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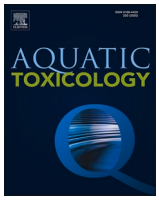

Temperature matters: Acute and latent toxicity of diluted bitumen to developing salmon is potentiated by a modest increase in water temperature

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ABSTRACT

Heavy crude oil, like bitumen, is used globally for plastics, petrochemicals and road surfacing. Canada's oil sands are the world's third largest crude oil reserve, and diluted bitumen (dilbit) is transported across North America primarily *via* pipeline and rail. Two environmentally-relevant concentrations of dilbit were used with a suite of toxicological endpoints to determine if a 3 °C increase in ambient temperature (T_a) water modulated the effects of dilbit to coho salmon (*Oncorhynchus kisutch*) when exposed from fertilization to swim-up. The 10–20 % increase in mortality and 25 % reduction in hypoxia tolerance with dilbit exposure was magnified by 18 % and 40 %, respectively, in warmer water. Consequences of dilbit exposure persisted after 6 weeks of additional rearing in clean T_a water but were greatest in fish exposed to dilbit at elevated temperature: additional 20 % mortality and 30 % decrease in mass relative to controls, and a residual 20 % reduction in hypoxia tolerance not seen with dilbit exposure alone. Relatively lower induction of the Phase I biotransformation enzyme *cyp1a* and greater tissue PAC content in warm-exposed coho suggests reduced PAC metabolism as a mechanism for the observed potentiation. Thus, seasonal fluctuations and baseline increases in water temperature from climate change can exacerbate the adverse effects of oil spills on developing fish.

Synopsis

A modest 3 °C increase in water temperature can exacerbate the adverse effects of oil spills on developing fish.

1. Introduction

Crude oil contaminates the environment on a global scale and constantly enters water through natural seeps, discharge, and accidental release (NRC, 2003). Increasingly, world leaders are promoting renewable energy strategies, including electric transportation, as a critical climate action strategy (United Nations, 2022) but this does little to impact immediate fossil fuel consumption and is unlikely to diminish the demand for unconventional heavy oils, like bitumen, that are used widely in asphalt, plastics, and other petrochemical products (Canadian Association of Petroleum Producers, 2025). For example, in North America alone, >4.8 million km of asphalt surfaced roadways will still need to be maintained for use by electric cars (Central Intelligence Agency, 2021). The toxicity of crude oil to diverse lifeforms (Lewis and Pryor, 2013; Varjani et al., 2017), including fish (Grosell and Pasparakis, 2021; Kennedy, 2015; Meador and Nahrgang, 2019), is not debated. Yet, abiotic factors, such as temperature, can influence the adverse outcomes of crude oil exposure (Lin et al., 2021; Serafin et al., 2019), which could then challenge the accuracy of environmental risk assessments and predictions of biological impacts of spills. Ultimately, this may undermine post-spill remediation efforts if estimated outcomes are undervalued; thus, the potential for prevailing environmental conditions to affect biological responses to crude oil exposure warrants immediate and comprehensive attention.

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Canada is recognized globally for its vast reserves of crude oil in the form of bitumen, ranking third after Saudi Arabia and Venezuela (National Energy Board of Canada, 2019). Bitumen is extracted from the oil sands region in western Canada by in situ and surface mining and then diluted ~3-fold with proprietary condensates for transport. The resulting product (diluted bitumen, dilbit) traverses North America primarily through pipelines to refineries in the U.S.A. and tidewater in Canada for export overseas. The extensive pipeline network includes the 1150 km Trans Mountain Pipeline that facilitates transport of dilbit from the oil sands to the Pacific coastline with a carrying capacity of 140 ML d^{-1}. Importantly, this pipeline corridor traverses the Fraser River Basin, an undammed watershed the size of Great Britain that contains critical spawning and nursery habitats for the five Pacific salmon species (*Oncorhynchus* sp.). Many subpopulations of wild Pacific salmon are listed as endangered or threatened (COSEWIC, 2016) due to anthropogenic and climate disturbances (Cohen, 2012); therefore, the risk of dilbit exposure in the event of a pipeline leak or failure may compromise the production and recruitment of these socioeconomically and ecologically important fish (Levy, 2009).

The spawning and nursery habitats for wild Pacific salmon are of special concern because embryonic and early larval stages of fish are especially vulnerable to crude oil exposure due to their relative immobility and inability to escape contamination zones. These early life stages are also hyper-sensitive to the contaminants in crude oil with low, environmentally-relevant concentrations causing increased mortality and a range of developmental abnormalities including edemas, impaired heart function, and skeletal deformities (Carls et al., 1999; Colavecchia et al., 2006; Jung et al., 2013; Perrichon et al., 2021; Sørhus et al., 2023). In addition, survivors of early life exposure to crude oil may experience latent mortality (Alderman et al., 2018; Brown et al., 2016; Johansen et al., 2017; Perrichon et al., 2021; Sørhus et al., 2023), or persistent physiological impairments like reduced cardiorespiratory capacity (Hicken et al., 2011; Incardona et al., 2015; Mager et al., 2014), but longitudinal studies are limited. Given the chemical complexity of dilbit and other crude oils, the mechanisms that underpin acute and long-term biological responses to exposure are understood to be multiple and varied.

Adding to this complexity is temperature, regarded as an ecological master factor in aquatic systems (Fry, 1971). For example, warmer water increases biochemical reaction rates, increasing the metabolic and physiological processes of ectotherms, like most fishes (Fry, 1971). Ecologically, this translates to faster progression through ontogeny such that hatching and exogenous feeding occur sooner in fish reared in warmer water. In the context of crude oil exposure, a spill at warmer temperature could therefore reduce total exposure time if fish are able to escape the contamination zone after hatch. Similarly, maturation of biotransformation pathways may be hastened in warmer water such that the capacity for exposed fish to metabolize contaminants is increased. However, temperature influences toxicokinetics in many ways, including contaminant uptake rates and monooxygenase activity (Kleinow et al., 1987). At the same time, a change in water temperature also alters the chemical and physical characteristics of crude oils, such that differences in weathering rate (i.e., evaporation of volatile constituents) and component solubilities could modify the relative proportions of dissolved and bioavailable oil constituents in the receiving environment (Lee et al., 2015). It is reasonable to posit that the combination of these temperature effects will shift the apparent toxicity and, by extension, adverse outcomes of an oil spill on exposed fish; however, the direction of this shift is uncertain and empirical evidence to support this hypothesis is sparse (Andersen et al., 2015; Lin et al., 2021; Pasparakis et al., 2017, 2016; Serafin et al., 2019).

Coho salmon (*O. kisutch*) spawn in coastal streams throughout the Fraser River Basin during late fall. Since adults die after a single spawning season (semelparity), the developing progeny represent the totality of recruitment efforts for a given cohort of spawners. Embryos develop in the gravel riverbeds throughout the winter and emerge as fry after hatching and resorption of the large external yolk sac in early spring. In addition to a progressive warming trend, year-to-year variation in seasonal water temperature in the lower Fraser River is considerable, with mean temperature differences as much as 3 °C in recent years (Fraser Basin Council and Municipalities, 2019). Therefore, this study tested whether a modest increase in water temperature during embryonic exposure to dilbit altered biological responses to crude oil in coho salmon, including mortality and growth as key fitness metrics. In addition, the bioaccumulation of polycyclic aromatic compounds (PAC), a majority component of oil hydrocarbons, was compared in fish tissues following exposure. PAC are known drivers of toxicity in fish owing in part to the capacity of certain PAC species to induce biotransformation pathways and generate reactive intermediates leading to cellular toxicity (Billiard et al., 2006; Hodson, 2017; Incardona, 2017). Therefore, the expression of *cytochrome p450 1a* (*cyp1a*; phase I biotransformation), *glutathione transferase* (*gst*; phase II biotransformation), and *heat shock protein 70* (*hsp70*; cell stress) were quantified as bioindicators of exposure and organismal toxicity (Bérubé et al., 2021; Madison et al., 2015; Santana et al., 2018).

2. Experimental methods

2.1. Fish

Fertilized coho salmon (*Oncorhynchus kisutch*) eggs were obtained from wild-caught parents supplied by the Capilano Fish Hatchery (North Vancouver, BC). Eggs were reared at Simon Fraser University (Burnaby, BC) where, upon arrival, they were evenly distributed amongst 12 heath trays (n = 250 per tray) supplied with flow-through dechlorinated municipal groundwater at ambient temperature (flow rate 6 L/min; dissolved >95 % O_2 saturation, hardness 6.12 mg/L $CaCO_3$, chlorine undetectable, <1 mg/L DOC, pH 7.0; 9 °C). Embryos were reared in the dark until swim-up, at which point the fry were transferred to one of twelve 250 L tanks supplied with flow through water, as above, maintaining experimental groups. Fry were reared for 6 weeks under a 12h:12 h light:dark photoperiod. Fish were fed salmonid fry feed (Skretting, Vancouver, BC) at a daily ration of 5 % body weight, adjusted weekly by batch weighing subsets of fish. Care and use of animals were approved by the Simon Fraser University Animal Care Committee, according to the guidelines of the Canadian Council for Animal Care.

2.2. Dilbit exposure

Dilbit exposures were conducted from approximately 2 dpf through to swim-up stage (fry) in duplicate heath trays at 3 concentrations (0 µg/L, 0.3 µg/L, 1.0 µg/L total initial PAC) and 2 temperatures (ambient temperature, T_a; T_a + 3 °C, heated), for a total of 6 experimental groups. All endpoints were assessed on stage-matched fish to account for the effects of temperature on developmental rate (Fig. 1A). Dilbit exposure followed previous studies (Alderman et al., 2020, 2018, 2017; Lin et al., 2022). Briefly, water-soluble fractions of dilbit were generated from fresh Cold Lake Summer Blend dilbit (supplied by COOGER, Canada) by soaking Siporax® ceramic beads in dilbit and then packing a proportional quantity of beads into PVC generator columns (15 cm diameter x 80 cm length), with clean beads used for the control tanks. Dechlorinated municipal groundwater flowed up through the generator columns and into 1 of 6 separate 2000 L header tanks (1 per experimental group). Water from the near-bottom of a header tank was pumped into the heath trays containing embryos (n = 250 per tray, duplicate trays per treatment). The header tanks supplying water to the heated group contained submersible heaters to increase the water temperature by 3 °C throughout the dilbit exposure (Fig. 1B), which represents the maximum difference between historical mean water temperature (1950–2018) and recorded values in 2019 (Fraser Basin Council and Municipalities, 2019). All exposures were flow-through with flow rates matched across all experimental units, and the generator columns were not recharged

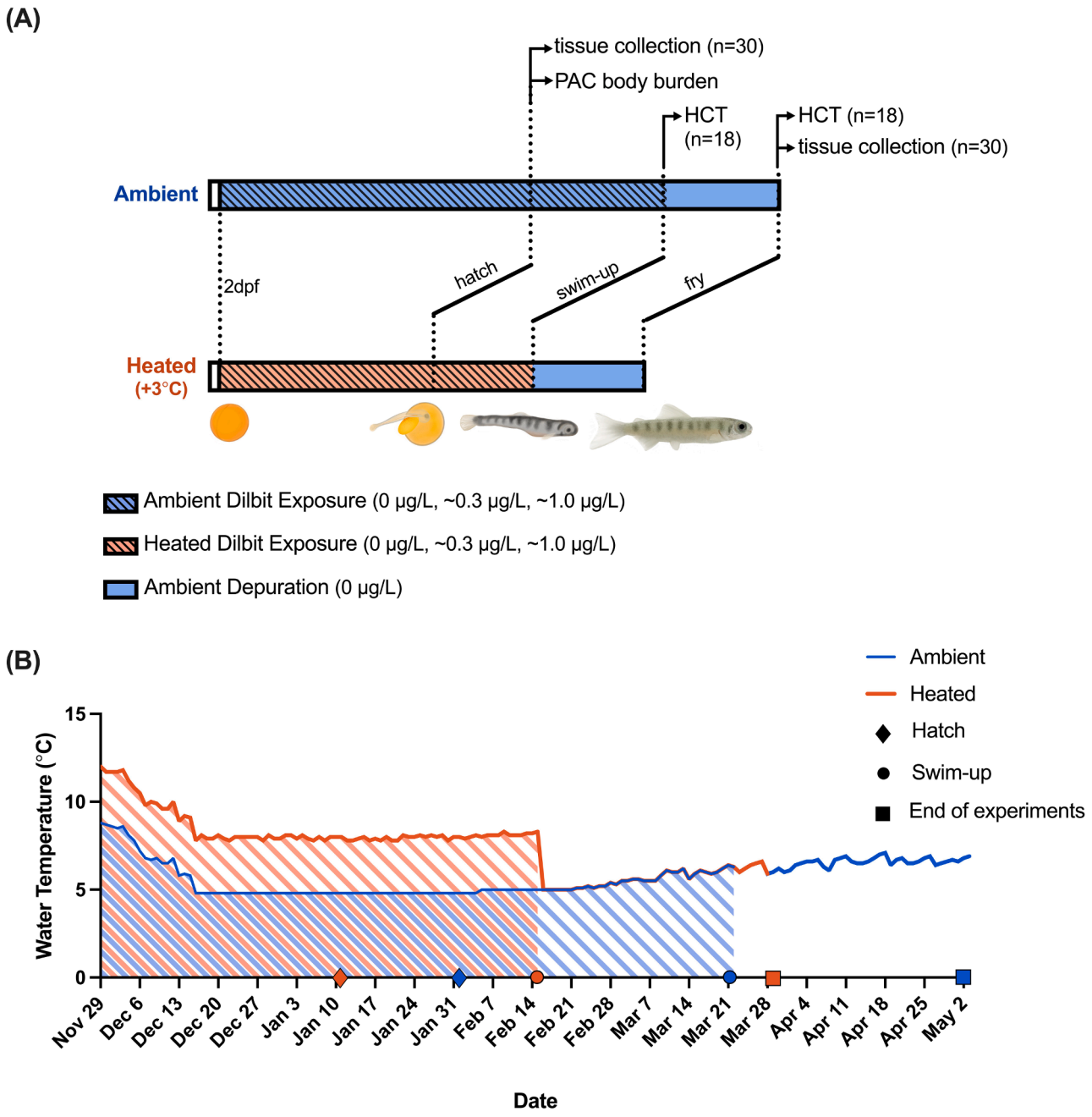

Fig. 1. (A) Schematic diagram of the experimental design and sampling times. The fish were exposed to three concentrations of water-soluble fraction of dilbit (0, 0.3, 1.0 µg/L initial TPAC) in duplicate trays containing 250 embryos each, starting at 2 days post fertilization (dpf) until swim-up. Tissue samples (n = 30 fish per tray or tank replicates) were collected at hatch and fry stage. Hypoxia challenges were conducted at swim-up and fry stage. Developmental points were stage matched across the two temperature groups, with the diamonds indicating hatch (ambient: 67 dpf; heated, +3 °C: 45 dpf) and circles indicating swim-up (ambient: 116 dpf; heated, +3 °C: 81 dpf). Both groups were kept for an additional 42 days after swim-up. (B) Temperature record of ambient and heated groups across the experimental timeline. For both panels, blue indicates ambient temperature group and orange indicates the heated group; cross-hatching indicates the dilbit exposure period.

after the initial addition of dilbit. Duplicate water samples from experimental tanks were collected into amber bottles with no headspace at 1, 2, 7, and 30 d of dilbit exposure, preserved with sodium azide, and stored for less than one week at 4 °C prior to analysis. A total of 75 individual parent and alkylated PAC were quantified by GC/MS (SGS-AXYS, Sidney, BC; data quality objectives exceed ISO/IEC 17,025 and QA/QC methods are certified) according to industry standards. For each heath tray, the exposure period ended when >95 % of the fish either died or reached swim-up stage, at which point the cohort was transferred to a 250 L tank containing uncontaminated municipal groundwater at ambient temperature for a 6-week depuration period (Fig. 1).

2.3. Mortality and growth

Cumulative mortality was recorded daily for each heath tray and tank throughout the experiment. Completion of the hatching period was decided when no new hatching events occurred in a tray for 5 consecutive days. At this time, any unhatched eggs were deemed non-viable and recorded as mortalities. Subsets of live fish (n = 30–36 per heath tray) were collected at the end of the hatching period, at swim-up, and in 6-wk fry by euthanizing in buffered MS-222 (1 g/L) and then snap-

freezing in liquid nitrogen before storing at −80 °C. Body mass and fork length were recorded for a subset of fish at swim-up (n = 14–18 per heath tray) and for remaining survivors at the 6-wk fry stage (n = 336–399 per treatment).

2.4. Hypoxia challenge test (HCT)

Hypoxia challenge tests (HCT) were conducted in contaminant-free water following previously described methods (Claireaux et al., 2013; Mauduit et al., 2019; Perugini et al., 2022). Briefly, at swim-up and after 6-week depuration, a subset of 18 fish from an experimental replicate were transferred to an aerated 250 L test tank filled with uncontaminated groundwater, temperature-matched with the origin tank for that replicate. Given differences in developmental rates and temporal flux in groundwater temperature, the realized temperature difference between the HCTs for the T_a vs T_a +3 °C experimental groups was 2 °C at swim-up (i.e., 6 °C vs 8 °C test temperature, respectively) and 0.5 °C at the fry stage (i.e., 7.0 °C vs 6.5 °C test temperature, respectively). Fish were acclimated in the test tanks for 1 h after which the HCT was initiated by stopping aeration and then bubbling nitrogen into the test tank through a ceramic diffuser. Dissolved oxygen content was reduced to 20 % saturation in 1 h, and then by ~4 % per hour until the end of the test. The dissolved oxygen content was continually monitored via a fibre optic oxygen sensor probe and a Witrox 1 minisensor oxygen meter (Loligo® Systems, Tjele, Denmark). When a fish was no longer able to maintain an upright position (loss of equilibrium, LOE) it was transferred into an aerated recovery tank and the time and dissolved oxygen (%) were recorded. The HCT was complete when the last fish reached LOE. This entire procedure was repeated for each experimental replicate at swim-up and in fry after a 6-week depuration period (12 HCT and 36 fish per treatment and per time point).

2.5. Gene expression

Alevin yolk sacs were resected from frozen alevins on an insulated frozen dissection stage, and then the bodies were homogenized in TRIzol™ reagent (Thermo Fisher Scientific, Waltham, MAS, USA) using a Precellys tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted according to the manufacturer guidelines, and purity and yield were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity numbers (RIN) were confirmed with Agilent TapeStation 4150 (Agilent Technologies, Santa Clara, CA, USA). Only samples with 260/280 and 260/230 ratios >2 and RIN values >8 were used for cDNA synthesis. One microgram of total RNA was treated with DNase I and then reverse transcribed to complementary DNA (cDNA) using the High-Capacity Multiscribe cDNA Synthesis kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer instructions. Transcript abundances of *cytochrome P450 1a* (*cyp1a*), *glutathione s transferase* (*gst*), and *heat shock protein 70* (*hsp70*), as well two reference genes (*ribosomal protein L8, rpl8; beta-actin, β-actin*), were quantified separately in duplicate reactions using gene-specific forward and reverse primers (Table 1), exactly as previously described (Perugini et al., 2022) including all negative and quality controls. The relative transcript abundance of each target gene was calculated from a 5-point calibration curve using the average threshold value of a sample and normalized to the mean expression of the reference genes.

2.6. Statistical analyses

Body mass and fork length measurements were used to calculate Fulton's body condition factor (K):

$$K = 100 \times \frac{W}{L^3}$$

Table 1
Forward (F) and reverse (R) primer sequences and assay specific final primer concentrations for target gene quantification, as well as the reaction efficiency (E) and R^2 for each calibration curve. *cytochrome P450 type 1a, cyp1a; glutathione S-transferase, gst; heat shock protein 70, hsp70; beta actin, β-actin; ribosomal protein L8, rpl8*.

Gene	Sequence (5′−3′)	Conc. (nM)	E (%)	R^2	References
cyp1a	F: agtgctgatggcacagaactcaa R: agctgacagcgcttgtgctt	400	98	0.99	(Matsuo et al., 2008)
gst	F: ctctgctccagttgcctggat R: gttgccattaatgggcagtttct	300	109	0.99	(Espinoza et al., 2012)
hsp70	F: ctgctgctgctggatgtg R: gctggttgtcggagtaagtg	400	105	0.99	(Yar Ahmadi et al., 2014)
rpl8	F: ttggtaatgttctgcctgtg R: gggttgtgggagatgactg	200	101	0.99	(Alderman et al., 2017)
β-actin	F: gacccacacagtgcccatct R: gtgcccatctcctgctcaaa	300	99	0.98	(Matsuo et al., 2008)

where W is the body mass (g), and L is the fork length (cm).

Effect of temperature and dilbit concentration on mortality and performance in the HCT were interpreted with Kaplan-Meier analyses, where the differences between treatment groups were established with log-rank curve comparisons. This was followed by a two-way ANOVA using cumulative mortality or median time to loss-of-equilibrium (TLOE) as the dependent variable (n = 2 experimental replicates/concentration/temperature). T-tests were used when data were collected on individual fish from the duplicate experimental replicates (growth parameters, n = 18/replicate; gene expression n = 6/replicate) to confirm an absence of tank effect (true in all cases). Subsequently, formal statistical analysis was carried out by pooling replicate data (i.e., growth parameters, n = 36; gene expression, or n = 12) as described below. The exception was PAC body burden, where sampling effort was necessarily restricted to 2 fish chosen at random per replicate due to prohibitive analytical costs, and data was pooled at the outset. A two-way ANOVA was used to determine the effects of the two independent variables (temperature and concentration) and their interaction on PAC body burden, growth parameters, and gene expression. Where statistical differences were found, these tests were followed by Tukey's multiple comparisons tests. Prior to ANOVAs, any existing outliers were removed at a conservative Q value of 0.1 % using the ROUT method. Data that did not satisfy the normality and equal distribution of variance were transformed using square root or log transformations. All analyses were conducted in GraphPad Prism 9 for MacOS (version 9.4.1).

3. Results

3.1. Water and tissue PAC

Chemical analysis of water samples collected during the dilbit exposure confirmed the presence of dissolved PAC at two distinct concentrations that were consistent between temperature groups (see Supplemental Data File S1 for component breakdown). Initial total PAC concentrations (TPAC) measured in duplicate heath trays were 0.29 µg/L and 0.78 µg/L at T_a, and 0.43 µg/L and 0.89 µg/L in heated heath trays (median values). Thereafter, PAC concentrations declined steadily throughout the experimental period, reaching 0.12 µg/L and 0.24 µg/L at T_a, and 0.15 µg/L and 0.40 µg/L in heated heath trays after 4 wk of flow-through exposure. Irrespective of temperature, the water PAC concentration in unexposed control heath trays was below 0.02 µg/L for the duration of the experiment. For simplicity, initial TPAC values are approximated at 0 µg/L (control), 0.3 µg/L, and 1.0 µg/L for both temperatures and used hereafter to define the dilbit concentrations in this study.

The effect of temperature on PAC bioaccumulation in embryos was determined at hatch. In general, the PAC that accumulated in fish tissues reflected the composition of dissolved PAC in the exposure water,

including a high proportion of napthalenes and naphthalene congeners (see Supplemental Data File S1). At T_a, PAC accumulation in fish exposed to 0.3 and 1.0 µg/L TPAC was 90 % and 148 % greater than in unexposed controls, respectively, reaching a maximal value of 213 ng/g. Temperature increased the bioaccumulation of PAC, reaching a maximum of 358 ng/g in fish exposed to 1 µg/L TPAC, which was 416 % greater than in the heated control fish and 68 % greater than in coho exposed to the same concentration at T_a (Fig. 2, $P_{interaction} = 0.0043$, $N = 4$).

3.2. Effect of dilbit and temperature on vital statistics

Cumulative mortality in unexposed control fish was low for the duration of the exposure (<10 %; Fig. 3A), irrespective of rearing temperature. Developmental exposure to dilbit increased mortality events between fertilization and the swim-up stage, with the majority of deaths occuring during the first half of the exposure period and prior to any hatching (Fig. 3A, log-rank $P < 0.0001$; combined data for duplicate heath trays, $N = 500$ embryos per treatment). Specifically, embryo mortality surpassed control mortality in a concentration-dependent manner, reaching 28 % and 32 % cumulative mortality under ambient and heated conditions, respectively (Fig. 3B; $P_{concentration} < 0.0001$; $N =$ 2 trays per treatment).

After being moved to clean T_a water at swim-up, cumulative fry mortality in all control and 0.3 µg/L PAC groups was ~5 % over the 6-wk depuration period. In contrast, mortality continued at a higher rate in fish that had been exposed to 1.0 µg/L PAC and was exacerbated by exposure in heated water (Fig. 3C, log-rank $P = 0.0124$; combined data for duplicate tanks; N= ~200 per tank). Specifically, cumulative mortality during the depuration period was ~15 % in fry exposed at T_a but reached ~22 % in fry exposed in heated water (Fig. 3D, $P_{interaction} = 0.0048$; $N = 2$ tanks per treatment). Therefore, considering cumulative mortality across the entire experimental timeline, the maximal net mortalities attributed to embryonic dilbit exposure were 28 % higher than controls at T_a and 45 % higher than heated controls.

When measured at swim-up, growth parameters of surviving fish were largely unchanged among experimental groups. Exceptions were a 6 % temperature-induced increase in body mass seen in the control groups (Table 2, $P_{interaction}$=0.027, $N = 36$), a temperature effect not seen with dilbit exposure. Also, condition factor of fish exposed to 0.3 µg/L total PAC significantly declined by 4 % in the heated group only (Table 2, $P_{interaction}$=0.0284, $N = 36$).

After the 6 week depuration period, latent effects of dilbit and temperature on growth of fry became evident. Specifically, fry in heated water that had been exposed to 0.3 and 1.0 µg/L TPAC during development were 6 % and 10 % smaller, respectively, than fry exposed to the same concentrations at T_a. Given the stimulatory effects of temperature on growth of unexposed control fish, the apparent inhibition of weight gain by dilbit exposure becomes 10 % at T_a and 30 % in the heated cohort (Table 2, $P_{interaction}$<0.0001, $N = 36$). In contrast, developmental dilbit exposure increased fork lengths of fry by ~4 % relative to unexposed controls at T_a, but decreased fork lengths by ~9 % in the heated cohort (Table 2, $P_{interaction}$<0.0001, $N = 36$). The combination of these changes to mass and length within individuals had varying effects on calculated body condition. Overall, the poorest overall body condition was observed in fry exposed to 1.0 µg/L TPAC during development, irrespective of temperature (Table 2, $P_{interaction}$<0.0001, $N = 36$).

3.3. Effect of dilbit and temperature on hypoxia tolerance

At the swim-up life stage, pronounced differences in the HCT were evident between experimental groups (Fig. 4A, log-rank $P < 0.0001$), including a significant ~20 % reduction in the median time-to-loss of equilibrium (TLOE) of coho exposed to 1.0 µg/L TPAC relative to their temperature-matched controls (Fig. 4B, $P_{interaction}$=0.0129, $N = 36$). Nevertheless, rearing temperature had an even greater effect on HCT performance, whereby a 30–40 % decrease in median TLOE was observed for coho raised at T_a +3 °C compared with T_a across all concentrations including controls (Fig. 4B, $P_{interaction}$=0.0129, $N = 36$).

The 6 week depuration period recovered performance in the HCT for some, but not all, experimental groups (Fig. 4C, log-rank $P < 0.0001$, $N = 36$). The median TLOE was similar for all fry reared at T_a, as well as for unexposed control fish reared in warmer water (Fig. 4D, $P_{interaction} = 0.0407$, $N = 36$). In contrast, fry exposed to dilbit at T_a +3 °C during development maintained a 20 % reduction in median TLOE relative to unexposed controls (Fig. 4D, $P_{interaction} = 0.0407$, $N = 36$).

3.4. Effect of dilbit and temperature on gene expression

The transcript abundance of *cyp1a* at swim-up increased in a concentration-dependent manner with dilbit exposure, and the magnitude of this increase relative to unexposed control fish was temperature-dependent (Fig. 5A, $P_{interaction}$=0.0002, $N = 12$). In coho exposed to 0.3 and 1.0 µg/L TPAC at T_a, *cyp1a* transcript abundance increased 10- and

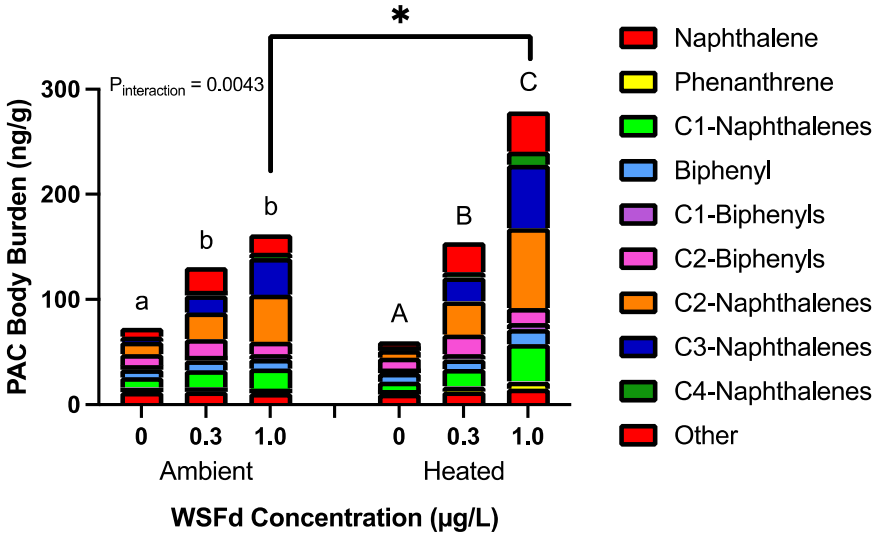

Fig. 2. Total PAC quantified in whole tissues of coho alevins sampled at hatch (mean ± *S*.E.M., ng/g wet weight). Differences in body burden were detected by two-way ANOVA and Tukey's post-hoc ($N = 4$ fish per exposure group, $\alpha = 0.05$). Data points with different letters indicate statistical significance within ambient (lower case) and heated (+3 °C, upper case) groups. Asterisks indicate significant effect of temperature.

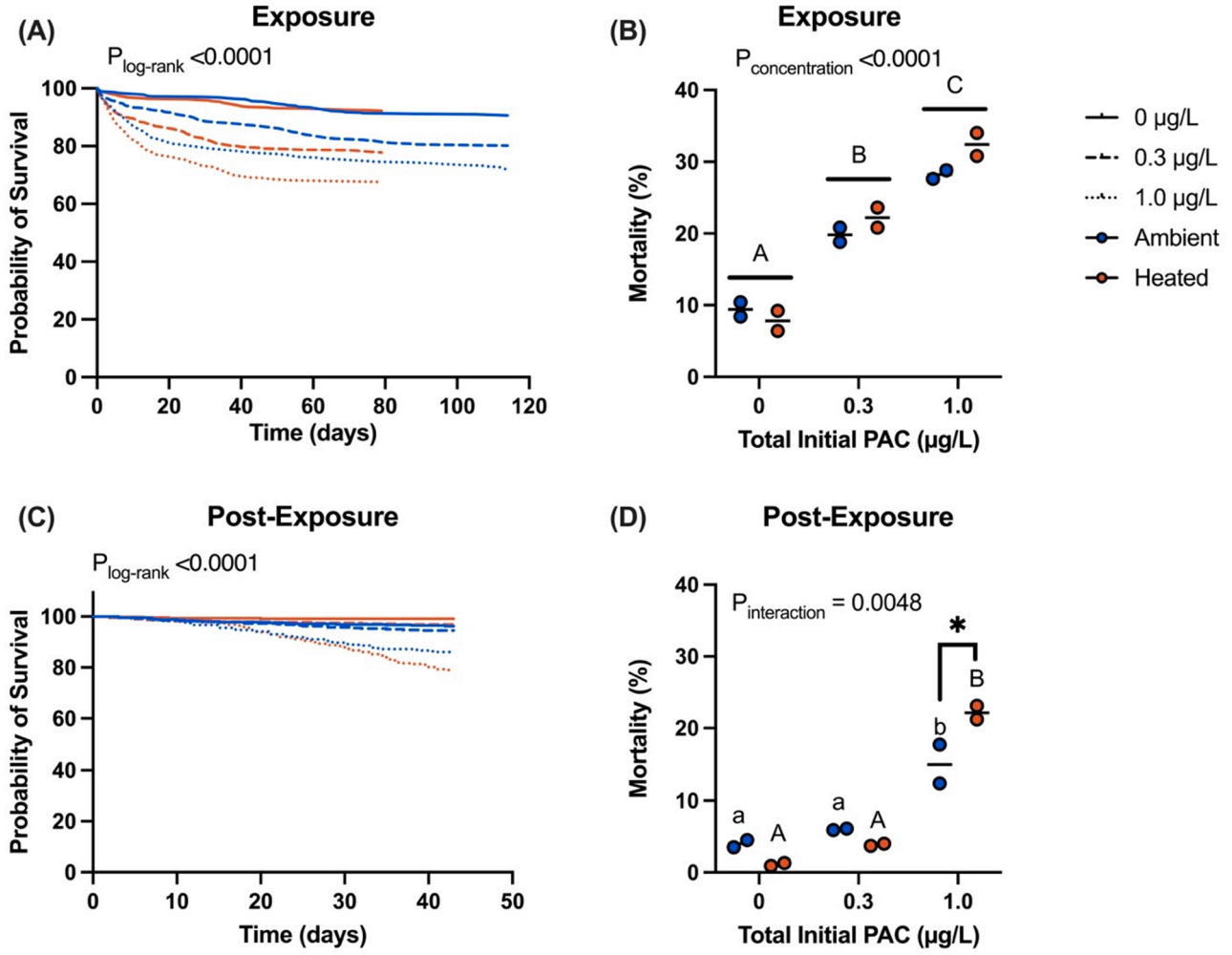

Fig. 3. Survival probabilities of coho salmon exposed to 0 (solid line), 0.3 (dashed line), 1.0 (dotted line) µg/L TPAC in ambient (blue) or heated (+3 °C, orange) temperatures, from fertilization to swim-up (A) and post-exposure period (C). Kaplan-Meier analysis of survival and log-rank comparison revealed significant differences between the survival probabilities of exposure groups. Total mortalities observed for the duration of dilbit exposure (B), and post-exposure period (D) were analysed with two-way ANOVA and Tukey's post-hoc tests (mean ± *S*.E.M, $N = 250$ fish per replicate exposure group, $\alpha = 0.05$). Data points with different letters indicate statistical significance within ambient (lower case) and heated (+3 °C, upper case) groups. Asterisks indicate significant differences between the mean mortalities observed across dilbit exposure groups in ambient and heated temperatures.

15-fold, respectively, above baseline expression. In contrast, whereas temperature did not affect baseline *cyp1a* expression, *cyp1a* abundance was 30–60 % lower in fish exposed to dilbit at elevated temperature relative to fish exposed at T_a (Fig. 5A, $P_{interaction}=0.0002$, $N = 12$). Transcriptional changes in *gst* and *hsp70* were modest and inconsistent across treatments. Specifically, there were significant effects of concentration ($P_{concentration}=0.0275$) and temperature ($P_{temperature}<0.001$), but not their interaction ($P_{interaction}=0.3163$), on *gst* mRNA abundance, but pairwise comparisons did little to help interpret these effects (Fig. 5B, $N = 12$). Similarly, an apparent interaction between the effects of concentration and temperature on *hsp70* mRNA abundance was driven by an increase in *hsp70* mRNA in fish exposed to 1.0 µg/L TPAC at T_a, but inter-individual variation in this response was substantial (Fig. 5C, $P_{interaction}=0.0206$, $N = 12$).

4. Discussion

Warmer temperature increases developmental and metabolic rates for ectotherms, including fish (Carter, 2005; Finstad and Jonsson, 2012; Pankhurst and Munday, 2011). In the event of an oil spill in an aquatic environment, elevated temperatures could result in multiple significant impacts. For example, faster development in warmer water may shift the ontogenic timing of when the fish are exposed to the highest concentrations of PAC, the bioavailability of various constituents, and the concomittant effects of temperature on chemical toxicokinetics within the organism. At the same time, water temperature at the time of a spill will influence weathering processes, including hydrocarbon solubility and volatility (Stoyanovich et al., 2019; Yang et al., 2016a). The present study showed that for developing coho salmon, a 3 °C increase in water temperature, a magnitude consistent with recorded year-over-year variation in the Fraser River Watershed and its general warming trend (Fraser Basin Council and Municipalities, 2019), amplified the toxic effects of dilbit exposure and increased adverse latent effects. It is possible that reduced induction of Phase I biotransformation ability was a potential contributing mechanism underlying the increased toxicity of dilbit in warmer water. Ultimately, the persistence of mortality, growth, and performance effects into the fry stage, despite a return to clean water at ambient temperature, emphasizes the added challenges of estimating population effects of a spill in some aquatic environments, such as temperate freshwater river systems. Thus the stochastic and warming weather patterns associated with the climate crisis may amplify the biological effects of oil spills in aquatic environments.

Table 2
Effects dilbit exposure and temperature on coho growth. Fish were exposed to one of two concentrations of dilbit (0.3 or 1.0 µg/L total initial PAC) or clean water (0 µg/L total initial PAC) at ambient temperature or ambient +3 °C (heated) from 2 days post fertilization until swim-up, and then transferred to clean water at ambient temperature and reared for an additional 6 wk. Body mass (g), fork length (cm), and Fulton's condition factor (K) were measured in a subset of fish at swim-up and 6-wk later (fry). At each life stage, differences in growth were determined by two-way ANOVA and Tukey's post-hoc tests (mean ± *S*.E.M, $N = 36$ fish, $\alpha = 0.05$). Significant differences between concentrations within temperature are denoted by letters in lowercase (ambient) or uppercase (heated). Values shown in bold italics indicate a significant difference from the ambient group at the same concentration.

	Body mass (g)		Fork length (cm)		Condition factor (K)	
	ambient	heated	ambient	heated	ambient	heated
Swim-up						
0 µg/L	0.277 ± 0.003	***0.293 ± 0.003^{A}***	3.343 ± 0.015	3.422 ± 0.016	0.743 ± 0.006	0.733 ± 0.01^{A}
0.3 µg/L	0.279 ± 0.002	0.280 ± 0.002^{B}	3.358 ± 0.015	3.414 ± 0.012	0.739 ± 0.009	0.704 ± 0.008^{B}
1 µg/L	0.273 ± 0.004	0.281 ± 0.003^{B}	3.351 ± 0.014	3.400 ± 0.010	0.732 ± 0.007	0.713 ± 0.005^{AB}
Statistics	$P_{interaction} = 0.027$		n.s.d.		$P_{interaction} = 0.0284$	
Fry						
0 µg/L	0.826 ± 0.007^{a}	***0.946 ± 0.008^{A}***	4.265 ± 0.012^{a}	***4.591 ± 0.011^{A}***	1.06 ± 0.005^{a}	***0.977 ± 0.003^{A}***
0.3 µg/L	0.824 ± 0.009^{a}	***0.775 ± 0.006^{B}***	4.452 ± 0.015^{c}	***4.283 ± 0.011^{B}***	0.925 ± 0.005^{b}	***0.985 ± 0.005^{A}***
1 µg/L	0.737 ± 0.009^{b}	***0.663 ± 0.008^{B}***	4.372 ± 0.013^{b}	***4.183 ± 0.014^{C}***	0.893 ± 0.004^{c}	0.893 ± 0.007^{B}
Statistics	$P_{interaction} < 0.0001$		$P_{interaction} < 0.0001$		$P_{interaction} < 0.0001$	

4.1. Embryonic dilbit exposure at elevated temperatures reduces coho vital statistics

The dynamics of fish populations are driven by variation in primary vital statistics (i.e., survival and reproduction) as well as secondary vital statistics, including growth. In Pacific salmon, the semelparous life history limits recruitment to a single lifetime spawning event and egg-to-fry survival in the wild is at best only 7–8 % (Bradford, 1995); thus large-scale disturbances to spawning habitats, such as an oil spill, have the potential to impose a massive population-level impact. Therefore, quantifying the effects of dilbit exposure on the vital statistics of early life stage salmon is a crucial step in estimating how contamination of Pacific salmon habitats by dilbit will impact population dynamics.

As anticipated, and consistent with previous work in freshwater fish (Alderman et al., 2018; Alsaadi et al., 2018; Bérubé et al., 2023, 2021; Lin et al., 2022; Madison et al., 2017; McDonnell et al., 2019), the exposure of coho to dilbit during development caused a concentration-dependent increase in mortality, reaching a cumulative maximum of ~30 %. These results are similar to a previous report of latent mortality from embryonic dilbit exposure (Alderman et al., 2018), which in the present study accounted for an additional ~20 % mortality during the 6-wk post-exposure rearing period in clean ambient water. Unexpectedly, the immediate and latent mortality observed in the present study for coho salmon was considerably greater than previously reported for sockeye exposed under similar experimental conditions but to higher concentrations (i.e., 13.4 µg/L initial PAC in Alderman et al., 2018). The underlying cause of latent mortality in salmon exposed to dilbit during embryonic development remains unknown.

Novel to this study, embryonic exposure at elevated temperature reduced the longterm viability of exposed fish. Temperature had a modest effect on dilbit-induced mortality during the exposure period, but latent mortality continued at a faster rate in coho exposed to 1 µg/L PAC in warmer water compared to fish exposed to the same concentration at T_a. Thus the greatest overall mortality occurred when coho embryos were exposed to higher concentrations of dilbit in warmer water. Similar to this study, a 5 °C temperature increase reduced the survival of juvenile pink salmon (*O. gorbushca*) exposed to dilbit for 3 months (Lin et al., 2021), and of post-hatch Gulf killifish (*Fundulus grandis*) larvae exposed to Deepwater Horizon crude oil for 48 h (Serafin et al., 2019). This means that the impact of crude oil spills on salmon populations may be more severe in warmer years or seasons, or in streams with higher temperature maxima.

Crude oil exposure impairs the growth trajectory of fish (Heintz et al., 2000; Holth et al., 2008; Rice et al., 2000; Schiano Di Lombo et al., 2021), including in salmonids exposed to dilbit (Alderman et al., 2018; Lin et al., 2022, 2021; Perugini et al., 2022). In the present study, where water PAC concentrations were considerably lower than those used in any of the aforementioned studies, ontogenic dilbit exposure manifested as smaller fry in both temperature groups, but the warm water treatment group demonstrated earlier growth restriction (i.e., swim-up) that was more pronounced after 6 wk at a common temperature in clean water (i.e., fry). Thus, developmental dilbit exposure in warmer water yielded the smallest fry (by mass and length) relative to all other treatment groups. Similarly, temperature exacerbated the inhibitory effects on growth by dilbit exposure in juvenile pink salmon (Lin et al., 2021). A smaller body size may correlate with increased mortality in juvenile salmon, reduced aerobic fitness, and as a result affect the recruitment of exposed fish (Carlson et al., 2011). Given the size-dependency of fish fitness, these latent impacts on the growth of fish exposed to dilbit at elevated temperature are a key finding that highlights the importance of considering abiotic factors at the time of a spill in population impact assessments.

The ability of a fish to withstand hypoxia is used as a high-throughput and repeatable measure of cardiorespiratory performance that serves as a proxy for aerobic fitness (Claireaux et al., 2013; Mauduit et al., 2016; Perugini et al., 2022; Zhang et al., 2017). A temperature-induced increase in metabolic rate and therefore a higher oxygen requirement is likely a key contributor to the difference in TLOE between the control swim-up fish raised at T_a and the control swim-up fish raised at T_a +3 °C. At the same time, the solubility of oxygen is temperature dependent, such that warmer water holds less oxygen, and this may be an additional factor contributing to the difference in TLOE between the control groups. The accelerated development of the T_a +3 °C groups meant the HCT for swim-up fish was performed ~5 wk earlier than for fish reared at T_a, resulting in a realized test temperature difference of 2 °C, which equates to ~0.6 mg/L difference in starting DO (estimated from oxygen solubility tables for freshwater at sealevel).

Developmental exposure to dilbit decreased TLOE at swim-up, indicating a reduction in hypoxia tolerance. This effect was magnified in coho exposed to dilbit in warmer water even above the substantial effect of temperature alone on TLOE. Rearing coho for an additional 6-wk in clean water was sufficient to recover the effects of dilbit on TLOE, but only for coho exposed at ambient temperature. In fact, a persistent impairment in TLOE was evident in the coho that were exposed under warm conditions even at 0.3 µg/L total initial PAC. Embryonic exposure to crude oil is known to impair cardiac development in fish (Alderman et al., 2017a; Incardona et al., 2004, 2015b) and contributes to the decline in cardiorespiratory performance that is frequently observed in oil-exposed fish (Alderman et al., 2017a; Avey et al., 2020; Incardona et al., 2015b). While cardiac morphology was not examined in the present study, it is possible that coho exposed to dilbit at elevated water temperature experience a greater teratogenic response in the heart, which may explain both the added mortality and persistent aerobic impairment of coho exposed to dilbit at higher temperatures. Irrespective of the mechanism, this finding emphasizes that sublethal effects of dilbit exposure can have a lasting impact on fish populations and can be amplified by a small increase in temperature at the time of a spill.

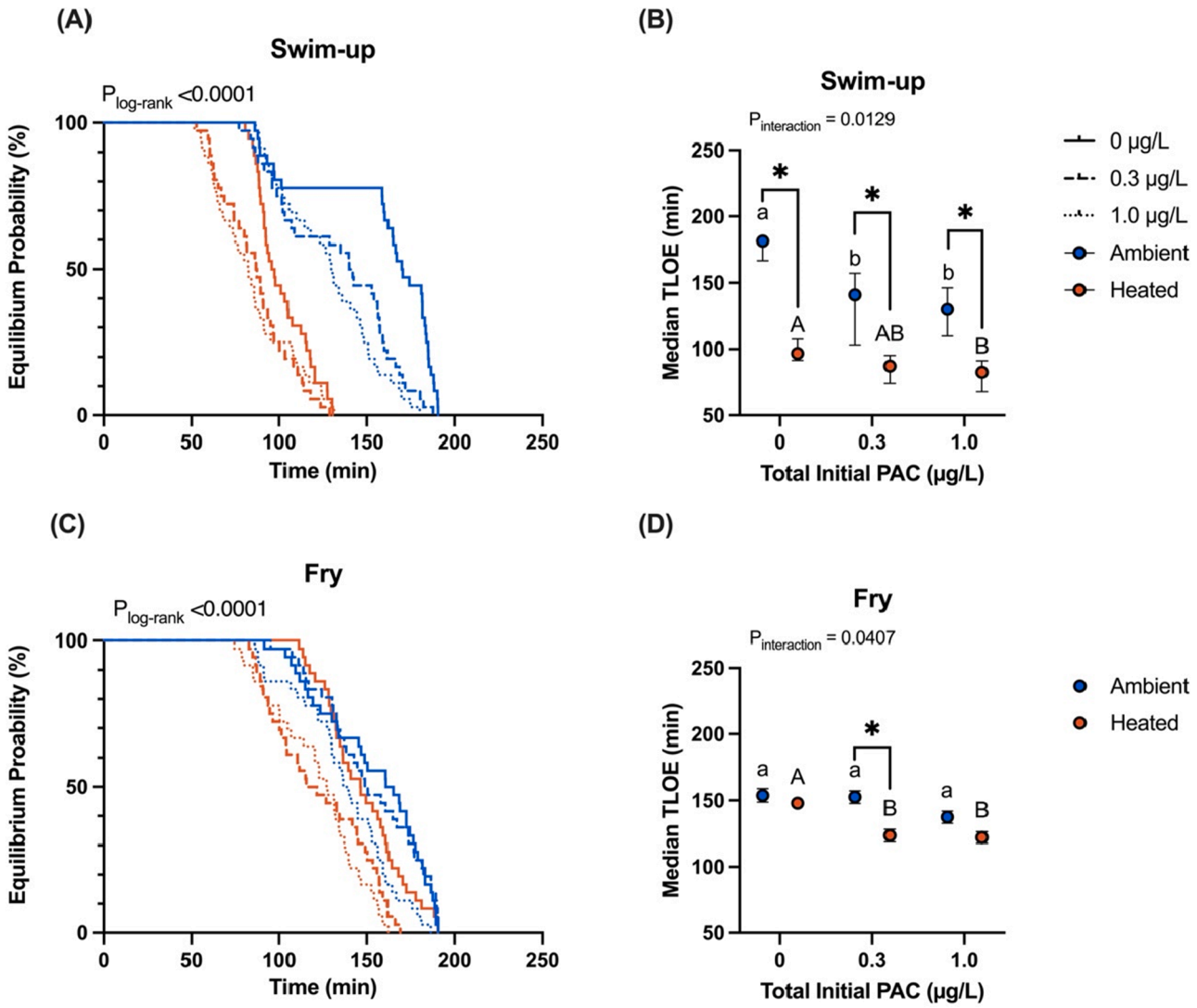

Fig. 4. Kaplan-Meier analysis of time (minutes) to loss-of-equilibrium (LOE) during hypoxia challenge tests (HCT) following exposure to control (solid line, 0 µg/L) or one of two water soluble fractions of dilbit (dashed line, 0.3 µg/L; dotted line, 1.0 µg/L), at ambient (blue) and heated (+3 °C, orange) temperatures. HCT were performed at swim-up (A, B) and fry (C, D) stages. Subsequent Log-rank tests on the data indicate significant differences between the concentration groups in each temperature ($N = 36$ fish per exposure group, $\alpha = 0.05$). Median time to LOE (TLOE) across exposure groups at swim-up (B) and fry (D) stages were compared with two-way ANOVA and Tukey's post-hoc tests (median ± 95 % confidence interval, $N = 18$ fish per replicate exposure group, $\alpha = 0.05$). Data points with different letters indicate statistically significant differences in exposure groups within ambient (lower case) and heated (+3 °C, upper case) temperatures. Asterisks indicate significant differences between median TLOE observed across temperature groups within an exposure group.

4.2. Dilbit × temperature interactions on salmon tissues

The bioaccumulation of PAC in coho tissues was concentration dependent and elevated at higher temperature. It is unclear if the higher PAC body burden is attributed to increased uptake, decreased elimination or both. Previous research has demonstrated the temperature dependency uptake of heavy metals (e.g., in rainbow trout [*O. mykiss*], MacLeod and Pessah, 1973) and organics (e.g., benzo[a]pyrene in gulf toadfish [*Opsanus beta*], Kennedy et al., 1989a, 1989b). Certainly, the high octanol-water coefficient values (log K_{ow}) of these PAC render them amenable to bioaccumulation in early life stages which have greater lipid proportions (Petersen and Kristensen, 1998). The predominant PAC species detected in fish tissues were napthalenes and their C1-C4 alkyl derivatives, reflecting the relative abundance of these PAC in dilbit (Alderman et al., 2017a; Avey et al., 2020; Perugini et al., 2022a) and other fossil fuels (Goto et al., 2021). The toxicity of these PAC to fish is not debated, and the additional body burden in coho exposed to dilbit at elevated temperature likely contributes to the greater toxicity observed in these fish.

The induction of Phase I and II hepatic biotransformation enzymes is a defense response to some environmental contaminants including components of crude oil. Certain PAC serve as agonists for AhR, leading to increased transcription of monooxygenases including Cyp1a (a Phase I enzyme). The subsequent increase in Cyp1a activity results in greater transformation of presiding PAC, in turn shifting the relative composition and concentrations of parent PAC and their metabolites in tissues (Hodson, 2017). Cyp1a induction contributes directly to the adverse outcomes of crude oil exposure by determining the rate of PAC elimination from the body and by extension, the duration of internal tissue doses (Hodson, 2017). At the same time, the presiding PAC in tissues in combination with Cyp1a induction determines the rate at which toxic intermediate metabolites are generated (Hodson, 2017). The concentration-dependent increase in *cyp1a* transcripts following crude oil exposure has been widely demonstrated in teleost fishes (Alderman et al., 2018, 2017; Billiard et al., 2006; Perugini et al., 2022; Timme-Laragy et al., 2007), whereas transcriptional responses in *gst* (a Phase II

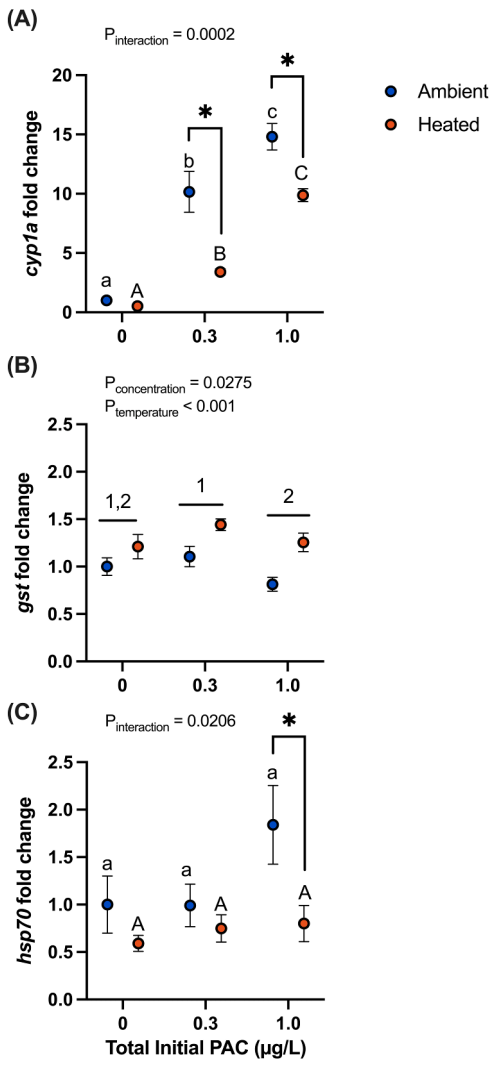

Fig. 5. mRNA abundance of several genes following developmental exposure to WSF dilbit (0.3 µg/L and 1.0 µg/L) or clean water (0 µg/L) at each of two temperatures (ambient and heated, +3 °C). Genes of interest were (A) *cytochrome P450 type 1a, cyp1a*; (B) *glutathione S-transferase, gst*; and (C) *heat shock protein 70, hsp70*. Gene expression was quantified using reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Transcript abundances were standardized to housekeeping genes *rpl8* and β-*actin*. Two-way ANOVAs and Tukey's post-hoc tests were used for statistical comparisons of temperature, dilbit concentration, and their interaction (mean ± *S*.E.M., $N = 12$ fish per exposure group, α=0.05). Data points that do not share a common letter denote statistically significant difference within each temperature group. Differences between concentrations are indicated by numbers. Asterisks (*) indicate significant difference across temperature groups in cases with significant interaction between the temperature and concentration factors.

enzyme), if observed, are often concentration independent (Lin et al., 2021; Madison et al., 2015; Perugini et al., 2022; but see Bérubé et al., 2021). Here, the reliability of *cyp1a* expression as a sensitive biomarker of dilbit exposure was confirmed, alongside evidence that the induction of *cyp1a* may be reduced at higher temperature. This is consistent with previous work showing lower *cyp1a* expression in fish exposed to crude oil in 5–7 °C warmer water (Andersen et al., 2015; Serafin et al., 2019), but is counterintuitive with the expected increased rate of contaminant uptake in warmer water (Kennedy et al., 1989b; Kennedy and Walsh, 1994) that would drive induction. Given the established thermal compensation of monooxygenases (Kleinow et al., 1987), whereby an inverse relationship between Cyp1a content and temperature exists (Kennedy et al., 1989a; Kennedy and Walsh, 1994; Sleiderink et al., 1995), the lower *cyp1a* transcript abundance may reflect down-regulation of Cyp1a protein in the face of higher temperature rather than an impaired induction response with warming. Taken together, the higher PAC body burden and reduced *cyp1a* expression in coho exposed to dilbit in warmer water supports the hypothesis that thermal modulation of Phase I induction effectively prolongs exposure of internal tissues to PAC. This may underscore the exacerbated adverse outcomes of crude oil exposure at elevated environmental temperatures (present study; Lin et al., 2021; Serafin et al., 2019). Additional studies on the interaction between temperature and toxicokinetics of contaminant mixtures, like dilbit, are clearly warranted.

4.3. Perspectives and implications

Decades of research on crude oil exposure and PAC toxicity in aquatic biota, including fish, confirms that biological responses are concentration- and time-dependent, and with respect to fish, life stage dependent. Thus the volume of spilt oil and the logistics of its clean-up are key players in the environmental impacts of any oil spill, and response measures to stop and contain a spill are of obvious priority to minimize ecosystem damage. Yet, oil spills are unpredictable, can happen at any time of the year, and as shown here, the biological effects of crude oil exposure may be underestimated if only time and concentration are considered in impact assessments. Both temperature and dissolved oxygen have recently been implicated as important abiotic variables that can intensify the toxic effects of crude oil exposure in marine and freshwater fish at multiple life stages (present study; Lin et al., 2021; Serafin et al., 2019). Increased temperature also enhanced the toxicity of representative PAC (anthracene, phenanthrene, naphthalene) on marine phytoplankton (*Tetraselmis chuii*) (Vieira and Guilhermino, 2012), highlighting the potential for enhanced multi-trophic impacts with a few degrees difference in water temperature at the time of a spill. This highlights the need to consider the thermal variability of the receiving environment during environmental risk assessments, as well as the prevailing conditions at the time of a spill when making mitigation and management decisions related to wild populations, including fish.

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CRediT authorship contribution statement

Derin M. Çalık: Writing – original draft, Visualization, Investigation, Formal analysis. **Feng Lin:** Investigation. **Mackenzie Edgar:** Investigation. **Anthony P. Farrell:** Writing – review & editing, Funding acquisition. **Christopher J. Kennedy:** Writing – review & editing, Supervision, Funding acquisition. **Todd E. Gillis:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Sarah L. Alderman:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sarah Alderman, Todd Gillis, Chris Kennedy, Anthony Farrell reports financial support was provided by Fisheries and Oceans Canada. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2025.107347.

Data availability

Data will be made available on request.

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