. 4D).

Transcript abundances of genes involved in biotransformation and cell stress were differentially affected by WSFd concentration, exposure duration, and life-stage (Fig. 5). In the liver of fry, *cyp1a* increased 3.8-fold relative to control fry after 48 h exposure to 6.3 µg/L TPAC, but was stable across all concentrations in the liver of parr; however, *cyp1a* expression was generally greater in fry than in parr ($P_{interaction}$ = 0.0004; Fig. 5A). After 4 wk of exposure, liver *cyp1a* increased 8.7 and 21.7-fold in parr exposed to 2.9 and 6.3 µg/L TPAC, relative to control parr, but no changes were observed in the liver of fry ($P_{interaction}$ = 0.0245; Fig. 5B). The transcript abundance of *gst* in the liver was greater in fry than in parr but unaffected by 48 h WSFd exposure ($P_{lifestage} < 0.0001$; Fig. 5C). After 4 wk of WSFd exposure, *gst* expression remained stable in the liver of parr but decreased in the liver of fry exposed to 6.3 µg/L TPAC ($P_{interaction}$ = 0.0032; Fig. 5D). The expression of *hsp70* increased ~2-fold after 48 h exposure to 6.3 µg/L TPAC in both fry and parr ($P_{concentration}$ = 0.01; Fig. 4E). There was a significant interaction between life-stage and concentration after 4 wk exposure, but pairwise differences in *hsp70* were not detected in the post-hoc analysis ($P_{interaction}$ = 0.0187; Fig. 5F).

Indicators of phase I and II biotransformation pathways were differentially expressed in fry and parr, independently of WSFd exposure. In unexposed control fish, the expression of *cyp1a* was ~12-fold higher ($P = 3.2 \times 10^{-5}$) and EROD activity was 1.5-fold higher ($P < 1 \times 10^{-6}$) in fry relative to parr (Fig. 6). Similarly, the expression of *gst* was 4-fold higher in unexposed fry relative to parr (not shown).

4. Discussion

By exposing two juvenile life-stages of coho in parallel to dilbit, this

Table 2
Effect of WSFd exposure on Fulton's condition factor (K) and packed cell volume (PCV). Fry and parr were exposed to uncontaminated water (0 μg/L) or to 2.9 or 6.3 μg/L initial TPAC for 48 h and 4 wk. Differences were determined using a two-way ANOVA and Tukey's post-hoc test ($N = 36$, $\alpha = 0.05$). * $P < 0.0001$; N.D = not determined; n.s.d. = no statistical differences.

	Fry			Parr			Statistics
	0 μg/L	2.9 μg/L	6.3 μg/L	0 μg/L	2.9 μg/L	6.3 μg/L	
Condition Factor (K)							
48 h	1.11 ± 0.02	1.12 ± 0.03	1.07 ± 0.02	1.01 ± 0.02	1.00 ± 0.01	1.01 ± 0.02	**Fry > Parr***
4 wk	1.17 ± 0.02	1.10 ± 0.02	1.06 ± 0.02	1.00 ± 0.02	0.94 ± 0.02	0.94 ± 0.01	**Fry > Parr*** **0 > 2.9 = 6.3***
Packed Cell Volume (PCV)							
48 h	N.D.	N.D.	N.D.	1.97 ± 0.23	2.05 ± 0.34	1.89 ± 0.19	n.s.d.
4 wk	N.D.	N.D.	N.D.	1.98 ± 0.22	2.05 ± 0.25	2.02 ± 0.23	n.s.d.

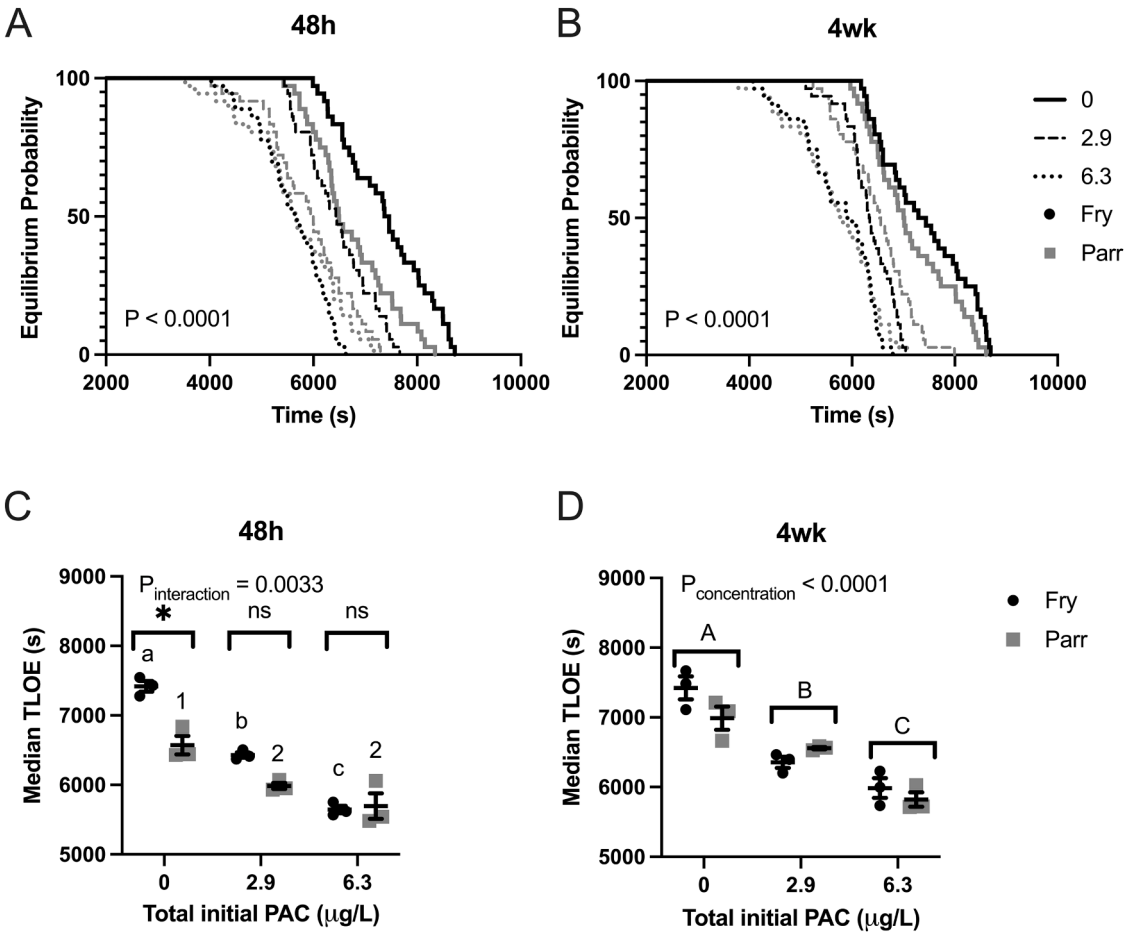

Fig. 3. Cardiorespiratory performance in hypoxia challenge tests for fry (black) and parr (gray) exposed to WSFd at total initial PAC concentrations of 0 μg/L (control; solid lines), 2.9 μg/L (broken lines) or 6.3 μg/L (dotted lines). A Kaplan-Meier analysis of time-to-loss-of-equilibrium (TLOE) revealed significant differences between experimental groups ($n = 36$ fish per treatment, pooled from 3 replicate tanks) after 48 h (A) and 4 wk exposure (B). The median TLOE was calculated for each tank replicate to determine differences in the effects of concentration and life-stage in fish exposed for 48 h (C) or 4 wk (D) using a two-way ANOVA and Tukey's multiple comparisons test ($n = 3$ per treatment; $\alpha = 0.05$). Within a life-stage, differences between concentrations are indicated by letters (fry) or numbers (parr). An asterisk denotes life-stage differences within a concentration. Concentration effects are denoted by uppercase letters.

comprehensive data set directly compared life-stage specific differences in the timing and magnitude of the resultant biological responses. We found that growth and performance metrics of fry and parr were similarly impaired by WSFd, but molecular indicators of exposure showed unique temporal- and concentration-specific changes for each life-stage. As discussed below, these results should assist spill managers and post-spill remediation efforts.

4.1. Organismal responses to WSFd exposure

Body condition and growth rates are important fitness metrics across taxa, including in fish. While individual growth rates were not determined in this study, differences in the mean mass, length, and condition factor (K) of fish sampled 26 d apart support a growth deficit induced by WSFd exposure for both fry and parr. More specifically, unlike the growth of unexposed control fish during this time, mass gain was reduced in WSFd-exposed fish and resulted in a significantly lower K. This result is consistent with previous studies demonstrating reduced growth in fish when exposed to water-soluble fractions of crude oil or oiled sediments during embryogenesis (Alderman et al., 2018; Heintz et al., 2000) or larval/juvenile life-stages (Claireaux et al., 2013; Lin et al., 2022b), and when fed oil-contaminated food (Meador et al., 2006;

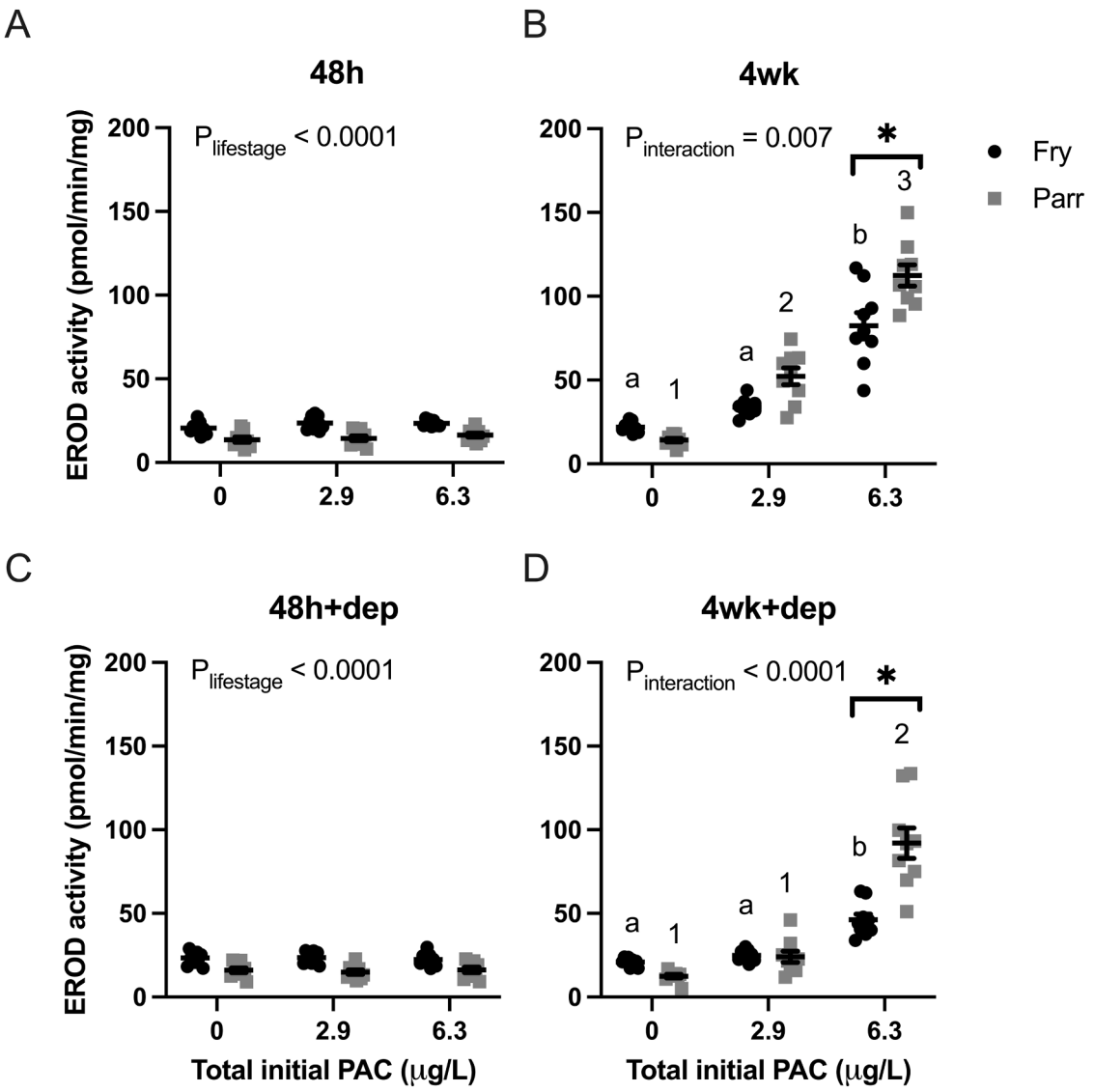

Fig. 4. Effects of WSFd exposure on CYP1 activation in the liver of fry (black circles) and parr (gray squares), measured as ethoxyresorufin-O-deethylase (EROD) activity following 48 h (A) and 4 wk (B) exposure to control (0 µg/L), 2.9 µg/L, or 6.3 µg/L total initial PAC, and following 1 wk depuration from each exposure duration (C, D). Differences were determined using a two-way ANOVA and Tukey's multiple comparisons test (n = 9; α = 0.05). Within a life-stage, differences between concentrations are indicated by letters (fry) or numbers (parr). An asterisk denotes life-stage differences within a concentration.

Rice et al., 2000; Saborido-Rey et al., 2007; Wang et al., 1993). The fact that growth reduction is observed across different life-stages and species of fish, as well as under a range of experimental scenarios that vary in oil type, contaminant concentration, exposure duration, and exposure mode, suggests there may be a common underlying mechanism contributing to growth impairment caused by exposure to petrogenic chemicals that is independent of life-stage.

Hypoxia challenge tests (HCT) are an effective, high-throughput, repeatable method for assessing fish health and performance in response to crude oil exposure (Claireaux et al., 2013; Mauduit et al., 2019, 2016; Zhang et al., 2017). Like swimming performance tests that determine critical swimming speeds (Brett, 1964; Farrell, 2008), they have been widely used to assess oil toxicity in fish. LOE in an HCT represents a failure of the cardiorespiratory system to match metabolic supply and demand, leading to system dysfunction. As such, individual performances in such tests are considered ecologically relevant metrics for predicting organismal and population-level outcomes (Claireaux et al., 2013; Cooke et al., 2012; Eliason et al., 2011). In the present study, there was a concentration-dependent decrease in TLOE for both fry and parr, and the magnitude of the response was similar in both life-stages and at both exposure durations. Packed red blood cell volume was unaffected by WSFd exposure in parr, thus it is unlikely that O_2-carrying capacity of the blood contributed to the performance decline. Rather, given that the heart sets the functional limits of the cardiorespiratory system in fish (Claireaux et al., 2005; Farrell, 2009), and that crude oil and PAC exposures impart broad effects on fish heart morphology and function (Incardona et al., 2011; Incardona and Scholz, 2016), it is a plausible conclusion that the hearts of both fry and parr have similar sensitivity limits to the contaminants in WSFd.

4.2. *Life-stage differences in molecular responses to WSFd exposure*

Many 3- and 4-ringed PAC present in petrogenic hydrocarbon mixtures are AhR ligands; therefore, the induction of phase I biotransformation enzymes is a widely used indicator of crude oil exposure in fish (Alsaadi et al., 2019b; Billiard et al., 2006; Blanc et al., 2010; Imbery et al., 2019; Incardona et al., 2006; Zhou et al., 2010). Adverse outcomes experienced by fish exposed to crude oils are at least partly attributed to CYP1-mediated PAC metabolism (Collier et al., 2013; Incardona et al., 2006; Jung et al., 2015). This is particularly true during early ontogeny,

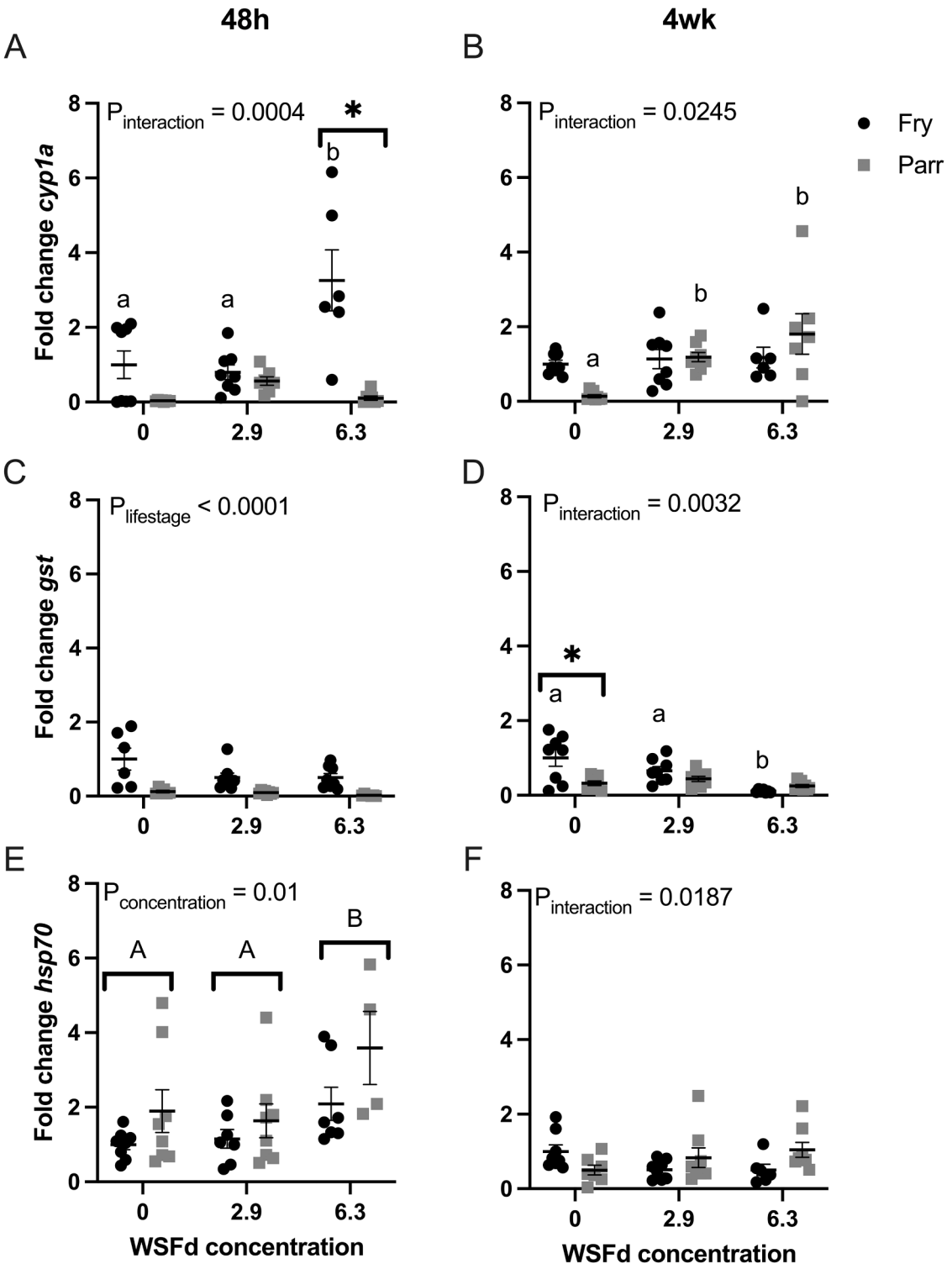

Fig. 5. Hepatic gene expression in fry (black circles) and parr (gray squares) exposed to WSFd at total initial PAC concentrations of 0 µg/L (control), 2.9 µg/L, or 6.3 µg/L for either 48 h (left) or 4 wk (right). The transcript abundances of *cyp1a* (A, B), *gst* (C, D), and *hsp70* (E,F) were quantified by RT-qPCR and normalized to the stably expressed reference gene, *β-actin*. To facilitate interpretation, data within each panel are presented as fold-change relative to fry control. Differences were determined using a two-way ANOVA and Tukey's multiple comparisons test ($n = 5–8$; $\alpha = 0.05$). Within a life-stage, differences between concentrations are indicated by lowercase letters. An asterisk denotes life-stage differences within a concentration. Concentration effects are denoted by uppercase letters.

when the incomplete maturation of biotransformation pathways coupled with increased chemical uptake across a relatively large surface area can drive a heightened sensitivity of fish embryos and larvae to contaminant exposure (Petersen and Kristensen, 1998). While the liver is the primary site of xenobiotic metabolism, extra-hepatic expression and activity of CYP1 is documented in a variety of fish tissues and throughout ontogeny (Otte et al., 2010), including tissues at the organism-environment interface like the gills (Alderman et al., 2020; Blanc et al., 2010; Jönsson et al., 2010). The specific contribution of extra-hepatic biotransformation to the biological response to crude oil

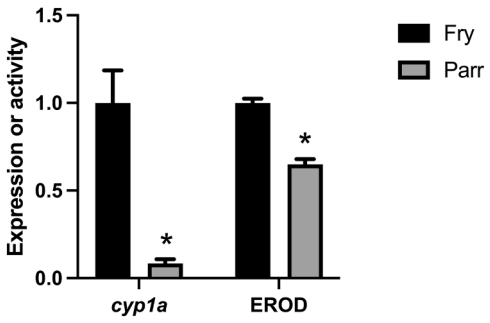

Fig. 6. Relative baseline levels of phase I biotransformation in unexposed control fry (black) and parr (gray), as indicated by mRNA abundance of *cyp1a* (A) and ethoxyresorufin-O-deethylase (EROD) activity (B). For each life-stage, data were pooled for all unexposed control fish across replicate tanks and sampling timepoints (*cyp1a* expression $N = 16$; EROD activity $N = 36$), and then differences determined by t-tests. Data are shown as mean ± s.e.m. and normalized to levels in fry. An asterisk denotes a significant difference between fry and parr (*cyp1a* expression $P = 3.2 \times 10^{-5}$; EROD activity $P < 1 \times 10^{-6}$).

exposure is not known. Here, we show that the timing and magnitude of biotransformation induction in the liver is distinct between coho fry and parr, which may be partly attributed to life-stage differences in baseline gene expression and enzymatic activity. In the absence of WSFd exposure, *cyp1a* and *gst* mRNA abundances as well as EROD activity were all greater in fry relative to parr. This result is consistent with previous work demonstrating greater *cyp1a* expression in the hearts of sockeye swim-up fry relative to 8-month parr (Lin et al., 2022b), and a decrease in hepatic EROD and GST activity during parr-smolt transition in coho (Seubert and Kennedy, 1997). Whether such differences in basal gene expression and enzymatic activity reflect ontogenic shifts in biotransformation capacity is unclear, but Seubert and Kennedy (1997) noted that variation in the accumulation of benzo[*a*]pyrene (B[*a*]P), a model PAC, across tissue compartments of juvenile coho corresponded with temporal variation in hepatic EROD and GST activity.

Temporal and concentration dependent changes in EROD activity and *cyp1a* and *gst* mRNA abundances following WSFd exposure were life-stage specific. Consistent with WSFd exposures in other salmonids (Alderman et al., 2017b; Avey et al., 2020), an increase in EROD activity was evident after 4 wk in fry exposed to 6.3 µg/L PAC and in parr exposed to 2.9 and 6.3 µg/L PAC, with some recovery occurring after 1 wk depuration. Notably, EROD activity was induced at a lower concentration and reached a greater activity level in parr relative to fry. The mRNA abundance of *cyp1a* showed a similar pattern as EROD induction in parr. In contrast, the early increase in *cyp1a* expression in fry was small and transient, with no detectable differences in *cyp1a* after 4 wk of WSFd exposure. These life-stage specific responses in *cyp1a* are obscured by the differences in baseline expression – the 20-fold increase in *cyp1a* in parr after 4 wk of exposure to 6.3 µg/L PAC relative to unexposed parr merely aligns *cyp1a* abundance to a level comparable with that of unexposed fry. Taken together, these results highlight the need for a contextual framework that accounts for ontogenic and species-level differences before interpreting phase I induction in fish exposed to dissolved components of crude oils (Kühnert et al., 2017; Lin et al., 2022b; Petersen and Kristensen, 1998).

Phase I biotransformation of PACs can result in the generation of toxic intermediate metabolites capable of inducing oxidative damage and cell stress. As such, changes in mRNA abundance of *gst* and *hsp70* have been explored as additional early molecular indicators of adverse outcomes of crude oil exposure, with mixed results. In larval fathead minnow (*Pimephales promelas*) exposed to the WSF of three different crude oils, *gst* expression was unchanged despite a concentration-dependent increase in measured GST activity (Bérubé et al., 2021); however, GST activity in the liver of the streaked prochilod (*Prochilodus lineatus*) decreased after a 96-h exposure to B[*a*]P (Santos and Bueno dos Reis Martinez, 2020). Neither *gst* nor *hsp70* were reliable predictors of malformations in embryonic Japanese medaka (*Oryzias latipes*) exposed to WSFd (Madison et al., 2020) despite previous observations of concentration-dependent increases in both genes following WSFd exposure in early life-stages of fish (Alsaadi et al., 2018b; Madison et al., 2015). A recent study in juvenile coho salmon exposed to WSF of low-sulfur marine diesel found no changes in hepatic *hsp70* expression (Imbery et al., 2019). In the present study, a modest decrease in *gst* occurred in fry exposed to 6.3 µg/L PAC for 4 wk and *hsp70* increased slightly in both life-stages after 48 h exposure to 6.3 µg/L PAC, suggesting that neither marker is a reliable indicator of dilbit exposure at very low PAC concentrations.

4.3. Implications for spill management

When oil spills occur in aquatic systems, water-soluble components can be carried beyond the borders of the visible spill zone and extend the margins of the impacted area. Employing biological indicators of exposure can help better define these margins through both space and time (Dubansky et al., 2013; Sammarco et al., 2013; Whitehead et al., 2012), which may facilitate informed decision making in post-spill monitoring and mitigation efforts (Beyer et al., 2016). This is particularly relevant amidst a growing body of evidence that very low, parts per billion concentrations of PACs are capable of eliciting ecologically-relevant sublethal responses in fish (Collier et al., 2013; Kennedy, 2015). In the present study, for example, exposure to an initial PAC concentration as low as 2.3 µg/L significantly impaired hypoxia tolerance in coho fry and parr, an effect that persisted through at least 4 wk of continuous flow-through exposure where PAC concentrations steadily declined. Of the bioindicators examined in this study, phase I induction – measured as changes in *cyp1a* gene expression and EROD activity – was most consistent with this organismal response; however, the distinct temporal and life-stage responses observed in each variable suggest a cautionary approach if relying on a single endpoint to confirm or negate potential exposure in a field scenario. The temporal profile of phase I induction in tissues that can be non-lethally biopsied, such as the caudal fin (Imbery et al., 2019) or gill (Santos and Bueno dos Reis Martinez, 2020), would be worthwhile investigating in the context of WSFd exposure.

4.4. Conclusions

Acute and sub-chronic exposure of juvenile coho salmon to the dissolved components of dilbit impairs cardiorespiratory performance and growth, highlighting the vulnerability of Pacific salmon stocks that depend on coastal freshwater environments along existing oil pipeline corridors. While the organismal responses to WSFd exposure were similar in fry and parr, common molecular indicators of PAC exposure were delayed and distinct between life-stages. Therefore, multiple lines of evidence of exposure and biological response should be considered when predicting or assessing the environmental impacts of an oil spill on salmon populations.

CRediT authorship contribution statement

Gabrielle Perugini: Investigation, Formal analysis, Data curation, Writing – original draft, Visualization. **Mackenzie Edgar:** Investigation, Data curation. **Feng Lin:** Investigation, Data curation. **Christopher J. Kennedy:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Anthony P. Farrell:** Writing – review & editing, Funding acquisition. **Todd E. Gillis:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sarah L. Alderman:** Conceptualization, Formal analysis, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106350.

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