The Biological Effects of Diluted Bitumen (dilbit) on Two Species of Pacific Salmonid: Sockeye (*Oncorhynchus nerka*) and Pink Salmon (*Oncorhynchus gorbuscha*)

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Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

a. human research ethics approval from the Simon Fraser University Office of Research Ethics

or

b. advance approval of the animal care protocol from the University Animal Care Committee of Simon Fraser University

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Abstract

The major petroleum product derived from the Canadian Alberta oil sands is bitumen, which is commonly mixed with diluents to produce several blends of diluted bitumen (dilbit). The prospected expansions of dilbit transportation capacity in coastal regions of British Columbia (BC) increase the risks of accidental releases of dilbit into freshwater and marine environments of particular concern are the potential risks of exposure to sensitive Pacific salmonids. The central goal of this research was to generate new empirical data to characterize the toxicity of the water-soluble fraction (WSF) of unweathered Cold Lake Blend dilbit to two Pacific salmon species: sockeye (*Oncorhynchus nerka*) and pink salmon (*Oncorhynchus gorbuscha*). A comprehensive suite of studies examined the acute and chronic toxic outcomes including lethality, effects on growth, swimming performance, exercise recovery capacity, body energetics, the interrenal stress response, iono-osmoregulatory ability, immune function, and genetic responses. Exposure of sockeye from the fertilized embryos to swim up stage resulted in increased mortality, impaired growth, as well as reductions in both critical (U_{crit}) and burst swimming speed (U_{burst}) in free-swimming fry. These effects correlated with alterations in energy substrate reserves at all stage and an interference in the utilization of lipid energy sources and the ability to mount a physiological stress response. Exposure of juvenile salmonids to the WSF of dilbit (at TPAC concentrations at the ppb level) resulted in sublethal effects that included a classic physiological stress response, and alterations in iono-osmoregulatory homeostasis and immunological performance. Reductions in swimming performance were correlated with a significantly diminished aerobic scope following exposure and recovery following burst exercise was altered. In experiments with juvenile pinks, A 3 month exposure at varying salinity and temperature showed that higher temperatures and salinities affected dilbit-induced mortality, growth, osmoregulation, and energy storage. In a larger context, the findings here provide necessary toxicological information required for the development of risk assessment plans for managing salmon populations and restoring habitat in the event of potential pipeline failures or tanker spill.

Keywords: Oil sands; diluted bitumen (dilbit); toxicity; Pacific salmon; sockeye salmon; pink salmon

Dedication

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List of Acronyms and Abbreviations

AH	Aromatic hydrocarbon
AS	Aerobic scope
API	American Petroleum Institute
AWB	Access Western Blend
BC	British Columbia
BSD	Blue sac disease
BTEX	benzene, toluene, ethylbenzene, and xylenes
CAPP	Canadian Association of Petroleum Producers
CER	Canada Energy Regulator
CLB	Cold Lake Blend
d	Day
Dilbit	Diluted bitumen
Dilsynbit	Diluted synthetic bitumen
DO	Dissolved oxygen
ECCA	Environment and Climate Change Canada
ELS	Early life stage
EROD	Ethoxyresorufin-O-deethylase
Fe	Iron
h	Hour
HMW	High-molecular-weight
KP	Keystone Pipeline
L	Liter
LAC	Library and Archives Canada
LC50	Lethal concentration to 50% of testing population
LMW	Low-molecular-weight
M	Month
min	Minute
MAH	Monoaromatic hydrocarbon
Mb/d	Million barrels per day
N	Nitrogen
NA	Naphthenic acid

NASEM	National Academies of Sciences, Engineering, and Medicine
Neatbit	Nearly solid bitumen
NGC	Natural gas condensate
Ni	Nickel
NRC	Natural Resources Canada
NTSB	National Transportation Safety Board
K_{ow}	Octanol–water partition coefficient
O	Oxygen
OSPW	Oil sands process-affected water
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
RCF	Raincoast Conservation Foundation
RMR	Routine metabolic rate
s	Second
S	Sulphur
SAGD	Steam assisted gravity drainage
SARA	Saturates, aromatics, resins, and asphaltenes
SFU	Simon Fraser University
SMR	Standard metabolic rate
Synbit	Synthetic bitumen
MMR	Maximum metabolic rate
TMP	Trans Mountain pipeline
TPAH	Total polycyclic aromatic hydrocarbon
U_{burst}	Burst swimming speed
U_{crit}	Critical swimming speed
U.S.	The United States
U.S. EIA	The U.S. Energy Information Administration
V	Vanadium
WAF	Water-accommodated fraction
WSF	Water-soluble fraction
WCSB	Western Canadian Sedimentary Basin

Chapter 1. Introduction

1.1. General background

Canada holds the world's 3rd largest proven oil reserves which are estimated to contain 171 billion barrels (bb); 97% of the reserves are contained in the oil sands from the Western Canadian Sedimentary Basin (WCSB) with the largest deposits found in the Athabasca, Peace River, and Cold Lake regions. Only 4.7 bb of oil are found in forms other than oil sands, such as conventional crudes, offshore, and shale oil (NRC, 2019a). As the 4th largest oil producer in the world, Canadian oil production reached 4.6 million barrels per day (mb/d) in 2018 with 2.9 mb/d (64%) generated from oil sands (NRC, 2019a). Canada supplies more petroleum products than its domestic needs. In 2018, greater than 60% of crude oils produced in Canada were exported to foreign markets, with 96% of the exports going to the U.S. and 4% destined for Europe, Asia, and the Caribbean (NRC, 2019b). Although the overall production volume in Canada is only expected to increase by 1.3 mb/d by 2035, the proportion from the oil sands sector is forecasted to grow exponentially, resulting in > 90% of the national crude oil production coming from this source, assuming that key transportation infrastructure is built for market assess (CAPP, 2019a; CER, 2018).

Oil sands, often referred to as tar sands and bituminous sands, are loose sand deposits that consist of approximately 1-18% hydrocarbons, 5-10% water, and 80–85% solid matter consisting of sand, clay and other minerals (Cleveland and Morris, 2014). The primary petroleum product derived from Canadian oil sands is bitumen, a highly viscous, tar-like mixture of hydrocarbons and significant content of nitrogen, oxygen, and sulfur-containing compounds, as well as trace amount of metals, organometals, naphthenic acids, water, and mineral particles (ECCC et al., 2013; Lee et al., 2015; Meyer and Attanasi, 2003). Raw bitumen is a solid or semi-solid material at room temperature, and it is recovered from deposit sites by either direct surface mining or *in situ* extraction (Read and Whiteoak, 2003). For deposits buried to $\leq$75 m below the surface, bitumen is trucked to processing plants and mixed with hot water; solid matter is subsequently separated by gravity (NRC, 2016). Deeper deposits are commonly

extracted *in situ* by a steam assisted gravity drainage (SAGD) method that injects steam into a drill well and the resulting diluted oil-water slurry pumped to the surface (NRC, 2016). Most extracted bitumen is diluted with natural gas condensate (NGC) at a typical ratio of 1:3, producing several blends of diluted bitumen (dilbit) that can remain as a consistent fluid and be transported to refineries *via* pipelines (Crosby et al., 2013). NGC is a low-density petroleum mixture of pentanes and heavier hydrocarbon fractions derived from raw natural gas. NGC remains as liquid under standard, ambient conditions, and is currently the most frequently used diluent for producing oil sands products (Bott, 2011). Another common diluent is synthetic crude oil that is essentially a form of bitumen after partial undegrading, coking, and hydrolysis processes that remove larger molecules (Crosby et al., 2013). Synthetic bitumen (synbit) is produced by mixing raw bitumen and synthetic crude at a ratio of 1:1, whereas diluted synthetic bitumen (dilsynbit) is the complex mixture consisting of unprocessed bitumen, NGC, and synthetic crude oil (variable percentage composition) (Dew et al., 2015). Railbit is a blend of a less diluted bitumen product (up to 15% diluent) that is typically shipped in heated rail tankers (Fingas, 2015a). Nearly solid bitumen (neatbit) specifically refers to undiluted bitumen that is transported *via* railway. For simplicity, the term dilbit used in this chapter encompasses all types of crude oil products that are engineered by diluting raw bitumen with lighter petroleum hydrocarbons. The proportions of bitumen and diluents may be adjusted according to the seasonal viscosity and density requirements for meeting pipeline transport specifications, resulting in seasonal differences in the hydrocarbon profiles of dilbit products (Dupuis and Ucan-Marin, 2015). Thus, the chemical composition of dilbit possesses a significantly higher dimension of variability when compared to those conventional crude oils from a given source region (Adams et al., 2013).

Crude oils are often characterized as light, medium, or heavy, based on three major quality criteria: density, viscosity, and sulfur content (CER, 2018; U.S. EIA, 2012). Crudes containing less than 0.5% sulfur are defined as sweet crude oils, while crudes with high sulphur content (≤1.99%) are referred to as sour crude oils (API, 2011). Lighter and sweeter oils are usually processed to yield high-valued end products, such as gasoline, kerosene, and high-quality diesel that are also highest in demand. In

comparison, heavy and sour crudes are considered lower-grade oils because they require far more complicated processing procedures to remove impurities (e.g., sulfur, carbon dioxide, solids) and therefore more expensive to refine. To reduce refining costs, heavy and sour oils are commonly used for producing diesel and heavier fuel oils, instead of processing further into lighter end products like gasoline. The majority of crude oil recovered from oil sands in the WCSB is sour heavy oil (CER, 2018).

The chemical composition and physical properties of crude oils can vary widely depending on their specific geological formation processes, methods of extraction and refining (NASEM, 2016). In general, crude oils are primarily comprised of thousands of petrogenic hydrocarbons that are commonly categorized into four main classes: saturates, aromatics, resins, and asphaltenes, as well as smaller proportions of compounds containing heteroatoms (e.g., N, S, and/or O) and trace metals (Ni, V, and Fe) (Lee et al., 2015; NASEM, 2016). The relative percentage composition of the four major classes of constituents together contributes to several important characteristics of oils, including viscosity, density, adhesive properties, and susceptibility to microbial biodegradation (Lee et al., 2015; NASEM, 2016). Saturated hydrocarbons, also known as alkanes or paraffins, are highly water-insoluble hydrocarbons consisting of only single bond between carbon atoms and may form structures as straight chains, branched chains, or rings (Kenney, 2015). The saturates are usually the most abundant and biodegradable fractions in petroleum products (Lee et al., 2015). Aromatic hydrocarbons (AHs), also known as arenes or sometimes aryl hydrocarbons, are cyclic, planar, unsaturated hydrocarbons containing one or more benzene rings in their structures (Lee et al., 2015). One-ringed AHs are often referred to as the monoaromatic hydrocarbons (MAHs) which include benzene, toluene, ethylbenzene, and the three isomers of xylene (BTEX) (Lee et al., 2015). The BTEX series is among the relatively more volatile, water-soluble, and biodegradable constituents in crude oils (Kennedy, 2015; Lee et al., 2015). Polycyclic aromatic hydrocarbons (PAHs) contain two or more fused benzene rings; they are progressively less volatile and water-soluble than BTEX, but more resistant to biodegradation. Unsubstituted or parent PAHs only represent 15% of the total PAHs (TPAHs) in most crude oils, while the majority of PAHs are found in alkyl-substituted forms (Lee et a., 2015). The aromatic fraction also includes some non-hydrocarbon

heterocyclic compounds such as furans, dibenzothiophenes, 2-methyldibenzo-thiophene, and their alkyl-substituted homologues (Lee et al., 2015; NASEM, 2016). A broader term, polycyclic aromatic compounds (PACs) is often used to include both the PAHs and polycyclic heterocycles. Resins and asphaltenes are complex high-molecular-weight (HMW) non-hydrocarbon compounds containing one or more heteroatoms (mainly nitrogen, sulfur, and oxygen), but the structure of individual compounds in these two classes are currently poorly understood. Resins and asphaltenes are known to be polar, resistant to the biodegradable action of microorganisms, virtually non-volatile and insoluble in water. The greater content of these larger molecules contribute to the higher viscosity, density, and adhesivity of heavy crudes and bitumen (Lee et al., 2015). Petroleum may also contain small and variable concentrations of non-hydrocarbon components derived from biological or geological sources, such as metals, organometals, sulphur, naphthenic acids (NAs), mineral particles and /or water (Lee et al., 2015).

Dilbits are engineered by mixing two major components: 1) the volatile diluents (e.g., NGC) that are rich in low-molecular-weight (LMW) saturates and BTEX; and 2) the viscous, dense bitumen fraction which provides abundant non-volatile HMW compounds (Lee et al., 2015; NASEM, 2016). Thus, dilbits and conventional crude oils generally contain the same classes of constituents, while the relative proportions of those constituents vary widely. In comparison, dilbits contain significantly lower amounts of lighter, more volatile saturates and BTEX, but an increased abundance of the heavy fractions that include HMW PACs, resins, and asphaltenes (Lee et al., 2015; NASEM, 2016). Dilbits also have lower percentages of TPAH, but an increased proportion of 3- to 5-ringed alkyl-substituted PAHs that are considered the primary contributors to the chronic toxicity observed in oil-exposed aquatic biota (Lee et al., 2015). Despite the differences in composition, the physical and chemical properties of dilbit are generally consistent with other commonly transported crudes. For example, the density of dilbit is approximately 0.92 to 0.94 g/cm^3, while most crude oils typically range within 0.77-0.94 g/cm^3 (Hollebone, 2015). The viscosity of dilbit is much greater than light and medium crudes, but comparable to heavy oils (Hollebone, 2015). Dilbits present markedly higher

adhesion properties than most other crude oils owing to its increased concentration of resins and asphaltenes (Hollebone, 2015).

Like other petroleum products, the vast majority of dilbit produced in Canada is transported from remote operating sites to domestic or international refineries and markets through an extensive oil pipeline network across North America (CAPP, 2019a). Currently, most existing transmission pipelines for exporting dilbit to the markets outside the WCSB, including the Trans Mountain pipeline (TMP) and the Keystone pipeline (KP), have reached their maximum available capacities (CER, 2018; CAPP, 2019a). Meanwhile, the growth in Canadian crude oil production has gradually outpaced the increase in pipeline takeaway capacity since 2016; the excessive oil supply is presently being moved by rail tanker shipments, a more expensive alternative to pipeline transportation (CER, 2018). Accordingly, multiple projects for new pipelines or expansions of existing infrastructure have been proposed by the Canadian energy sectors, aiming to provide sufficient takeaway capacity and convenient market access to global markets (e.g., Asia-Pacific regions and the US) where anticipated growth in energy demands are significant (CER, 2018; CAPP, 2019b). These proposals include the Keystone XL replacement project, the Energy East Pipeline, the Northern Gateway Pipeline, and the expansion plan for the existing TMP. Pipelines are designed to traverse great geographical distances, and many traverse a wide variety of landscapes, environments, and ecosystems including watersheds, streams, rivers, and lakes. Although pipelines are a relatively safe and cost-efficient method for transporting crude oil, increasing shipment volumes inevitably poses higher risk of pipeline failure or rapture (NASEM, 2016). Therefore, the proposed pipeline projects have increased awareness and environmental concern regarding the potential impacts of accidental oil spills in the adjacent terrestrial and freshwater ecosystems through which they pass.

To date, only the TMP expansion project has been approved, and it is currently under construction, while other the other proposed projects mentioned above have all been cancelled (CAPP, 2019c). The TMP delivers crude oil and refined petroleum products, including dilbit from Strathcona County, Alberta to Weigh Anchor Terminal in Burnaby, British Columbia (BC) (RCF, 2018). Oil tankers currently depart from the terminal and transit through costal waters in the Salish Sea, exporting oils to both the US

and overseas markets in Asia. The expansion project twins the existing pipeline, and will nearly triple the capacity from the current 300,000 to 890,000 b/d (CER, 2018). The existing route and new pipeline will cross > 250 rivers and streams in the Fraser River watershed, many of which are spawning habitat for multiple unique populations of Pacific salmon including Chinook (*Oncorhynchus tshawytscha*), chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*), pink (*Oncorhynchus gorbuscha*), sockeye (*Oncorhynchus gorbuscha*), as well as interior and coastal populations of steelhead and trout (genus *Salmo*) (Levy, 2009; RCF, 2018). All Fraser River salmon populations (genus *Oncorhynchus*) are destined to transit its estuary and the Salish Sea twice in their lifetime: once as the seaward migrating juveniles and once are the mature spawning-ready adults returning to their natal streams (RCF, 2018). In addition, these estuaries also provide important rearing grounds for some species of ELS salmon (e.g., pink salmon) prior to their seaward migration; the health and growth during this early nursery period can directly determine their future survival in open ocean (Godin, 1981). Many of these species and populations are currently facing a crisis due to decades of habitat loss, fishing, climate change, and contamination from anthropogenic sources (Boldt et al., 2019; Cohen, 2012). The expansion in pipeline capacity will necessitate a significant increase in oil tanker traffic in the estuaries of the Salish Sea that provides important migratory routes and rearing grounds for young salmon (Levy, 2009; RCF, 2018). While spills from pipelines have been relatively infrequent to date, the increased transport and shipment of dilbit and other petroleum products in these ecologically sensitive aeras inevitably poses a higher risk for multiple species of Pacific salmon and other species.

When released into the aquatic environment from a transmission pipeline, crude oils will immediately undergo a series of chemical and physical processes that are collectively called weathering (Wang and Fingas, 2003). Chemical processes include photooxidation and biodegradation, during which the molecular structures of oil constituents are altered and potentially enhancing or decreasing the toxicity of various compounds (Baron et al., 2003; NASEM, 2016). These chemical processes tend to occur slowly over a period of weeks to years and represent the breakdown of oil at the molecular level (NASEM, 2016). Physical-chemical partitioning processes include the interaction with particulates or dissolved organic matter (DOC), the uptake by aquatic

biota *via* bioaccumulation, the evaporation of volatile components (e.g., BTEX), and the aqueous dissolution of water-soluble fractions (Alsaadi et al., 2018a; NASEM, 2015). These processes do not directly cause alteration to the molecular structure of oil components and their partitioning between different phases; they tend to occur rapidly and can significantly affect the chemical composition and behavior of spilled oil (NASEM, 2016). Physical processes include spreading, dispersion, emulsification, adhesion, and sedimentation. While these physical processes do not directly alter the molecular structure of oil components, they can result in complex, environment-dependent interactions with the chemical and physical-chemical partitioning processes. In general, conventional crude oils undergone extensive weathering processes are observed with significant losses of the relatively more volatile, water-soluble LMW compounds (e.g., <C10 saturates, mono- or di-aromatics), but a markedly increased proportion of the heavier, viscose HMW compounds (e.g., resins and asphaltenes), resulting in strongly enhanced density, viscosity, and adhesiveness (Lee et al., 2015; NASEM, 2016). Weathering can substantially alter the environmental fate and behaviours of spilled oils and the residues, resulting in profound effects on the potential environmental and ecological consequences of that release (NASEM, 2016). Fate and behavior of crude in aquatic systems oil are also subject to a host of environmental factors that are specific to the actual spill site, such as temperature, salinity, turbidity (Lee et al., 2015; Tsaprailis, 2013).

Spilled dilbit may exhibit different environmental fates and behaviours when compared to other conventional heavy crudes owing to unique physical and chemical properties (Dupuis and Ucan-Marin, 2015). These differences can be further enhanced by weathering processes, as indicated by data from the dilbit spill that occurred in a tributary of the Kalamazoo River near Marshall, Michigan in 2010 (Alsaadi et al., 2018b; NASEM, 2016). An estimated 3.2 million L of dilbit was released into the local aquatic habitats following rupture of the Enbridge Line 6B pipeline. This has been characterized as one of the largest inland oil spills in North America with an estimated > $1B costs for clean-up and habitat recovery (Dollhopf et al., 2014; NTSB, 2012). Like most crude oils, fresh dilbit has a lower density (0.92-0.93 g/mL) than both freshwater and seawater, therefore was found to float upon entry to the water column (Lee et al., 2015). Spilled

dilbit underwent immediate weathering processes during which the LMW from the oil-gas condensates rapidly evaporated, leaving residuals with an increased proportion of HMW compounds (NASEM, 2016). The weathered residues initially had a density near, or slightly lower than freshwater, and subsequently formed oil globules which continued to absorb suspended particulates (at least 2-fold higher density than water), yielding oil-aggregates that were neutrally or negatively buoyant in water. A large volume of the persisting aggregates eventually submerged below surface and became associated with bottom sediments, resulting in a prolonged release of toxic hydrocarbons (NASEM, 2016). The higher predominance of resins and asphaltenes in dilbit compared to conventional oils further increases the density and viscosity of residual oil, making spill remediation challenging and expensive (NASEM, 2016). An estimated 302,000 L of sunken residuals remain in the sediments in the Kalamazoo system years following recovery efforts (NASEM, 2016). In contrast, although weathered heavy oils also exhibit a similar density to freshwater, they are less likely to sink than dilbits because of their relatively lowered levels of adhesion, density, and viscosity (NASEM, 2016). Due to the potential for dilbit to persist in the sediments following a spill scenario in some freshwater environments, there is a data gap regarding the long-term effects of persistent exposure of dilbit components to aquatic biota (Alderman et al., 2018).

Crude oil is composed of a multitude of petrogenic hydrocarbons that are known to cause various adverse effects on teleosts *via* diverse mechanisms of action (Kennedy, 2014). The adverse outcomes following oil exposure at the organism level may include, but are not limited to: lethality, genotoxicity, histopathological changes, embryotoxicity and ELS developmental defects, altered bioenergetics, disturbances to the neurological and endocrine systems, suppressed immune functions and resistance to disease, impairment to exercise-related physiological performance, behavioral disruptions, as well as reduced growth and reproductive capacity (Dupuis and Ucan-Marin, 2015; NASEM, 2016; Pasparakis et al., 2019). While crude oils are complex mixture of compounds, oil-induced toxicity to fish is primarily attributed to non-polar, lipophilic alkanes and aryl hydrocarbons which include both monoaromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs), as well as their alkylated homologs (Khursigara et al., 2019). The LMW hydrocarbons, including < C10 saturates,

MAHs, and PAHs containing three or less benzene rings are often linked to the acute and lethal toxicity seen in aquatic organisms, mainly owning to their relatively higher water solubility than HMW hydrocarbons (e.g., > C10 saturates and > 3-ringed PAHs), and therefore their concentrations in saturated aqueous solution can exceed the typical LC50 values to fish (Kennedy, 2014). However, these LMW hydrocarbons are typically more volatile, readily bio- or photo-degradable, and not bioaccumulative, and thus they are generally not associated with chronic effects observed during oil exposure (Kennedy, 2014; NASEM, 2016). The HMW PAHs (both unsubstituted and alkylated) are demonstrated to cause chronic toxicity that can result in delayed responses and long-term residual effects, such as carcinogenesis, reproductive failure, developmental deformities, and immune suppression (NASEM, 2016). HMW PAHs are generally not acutely toxic due to limited water solubilities (Kennedy, 2014), but their higher persistence in aquatic environmental can lead to a greater potential for prolonged exposure to local biota following oil spills (NASEM, 2016). In particular, the chronic toxicity of ≥ 3-ringed PAHs to ELS fish tends to increase with hydrophobicity, as expressed by the octanol–water partition coefficient (log K_{ow} value [Hodson, 2017]). Hydrophobicity generally increases with molecular size and determines the rate of water–lipid partitioning of PAHs, the accumulation by fish, and their apparent toxicity (Alsaadi et al., 2018a). However, the acute and chronic toxicity of larger PAHs (> 5 rings) may not increase at log Kow values > 6. For persistent organic compounds, low water solubility and large molecular size limit the rates of water-membrane partitioning and the dose accumulated (e.g., toxicity cut-off values; [Veith et al., 1983]). Larger alkanes (> C10), resins, and asphaltenes are almost insoluble in water, and they typically represent greater log K_{ow} values; these major oil components are therefore often considered non-toxic because they are virtually not bioavailable to aquatic animals (Adams et al., 2014; Alsaadi et al., 2018a; Hodson et al., 2017). Accordingly, the summed concentrations of for each individual PAH quantified, or the total PAHs (TPAHs) are frequently reported in filed or laboratory studies as the main causative agents for crude oil toxicity (Meador and Nahrgang, 2019). In addition to direct, chemically-based mechanisms, crude oils can also result in acute and sublethal effects *via* physical mechanisms. The physical coating of biological surfaces impedes an organism's movement and can alter behavior and/or

hamper respiration (e.g., coating of gills and permeable skin surfaces of fish) (NASEM, 2016).

Dilbits are composed of similar classes of constituents to conventional crude oils, and therefore similar toxic properties to fish are to be expected (NASEM, 2016). Although dilbit has been transported in North America for decades, limited laboratory studies exist that have specifically investigated the acute or long-term toxicity of dilbit to fish species in general (Dupuis and Ucan-Marin, 2015; NASEM, 2016). Most existing literature describes studies focused on the developmental and molecular effects in sensitive ELS teleosts following exposure to prepared water-accommodated fractions (WAFs) or water soluble fractions (WSFs) of dilbit (Alderman et al., 2018; Alsaadi et al., 2018b; Madison et al., 2015, 2017; McDonnell et al., 2019; Philibert et al., 2016). WAFs are commonly prepared by low-energy mechanical stirring of a water-oil mixture in a closed container with headspace. WAFs are presumed to contain both dissolved constituents and small amounts of suspended oil particulate (Hodson et al., 2018). Water-soluble fractions (WSFs) can be generated by continuously passing water through petroleum-coated passive diffusers which can become depleted as exposure is prolonged. Since no artificial mixing process is incorporated in this exposure method, it delivers time-dependent hydrocarbon profiles that that aquatic organisms may directly encounter in oil spills (Rodrigues et al., 2010). Previous studies using standard laboratory test species, such as fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), silverside (*Menidia beryllina*), yellow perch (*Perca flavescens*), zebrafish (*Danio rerio*), and hatchery-reared sockeye salmon (*O. nerka*) have demonstrated that exposure to the WAFs or WSFs of dilbit can result in acutely lethal effects, increased mortality during embryonic development, a higher prevalence of post-hatching deformities, abnormal behavior patterns, changes in bioenergetics, alterations in gene expression in ELS fish, and latent effects in body morphology (Alsaadi et al., 2018b; Barron et al., 2018; Madison et al., 2015, 2017; McDonnell et al., 2019; Philibert et al., 2016). Fewer studies by far have attempted to investigate dilbit toxicity to teleosts at juvenile and adult stages. Alderman et al. (2017a, b) reported that subchronic exposure (1-4 weeks) to the WSFs of dilbit resulted in impairment to the swimming performance, histopathological changes in cardiac tissues, alterations in serum

proteome as well as significant elevation of biomarker enzyme in 1+ year juvenile sockeye. Similar reductions in swimming speed were also demonstrated in WSF-exposed Atlantic smolts, coupled with the upregulation of biomarker responses, as well as altered enzyme activities and gene expression in muscles (Avey et al., 2020). Based on the available data, the nature of dilbit toxicity to the vast majority of North American freshwater and/or marine teleost species represent a remarkable knowledge gap. The immediate and latent impacts of dilbit exposure to fish different at life stages also remain poorly characterized.

Although abundant studies using crude oils or individual PAHs have provided key toxicological information which may be extrapolated to predicting dilbit toxicity, the unique hydrocarbon profile and environmental fate and behavior of dilbit highlights the scientific uncertainties of using generalizations of conventional crude oil toxicity and the application of this information to predicting dilbit toxicity. In particular, the relative proportions of oil components vary widely across different oil products and blends, and thus generalizations from data from existing studies using other petroleum hydrocarbons may prove unreliable. Comparisons of several blends of dilbit to other commonly transported heavy crudes in North America using SARA extraction demonstrated that dilbit products generally have fewer saturates (12-20% vs. 38%), more resins (33-60% vs. 20–33%) and asphaltenes (13-18% vs. 2-13%), whereas less aromatics (10-35% *v.* 29-42%) than heavy oils (Hollebone, 2015; King et al., 2017). Dilbits contain a lower relative abundance of BTEX and PAHs, but an increased percentage of 3- to 5-ringed alkyl PAHs (Lee et al., 2015; King et al., 2017) which have been identified as the most embryotoxic oil constituents to sensitive ELS teleosts (Hodson et al., 2017). Noteworthy is that dilbit contains a comparable or slightly higher content of naphthenic acids (NAs; Been, 2011) which represents a large naturally occurring group of saturated aliphatic and alicyclic carboxylic acids found in hydrocarbon deposits like crude oils and bituminous oil sands (Headley and McMartin, 2004). NAs are conventionally identified as a major component in the toxic fraction of oil sands process-affected water (OSPW; Clemente and Fedorak, 2005); their contribution to the overall toxicity of crude oils has not been specifically investigated (Kennedy, 2014). Few studies have reported that exposure to the OSPW or directly to pure NAs have been associated with lethal effects,

developmental toxicity, and histopathological and molecular alterations in fish (Lyons et al., 2018; Nero et al., 2006; Peters et al., 2007). In addition, dilbits consist of significantly higher vanadium (V) content and similar level of nickel (Ni) as compared to the conventional heavy sour crudes (Been, 2011). These metals form metalloporphyrin structures which are usually associated with the asphaltenes; therefore, metals are more abundant in heavier petroleum fractions and in heavy bituminous crudes at concentrations up to hundreds of parts per million (Fingas 2015b; Lee et al., 2015). Previous field studies show evidence that metal atoms may disassociate from their organic ligands in petrogenic compounds and become water-soluble and bioavailable after oil spills into the aquatic environment (reviewed in Lee et al., 2015). While V and Ni are known toxicants to fish (Blewett and Leonard, 2017; Sprague et al., 1978), a significant knowledge gap currently exists pertaining to the mixture toxicity of these metals and PAHs or other oil components to aquatic organisms (reviewed in Gauthier et al., 2014), making it extremely difficult to assess how increased metal content in dilbit might impact aquatic systems.

1.2. Project Overview and Objective

The potential risk from spills of dilbit is the major source of scientific uncertainty with respect to fresh and seawater salmon habitat quality in western Canada. To address this uncertainty, the primary objective of this work was to investigate the toxicity of a commonly transported dilbit (the Cold Lake Blend [CLB] dilbit) to two Pacific salmonid species: sockeye and pink salmon which are of great ecological, economic, and cultural importance to BC and Canada. The unique life history of these two species makes them particularly vulnerable receptors to oil exposure in the case of a dilbit spill. In general, Pacific salmon have a complex life history consisting of four main phases culminating in recruitment to the mature population: 1) as eggs and alevins in the gravel of rivers and lakes; 2) as young juveniles in streams and lakes; 3) as older juveniles in the ocean; and 4) as grown adults that are ready for spawning migration (Bradford, 1995). The Fraser River sockeye salmon commonly have a four-year lifespan, whereas the pink salmon have a fixed two-year life cycle. Reproduction-ready adults usually return to their natal habitats for spawning in summer and fall months, the fertilized

embryos and hatched alevins reside in bottom gravels overwinter until emergence (Bradford, 1995). The swim-up fry of pink salmon typically migrate immediately downstream to the estuaries of BC, and juveniles typically reside in nearshore areas of estuaries for a fast-growing period up to several months, then migrate further offshore into open ocean until adult stage (Godin, 1981). In contrast, emerged sockeye fry normally take up residence in streams or lakes for a year or more before migrating to sea as larger smolts (Groot and Margolis, 1991). The salmon embryos and alevins are relatively immobile, and therefore have limited ability to avoid the presence of contaminants in their habitat. As for sockeye, fry to pre-smolt fish inevitably rely on the freshwater habitats for early nursery before seaward migration. Both species are destined to transit the Fraser Watershed and the estuaries of the Salish Sea twice in lifetime: once as young juvenile and once as spawning-ready adult. Thus, in a dilbit spill event, it is likely that salmon across various life stages may be exposed to the toxic constituents of dilbit, and exposure durations may be prolonged if spilled oil is not immediately recovered (Dew et al., 2015; Dupuis and Ucan-Marin, 2015). The experiments included in this research aim to generate new empirical data that directly addresses how acute and sublethal exposures to dilbit at environmentally realistic concentrations affect a wide array of important physiological functions that are relevant to the life history and fitness of salmonids. It is believed that the results of these studies will apply to other salmonid species due to overlapping similarities in their physiology and life cycles.

In Chapter 2, we investigated the effects of exposure to WSFs of CLB dilbit on three critical physiological systems in juvenile sockeye salmon: the iono-osmoregulatory system, the hypothalamic-pituitary-interrenal axis and the physiological stress response, and the immunological system. These systems have previously been reported to be adversely affected in Pacific herring (*Clupea pallasii*) exposed to the WSFs of Alaska North Slope crude oil (ANSCO; Kennedy and Farrell, 2005, 2006; 2008). Pre-smolt sockeye were acutely (24 h) or subchronically (96 h, 21 d) exposed to the WSFs of CLB dilbit at 3 concentrations. Following exposure, the effects on iono-osmoregulation were assessed by measuring gill Na^+-K^+-ATPase activity, plasma osmolality, and serum concentrations of Na^+ and Cl^-. Alterations in stress-related endpoints, including liver

glycogen content, and plasma [glucose], [lactate], and [cortisol] were also quantified. The function of the innate immune system was assessed by challenging exposed fish to a seawater pathogen *Vibrio* (*Listonella*) *anguillarum*, in addition to several hematological measurements related to the immune system. The stage of salmonid examined here, the pre-smolt (parr), undergo a series of physiological changes collectively called smoltification which prepare them for their upcoming seaward migration and the transition from life in freshwater to life in seawater (Cohen, 2012). Clearly, any dilbit-induced impairments in these physiological systems will result in long-term fitness effects and therefore conservation concerns for affected salmonid populations.

In Chapter 3, we investigated the immediate effects of prolonged exposure to WSF of CLB dilbit on ELS development, body energetics, and gene expression changes in fertilized sockeye salmon embryos. Subsets of phenotypically normal survivors were subsequently reared in uncontaminated water for up to 8 months, the latent effects on their survival, growth, morphological alterations (brain and heart), and swimming performance were also determined. Exposure to crude oils or individual PACs are known to impair hatching success, embryonic development, growth, as well as the induction of molecular responses that are involved in xenobiotic biotransformation in salmonids (Carls et al., 2005; Carls and Thedinga, 2010; Heintz, et al., 1999; Hodson, 2017). Similar toxic outcomes were also demonstrated in recent dilbit studies using standard laboratory fish species. Specifically, WAF-exposed ELS zebrafish, Japanese medaka, and fathead minnow exhibited reduced survival, increased incidence of blue sac disease and post-hatching malformations (e.g., skeletal and cardiac deformities, presence of edema, abnormally inflated or un-inflated swim bladder), and molecular responses in genes that are associated with phase I and II xenobiotic metabolism, oxidative stress, cell cycling and mutagenesis (Alsaadi et al., 2018b; Madison et al., 2015, 2017; McDonnell et al., 2019). While these studies provide important baseline toxicity information regarding the WAF of dilbit, the sensitivity of developing salmon embryos to dilbit WSF is unknown. The results of this study provide direct comparison for the sensitivity of local species like sockeye salmon to typical testing species, aiding in furthering the characterization of the risk associated with a dilbit spill to potential receptors in the waters of Pacific Northwest. Meanwhile, although ELS fish may survive

chronic exposure to crude oil, some oil-induced sublethal effects, such as such as deformities in vital organs (e.g., Hicken et al., 2011), suppressed development and growth (e.g., Heintz et al., 2000) may persist throughout their later life stages, resulting in increased susceptibility to predation, and reduced survival to adulthood (Carls and Thedinga, 2010). For example, pink salmon embryos incubated in PAHs-contaminated water had significantly lowered spawning return rates by 40% as compared to the controls (Heintz et al., 2000). To our knowledge, the present study is the first attempting to investigate the potential latent impacts on dilbit-exposed fish.

In Chapter 4, we investigated the combined effects of a chronic CLB dilbit WAF exposure (90 d) on pink salmon fry survival, growth, burst swimming, muscle energy stores, and hepatic gene expression relevant to biotransformation under different temperatures and salinities. Coastal estuaries are productive, yet highly dynamic habitats where periodic and stochastic fluctuations in environmental parameters occur regularly (Whitehead, 2013). Juvenile pink salmon inhabiting in these aquatic environments are constantly challenged by a variety of environmental stressors, particularly, temperature, salinity, and dissolved oxygen levels, each of which can potentially interact with the toxic stress posed by exposure to dilbit during a spill event. While there have been some studies attempting to explore the potential additive and synergistic effects of oil exposure and natural stressors on similar toxicological endpoints (e.g., survival, growth, reproduction, and cellular damage) (Khursigara et al., 2019; Whitehead, 2013), to our knowledge, there have been no studies that can provide data for the effects of dilbit on salmonid species under these conditions. The combined effects of these stressors may amplify the damage of oil-exposure to organisms in the real world, and contribute to impacts on fitness, populations, and communities, that may not have been predicted from direct toxicity of hydrocarbons alone (Whitehead, 2013).

In Chapter 5, fertilized sockeye salmon embryos were exposed the WSFs of CLB dilbit throughout embryonic development until swim-up fry stage. Exposures were also continued until fish were 8-months-old, with the aim of investigating adverse outcomes under chronic exposure scenarios. Potential dilbit-induced alterations on hatching success, survival, growth, critical swimming speed (U_{crit}) and U_{burst}, pre- and/or post-exercise body energetic substrate levels, and molecular responses were investigated.

While these ecologically relevant endpoints were previously found to be affected by oil exposure in various teleosts (e.g., reviewed in Kennedy, 2014; Laurel et al., 2019; Pasparakis et al., 2019), few investigators have specifically focused on dilbit, and the sensitivity of salmonids at this particular embryo-to-fry window in their life cycle. It is known that ELS and fry are often considered the most sensitive life stages in a population (e.g., Hodson et al., 2017), and that disturbances to normal embryonic and early larval development may directly impact fish's near-term and latent growth and survival at later life stages (Laurel et al., 2019); these effects may compromise individual fitness and long-term population recruitment. These data may provide the baseline toxicological information required for estimating the risk of dilbit spill to salmon habitats, as well as post-spill restoration plans for salmonid habitat. These experiments may also provide some insight into the potential impacts on local biota in scenarios where spilled dilbit is not be immediately removed, leading to the long-term release of contaminants.

In Chapter 6, juvenile sockeye salmon were either acutely (96 h) or subchronically (28 d) exposed to WSFs of CLB at 3 concentrations. The effects of dilbit exposure on sockeye prolonged swimming performance (Ucrit), respirometry endpoints including standard metabolic rate (SMR), maximum metabolic rate (MMR), and aerobic scope (AS) were evaluated. The metabolic and ionic recovery capacity of exposed individuals was also assessed by measuring pre- and post-exercise plasma concentrations of cortisol, lactate, Na^+, Cl^-, and muscle glycogen concentrations. Oil- or PAH-induced impairments to swimming speed are widely documented in many freshwater and marine teleosts (reviewed in Kennedy et al., 2014; Pasparakis et al., 2019), whereas few studies exist for salmon and dilbit (Alderman et al., 2017a; Avey et al., 2020). No research, to our knowledge, has been dilbit exposure effects on metabolism in fish. Locomotory performance and aerobic capacity are commonly adopted as good indicators of fish fitness; these endpoints carry high ecological relevance to anadromous teleosts such as salmon because they are involved in many critical life events, such as long-distance migration between habitats, avoidance of predation, and food capture (Hammer, 1995; Johansen and Esbaugh, 2017). Even fewer studies have attempted to examine oil effects on the ability of to recover from exhaustive exercise. The ability to efficiently recover from intense exercise is considered an

ecologically relevant endpoint as avoidance from predators and advancing through rapid currents often involve several repeated swimming episodes (Kieffer, 2000). The integration of these crucial fitness traits may help better characterize the potential disturbances of dilbit to multiple physiological functions in juvenile salmonids following a spill.

In Chapter 7, we briefly reviewed the critical findings in the present work, and suggested potential directions for future research.

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Chapter 2. Physiological disturbances in juvenile sockeye salmon (*Oncorhynchus nerka*) exposed to the water-soluble fraction of diluted bitumen

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Abstract

Current and proposed transcontinental pipelines for the transport of diluted bitumen (dilbit) from the Canadian oil sands traverse the coastal watersheds of British Columbia, habitat essential to Pacific salmonids. To determine the potential risks posed to these keystone species, juvenile sockeye (*Oncorhynchus nerka*; 1+ parr) were acutely (24–96 h) or subchronically (21–42 d) exposed to 4 concentrations of the water-soluble fraction (WSF) of unweathered Cold Lake Blend dilbit (initial total PAC concentrations: 0, 13.7, 34.7 and 124.5 µg/L) in a flow-through system. Dilbit effects on iono-osmoregulation, the physiological stress response, and the immune system were assessed by both biochemical and functional assays. Hydrocarbon bioavailability was evidenced by a significant induction of liver ethoxyresorufin-O-deethylase (EROD) activity in exposed fish. Acute and subchronic exposure significantly reduced gill Na^+, K^+-ATPase (NKA) activity and resulted in lower plasma osmolality, Cl^-, and Na^+ concentrations. Acute exposure to dilbit resulted in a classic physiological stress response, however at 21 d of exposure, plasma cortisol remained elevated while other measured parameters had returned to baseline values. A compromised immune system was demonstrated by a 29.5 % higher mortality in fish challenged with *Vibrio* (*Listonella*) *anguillarum* following dilbit exposure compared to unexposed controls. Exposure of juvenile salmonids to the WSF of dilbit (at TPAC concentrations at the ppb level) resulted in sublethal effects that included a classic physiological stress response, and alterations in iono-osmoregulatory homeostasis and immunological performance.

Keywords: Oil sands; diluted bitumen (dilbit); crude oil; toxicity; fish; sockeye salmon; stress; osmoregulation; immune system; physiology

2.1. Introduction

Canada is currently the 4th largest oil producing country and 4th largest oil exporter in the world, producing an average of 4.2 million barrels of crude oil per day (Mb/d) in 2017 (Natural Resources Canada, 2019). The majority of this originates as raw bitumen in the oil sands deposits of the Western Canada Sedimentary Basin, accounting for 64 % of oil production in 2017 (National Energy Board of Canada, 2018). Extraction rates in the Western Canadian oil sands are projected to increase from the current 2.7 Mb/d [(Natural Resources Canada, 2019)]) to 3.7 Mb/d over the next decade (Canadian Association of Petroleum Producers, 2016). Similar to common crude oils, dilbit is a complex mixture of hydrocarbons that include saturates (16–17 %), aromatics (25–32 %), resins (37 %) and asphaltenes (18–21 %) (Strausz et al., 2011; Woods et al., 2008). To facilitate pipeline transportation, bitumen-derived products contain proprietary diluents such as low molecular weight saturates and mono- and di-aromatics derived mainly from oil–gas condensates (Alsaadi et al., 2018a) (e.g. dilbit: 3:1 bitumen to diluents; synthetic oil or synbit: 1:1 bitumen to diluents; dilsynbit: a combination of the two [Canadian Association of Petroleum Producers, 2016; Dew et al., 2015]). The hydrocarbon profiles of different dilbit blends varies slightly due to the location of the source deposit, the process used for extraction, as well as adjustments of diluent concentration that correspond to seasonal requirements for viscosity (ECCC et al., 2013; Lee et al., 2015).

The transportation of Canadian crude oils *via* an extensive and expanding pipeline and rail network (current and proposed) traverses diverse ecosystems over long distances (Canadian Association of Petroleum Producers, 2018; Levy, 2009; Natural Resources Canada, 2019), some of which contain critical spawning habitats and migration routes for multiple species of Pacific salmon (Natural Resources Canada, 2019; Raincoast Conservation Foundation, 2018). For example, expansion of the existing Trans Mountain pipeline crosses Canada's largest salmon-bearing river system (Henderson and Graham, 2006), the Fraser River Watershed, and puts millions of early life stage (ELS) salmon at risk. While pipelines are a relatively inexpensive and safe

means of transportation, according to the Transportation Safety Board of Canada (2012), 28 % of 67 oil spills between 2007 and 2016 were from pipelines.

Previous research on conventional crude oils have identified several classes of compounds including low molecular weight aliphatics and aromatics (< C10; e.g., benzene, toluene, ethylbenzene and xylene [BTEX]), heterocyclic aromatics (e.g., dibenzothiophene), naphthenic acids (NAs), and polycyclic aromatic hydrocarbons (PAHs) (Alsaadi et al., 2018a; Kennedy, 2014a; Rhodes et al., 2005) that are toxic to fish at relatively low concentrations. A wide range of toxic outcomes in fish include mortality, morphological and histopathological alterations, iono-osmoregulatory disturbances, stimulation and the abolishment of the interrenal stress response, genotoxicity, immunotoxicity, endocrine disruption, as well as developmental and reproductive effects (Kennedy, 2014a).

Limited data on dilbit toxicity currently exists and the utility of extrapolating data from current knowledge on conventional crude oil to dilbit is unknown. To date, the majority of data on dilbits effects in freshwater organisms has been through information gathered from the Kalamazoo River spill (July 2010), several reviews on the toxicity of major constituents of dilbit (Ball and Truskewycz, 2013; Brown and Ulrich, 2015; Gauthier et al., 2014; Headley and McMartin, 2004; Manzetti, 2012), and several published studies on dilbit specifically (Alderman et al., 2017a, b; Alderman et al., 2018; Alsaadi et al., 2018b; Barron et al., 2018; Madison et al., 2015, 2017; Philibert et al., 2016; McDonnell et al., 2019).

Studies published in recent years have focused on the developmental effects of dilbit in the ELS of fish. Delayed hatch time, higher prevalence of post-hatch deformities, increased mortality during embryonic development, and alterations in body composition in addition to latent effects on brain morphology have been reported in sockeye exposed to the WSF of Cold Lake blend (CLB) dilbit (Alderman et al., 2018). Embryo stage fathead minnow (*Pimephales promelas*) and yellow perch (*Perca flavescens*) exhibit increases in post-hatch malformations following exposure to the water-accommodated fraction (WAF) of both Access Western Blend (AWB) and CLB dilbit, and the observed developmental effects correlated well with significantly upregulated *cyp1a* (Alsaadi et al.,

2018b; McDonnell et al., 2019). Japanese medaka (*Oryzias latipes*) exposed to the WAF of AWB dilbit show an increased prevalence of blue sac disease (BSD) and post-hatching malformations (Madison et al., 2015, 2017). Developing zebra fish (*Danio rerio*) exposed to the WAF of dilbit exhibit increased mortality and altered patterns in their shelter seeking and swimming behaviors (Philibert et al., 2016). Molecular responses relevant to Phase I and II biotransformation, cellular cycling, mutagenesis, and oxidative stress have also been documented following dilbit exposure in ELS fish (Alderman et al., 2018; Alsaadi et al., 2018b; Madison et al., 2015, 2017).

Fewer studies have been conducted using juvenile fish. Alderman et al. (2017a) exposed juvenile sockeye to the WSF of CLB dilbit and reported reductions in swimming performance, histological and molecular/ biochemical responses (e.g., induction of hepatic EROD activity, up regulation of aryl hydrocarbon receptor (AhR) and *cyp1a* in cardiac tissues), as well as alterations in the serum proteome (e.g. proteins involved in immune and inflammatory responses, coagulation, and iron homeostasis [Alderman et al., 2017b]).

Due to sockeye population declines, approximately 40 % of these populations currently monitored by the Canadian government are listed as being a conservation concern (Alderman et al., 2017b); these include stocks from watersheds crossed by existing (e.g., Fraser River, BC) and proposed (e.g., Skeena River, BC) pipeline routes (Levy, 2009). Sockeye juveniles were selected for use in the present study because: 1) fry and parr are found in freshwater tributaries, and 2-year-old juveniles traverse the Fraser River watershed *en route* to the Pacific Ocean during migration, 2) these stages are unable or unlikely to be able to avoid contaminated water following a spill, and 3) evidence suggests that ELS and juvenile fish are the most sensitive life stages. In the current study, two separate experiments were conducted to examine the sublethal effects of acute and subchronic dilbit exposure on: 1) iono-osmoregulation and the physiological stress response and 2) the immune system and disease resistance. Alterations in these critical systems have been previously reported in crude oil-exposed juvenile Pacific herring (*Clupea pallasii*) (Kennedy and Farrell, 2005, 2006, 2008). Information regarding dilbit toxicity to juvenile salmon is needed for ecological risk

assessments, environmental impact assessments, and in the implementation of post-spill remediation plans.

2.2. Materials and Methods

2.2.1. Fish

The care and use of fish in the present study were approved by the SFU University Animal Care Committee that follows guidelines set by the Canadian Council on Animal Care. In the first experiment, juvenile sockeye salmon (1.5 year old, body weight 172.9 ± 10.2 g (x ± SE) and fork length 24.1 ± 1.8 cm) were obtained from LSL Living Seafoods Ltd. (Burnaby, BC). Fish were acclimated in 200-L fiberglass tanks supplied with aerated, flow-through dechlorinated municipal water (DO > 95 % saturation; water hardness 6.12 mg/L $CaCO_3$; DOC < 1 mg/L; pH 7.0; temperature 12.5 °C) for 3 weeks. Photoperiod was set to 12h:12 h (light:dark). Fish were fed twice daily *ad libitum* with commercial salmon feed (Skretting, Vancouver, BC).

Sockeye gametes were provided by the Upper Pitt River Hatchery (Fisheries and Oceans Canada) and fertilized according to standard procedures (Ontario Ministry of Natural Resources, 2009). Fertilized embryos were incubated in Heath trays supplied with a continual flow of dechlorinated municipal water without light exposure until fish reached the swim-up fry stage. Phenotypically normal swim-up fry were transferred to 200 L fiberglass tanks with a rearing photoperiod of 12h:12 h, and were fed ad libitum 3 times a day with commercial salmon feed until they were 8 months of age (mass 12.7 ± 1.6 g, fork length 9.8 ± 0.8 cm) when they were used for the second experiment.

2.2.2. Exposure

Prior to dilbit exposure in the first experiment, juvenile sockeye were acclimated for 1 week in 200-L aerated exposure tanks and then exposed to one of 4 concentrations of the WSF of unweathered CLB summer dilbit (COOGER, Fisheries and Oceans Canada) for 24 h, 96 h, or 21 d (4 replicate tanks for each WSF treatment group or control at each time point, n=5 fish per replicate tank). Different WSFs were generated

by continuously passing dechlorinated water through PVC columns (16 cm diameter x 80 cm length) containing Siporax® ceramic beads that had been pre-soaked in dilbit (Kennedy and Farrell, 2005). Water containing the WSF of dilbit flowed into a 500-L distribution tank and was then distributed into replicate exposure tanks at a flow rate of 6 L/min. Different WSF concentrations were achieved by varying the bead content in each column (Kennedy and Farrell, 2005; Alderman et al., 2017a, b). In control tanks, water flowed through columns containing uncontaminated beads. All fish were fed once daily, and tanks were cleaned of any uneaten food within 15 min.

For the second experiment, 8-month-old juveniles were randomly selected from grow-out tanks and fin clipped for identification in several treatment groups (Kennedy and Farrell, 2008), no mortality occurred following fin clipping following 1 week of recovery. Fish were then randomly distributed into 200-L exposure tanks (4 replicate tanks for each WSF treatment group or control at each time point, n=50 fish per replicate tank) and acclimated for 1 week. Fish were exposed to uncontaminated water or the highest WSF of dilbit as above for 24 h or 42 d. All fish were fed as above.

2.2.3. Sampling

Following exposure to the 4 concentrations of WSF for 24 h, 96 h, and 21 d, fish were rapidly netted from exposure tanks and immediately euthanized in buffered MS-222. To avoid potential diurnal variations in measured parameters, the collection of plasma and tissues was conducted at 10:00 am at each sampling time point. Body mass and fork length measurements were taken, followed by blood samples taken from the caudal vasculature using micro capillary tubes pre-coated with heparin. A subsample of whole blood was stored in Drabkin's reagent, vortexed and centrifuged for 5 min at 2800 x g (Drabkin and Austin, 1932). Another subsample of whole blood was stored in Dacie's solution for blood cell counts (Blaxhall and Daisley, 1973). Blood was also centrifuged at 13,000 x g and hematocrit and leucocrit determined using digital calipers. Plasma was then separated and snap-frozen on dry ice. Fish were dissected, the livers were then weighed and flash-frozen in liquid nitrogen. Gill filaments were collected and stored in ice-cold SEI buffer containing 150mM sucrose (Sigma, Oakville, ON, CA), 10mM EDTA,

50mM imidazole (Sigma) at pH 7.3 (McCormick, 1993). Gill samples were snap-frozen on dry ice. Plasma, liver, and gill samples were stored at −80 °C for further analysis.

2.2.4. Biochemical and hematological analysis

Plasma glucose, L-lactate (Catalog# 120003400P; 120001400P; Eton Bioscience, San Diego, CA), and cortisol (Catalog# 402710; Neogen Corp., Lexington, KY) concentrations were measured spectrophotometrically using the respective assay kit protocols provided by the manufacturers. Liver glycogen content was quantified using a commercial glycogen colorimetric assay kit (Catalog# K646-100; BioVision, Milpitas, CA). All measurements were performed in triplicate. Plasma osmolality was measured using a vapor pressure osmometer (Model Wescor Vapro 5520; EliTech Group, Logan, UT). Plasma Cl^- concentrations were measured spectrophotometrically according to Osachoff et al. (2014). Plasma Na^+ concentrations were determined using an atomic absorption spectrometer (Model # Varian AA240FS; Palo Alto, CA). All ion concentration and osmolality measurements were performed in triplicate.

Liver microsomes were isolated according to the method outlined in Gourley and Kennedy (2009). The prepared microsomal fraction was used to measure EROD activity according to Hodson et al. (1991). Microsomal protein concentrations were measured using a Bradford protein assay kit (Catalog# 23200; Thermo Fisher Scientific, Mississauga, ON).

Frozen gill filaments were thawed on ice, 25 µL SEID buffer (0.5 g of sodium deoxycholate (Sigma) in 100 mL of SEI buffer) was added and samples homogenized using a Mixer Mill homogenizer (Model: MM 300; Qiagen, Mississauga, ON) at 25 Hz for 30 s, and then centrifuged (5000 *x g*) at 4 °C for 30 s to remove insoluble debris. Gill Na^+, K^+-ATPase (NKA) activity was measured in the homogenate using a standard protocol (McCormick, 1993). Gill protein was quantified using a Bradford protein assay kit and bovine serum albumin as a standard (Thermo Fisher Scientific).

Whole blood hemoglobin concentration was quantified using a modified version of the cyanomethemoglobin method (Drabkin and Austin, 1932). In brief, 5 µl whole

blood was diluted in 1.25 ml Drabkin's solution (Drabkin's reagent [Sigma] and 30 % Brij 35 solution [Sigma]). Absorbance was measured at 540 nm on a microplate reader (BioTek Epoch™ 2, Winooski, VT) using cyanmethemoglobin as a standard (Stanbio Laboratory, Boerne, TX). For blood cell counts, whole blood samples were diluted up to 6-fold in Dacie's solution and enumerated on a Neubauer hemocytometer (www.hausserscientific.com). Total areas of 0.2mm2 and 4mm2 were counted for erythrocytes and leucocytes, respectively (Bastidas, 2013). Ratios of hematological measurements were calculated and used to provide additional interpretations of hematological parameters (Velenzuela et al., 2007). These secondary indices (mean corpuscular volume [MCV] of erythrocytes, mean corpuscular hemoglobin [MCH], and mean corpuscular hemoglobin concentration [MCHC]) were calculated using hematocrit, hemoglobin concentration and erythrocyte counts (Valenzuela et al., 2007).

2.2.5. Disease challenge

In experiment 2, fish were challenged with *Vibrio* (*Listonella*) *anguillarum* following exposure to dilbit; 25 fish from each control tank and 25 fish from each dilbit exposure tank were randomly pooled into one 500-L challenge tank supplied with aerated flow-through dechlorinated municipal water (water temperature 12.5 °C). For either 24 h or 42 d exposure, there were 4 replicate challenge tanks per exposure time, with n=50 fish per replicated challenge tank. Fish were allowed to acclimate to new tank conditions for 12 h, followed by exposure to *V. anguillarum*, a common marine opportunistic pathogen and the main causative agent of vibriosis disease (Egidius, 1987). Fish were reared exclusively in freshwater and therefore had no previous exposure to this marine pathogen, assuring that the sockeye were immunologically naïve to *V. anguillarum* and had no specific immune resistance mechanisms to vibriosis (Balfry et al., 2001; Wood et al., 1996). During the disease challenge, water volumes in challenge tanks were reduced to 150 L, and the salinity of the water adjusted to approx. 30‰ using sea salt (Fluval Aquatics, Baie-d'Urfé, QC) (Kennedy and Farrell, 2008; Shelley et al., 2009). A previously cultured bacterial suspension was then added to the tanks; fish were exposed to a nominal concentration of 1×10^7 cfu/ml of *V. anguillarum* for 1 h. Tank water volumes were increased and fish were monitored daily for mortality and

signs of disease for a 3-week period following the challenge. The monitoring of post-challenge mortality was terminated when no dead fish were found for 3 consecutive days or to a maximum 3 weeks. Dead fish were collected and frozen for subsequent necropsy and examination for the clinical signs of *V. anguillarum* infection to confirm that vibriosis was the cause of death (Balfry et al., 2001). The *V. anguillarum* used in this study was obtained from wild coho salmon (*Oncorhynchus kisutch*) that had died from previous infection with the pathogen. The culture of *V. anguillarum* and the confirmatory microbiological techniques were performed by DFO West Vancouver Laboratory (West Vancouver, BC, Canada) following standard protocol (Balfry et al., 2001).

2.2.6. Hydrocarbon analysis

Water samples were collected from duplicate exposure tanks at 12 h, 12 d, and 25 d after initiating WSF generation. Hydrocarbon analysis was performed by Axys Analytical Services Ltd. (Sidney, BC); concentrations of individual polycyclic aromatic compound (PAC) in water samples were measured following standard procedures as described in Alderman et al., 2017a. Final total PAC (TPAC) concentrations were expressed as the average of the two replicates at each of the 3 sampling time points.

2.2.7. Statistical analysis

The data sets were first examined for homogeneity of variance and normality; no violation to these assumptions was detected. All biochemical measurements were analyzed to determine the effect of exposure concentrations and exposure lengths, as well as the interactions between these two factors, using a two-factor ANOVA followed by a Tukey-Kramer multiple comparison test. The mortality data from the disease challenge were analyzed using the Chi-square test. All data analyses were conducted with a fiducial limit of significance at $p < 0.05$ or $p < 0.01$, using JMP 14 program (SAS Institute Inc., Cary, NC).

2.3. Results

2.3.1. Water chemistry

Initial aqueous TPAC concentrations in exposure tanks were: control (0.08 µg/L), low (13.7 µg/L), medium (34.7 µg/L) and high (124 µg/L). In previous studies using the WSF-generating apparatus for crude oil and diluted bitumen, TPAC concentrations declined with time in a similar manner across all concentrations (Kennedy and Farrell, 2005, 2006, 2008; Alderman et al., 2017a, 2018). Comparable aqueous concentrations were reported by Carls et al. (1995) using a similar design. In addition, values obtained using this apparatus and CLB summer dilbit show that the relative contributions individual PAC change over the exposure starting with predominantly smaller and less substituted (e.g., naphthalenes) PACs at the start of WSF generation which progressively shifts to larger and more highly substituted PACs (e.g., phenanthrenes; Figure 2.1, Table 2.1).

2.3.2. Biochemical and hematological parameters

Hepatic microsomal EROD activity in control fish and those exposed to dilbit is shown in Figure 2.2. No significant differences were seen in EROD activity in fish between treatments at 24 h, however significantly higher EROD activities were observed in the medium and high exposure groups at 96 h (2.5- and 3.0-fold higher than controls, respectively). Maximum EROD induction was seen at 21 d of exposure, with the activity levels 4.5-fold higher in fish exposed to the highest WSF concentrations compared to controls.

Plasma cortisol concentrations were significantly elevated over controls in fish exposed to the medium and high concentrations of WSF at 24 h, 96 h and 21 d, with the maximum concentrations between 86.6 ± 3.7 and 111.7 ± 7.2 ng/mL consistently seen in the highest WSF concentration (Figure 2.3a). At both 24 h and 96 h, plasma glucose concentrations were significantly higher in fish exposed to the two higher WSF concentrations compared controls; plasma glucose concentrations were not significantly different between any treatment groups at 21 d (Figure 2.3b). At 24 h, dilbit exposure

significantly elevated plasma lactate concentrations in a concentration-dependent manner; however, by 96 h and 21 d, elevated plasma lactate concentrations were only seen in the medium and high exposure groups (Figure 2.3c). Liver glycogen concentrations decreased in a concentration-dependent manner following exposure to dilbit at 24 h (maximum reduction by 39 % compared to controls) (Figure 2.3d). In longer exposures, alterations in liver glycogen concentration in both the low and medium exposure groups were reduced or were not statistically different from controls (Figure 2.3d).

Exposure to dilbit reduced gill NKA activity at 96 h and 21 d, while no significant alteration was found at 24 h (Figure 2.4a). By 96 h, fish exposed to the medium and high concentrations exhibited significant reductions (approximately 45 % compared to controls). At the longest exposure duration, activity was reduced at the low concentration as well, and was 51 % lower in the high concentration group compared to controls.

Plasma osmolality was decreased at all exposure durations in fish in the two higher concentration treatment groups. The greatest reduction in plasma osmolality was seen in the highest concentrations at 96 h (20 %) and 21 d (21 %) compared to the controls (Figure 2.4b). At 24 h, only fish in high exposure group showed a significant decrease in plasma [Na^+] compared to controls, however, as the duration of exposure increased, the medium (96 h) and then in the low exposure group (21 d), also exhibited significant reductions (Figure 2.4c). No significant changes were seen in plasma [Cl^-] in fish in the low group at any exposure duration, while fish in the medium and high groups exhibited decreased plasma [Cl^-] at all three time points (Figure 2.4d).

Hematocrit was not affected by exposure to the WSF of dilbit (Figure 2.5a). Hemoglobin concentration in the blood of fish from the high exposure group was significantly higher by 96 h and 21 d exposure (Figure 2.5b). Erythrocyte counts were not affected by exposure (Figure 2.5c). Leukocyte concentrations were significantly lower at the 96 h time point for the medium and high exposure groups compared to controls (Figure 2.5d). Mean corpuscular volume (MCV) was lower at 21 d of exposure in the medium and high exposure groups (Table 2.2). Mean corpuscular hemoglobin content (MCH) was significantly higher at 21 d in the high treatment group compared to

controls. Mean corpuscular hemoglobin concentration (MCHC) in fish in medium and high exposure groups were significantly elevated at both 96 h and 21 d.

2.3.3. Disease challenge

No significant difference in cumulative mortality from *V. anguillarum* between control and 24-h dilbit-exposed fish was seen. Post-challenge mortality was 53.0 ± 3.5 % and 50.0 ± 2.2 %, respectively in control and exposed groups of fish at the end of the experiment (Figure 2.6a). Fish exposed to the highest WSF of dilbit for 42 d showed significantly higher (29.5 %) cumulative pathogen-induced mortality 16 d post-challenge compared to control fish (Figure 2.6b).

2.4. Discussion

The ELS and juvenile salmonids are particularly vulnerable to potential dilbit spills due to their inability to avoid exposures spatially, and their apparent high sensitivity to some dilbit components (Alderman et al., 2018; Alsaadi et al., 2018b; Carls et al., 1999; Madison et al., 2015, 2017; McDonnell et al., 2019; Philibert et al., 2016). Other than physical effects that could occur from insoluble dilbit matrices, toxicity results from the bioavailable fraction of the components dissolved in the water phase. The exposure system used here generates an aqueous exposure medium containing the WSF of dilbit with initial TPAC concentrations ranging from 0.002 µg/L to 124 µg/L, representing total dissolved hydrocarbons in dilbit that are bioavailable to freshwater biota. Initial concentrations declined with time and the concentrations of relatively smaller, lightweight, and more volatile hydrocarbons (e.g., naphthalenes) predominate initially, and with time the proportion of larger higher molecular weight compounds (e.g., 3–5 ringed PAHs) increases. The exposures to those special hydrocarbon profiles in present study provide important information regarding the acute toxicity that juvenile salmon may experience at the initial of a potential dilbit spill event, as well as the subchronical effects and latent impact of dilbit exposure if the residual oils become associated with sediment particles, causing prolonged environmental contamination. This exposure system allowed for acute and subchronic exposures with the appropriate chemical composition

that may more closely resemble those in the environment following an accidental spill of dilbit in freshwater ecosystems.

Hepatic EROD activity is an established biomarker for bioavailable PAHs in fish (McCarty et al., 2002). A TPAC concentration-dependent induction in EROD activity was seen by 96 h which remained elevated at 21 d. EROD activity is induced following exposure to the WSF of dilbit and WAF of crude oils; other studies with Pacific herring, polar cod (*Boreogadus saida*), and Atlantic salmon (*Salmo salar*) showed similar induction patterns and levels following exposure to several petroleum sources at TPAH concentrations in the µg/L range (Gagnon and Holdway, 2000; Kennedy and Farrell, 2005; Nahrgang et al., 2010). Exposure of sockeye juveniles to the WSF of CLB dilbit (TPAH concentration 3.5 µg/L for 28 d) showed EROD induction to similar levels as seen in the present study (Alderman et al., 2017a).

The acute toxicity of crude oil, chemically dispersed oil, WAF and WSF of oil or its components to various fish species has been previously reviewed (Kennedy, 2014a). The WSF of dilbit was not lethal to juvenile sockeye salmon in the present study, supporting limited data from other studies (sockeye salmon juveniles [Alderman et al., 2017a] and larval fathead minnow [Barron et al., 2018]). An exception is the Inland silverside (*Menidia beryllina*: 10–14 d old) where dilbit was acutely lethal with an estimated 96-h LC50 value of 24.1 µg/L TPAH (Barron et al., 2018). Similarly, other recent dilbit studies generally reported no acute necrotic effect to developing embryos and early larvae of several freshwater fish species at stage at TPACs up to 100 µg/L (Alsaadi et al., 2018b; Madison et al., 2015, 2017; McDonnell et al., 2019). In comparison, the acute lethal toxicity of conventional crude oils to fish is generally reported at concentrations above the solubility values for most oil components (Kennedy, 2014a).

The sublethal toxicity of conventional crude oil includes effects ranging from alterations in gene expression (Edmonds et al., 2015), genotoxicity, biochemistry, histopathological changes (Alderman et al., 2017a), developmental, behavioral, and reproductive effects, and effects on other indicators of performance (e.g., growth, swimming ability, immunocompetence) (Kennedy, 2014a, Alderman et al., 2017b) and

behaviour (Philibert et al., 2016). Recent studies to determine if dilbit toxicity is similar to that of conventional oils has primarily focused on developmental effects; limited data is available regarding other potential sublethal effects, particularly in juvenile life stages.

The ability to initiate an appropriate physiological stress response is adaptive (Gorissen and Flik, 2016) and occurs through the activation of the hypothalamus–pituitary–interrenal axis and sympathetic chromaffin system leading to the release of cortisol and catecholamines. Downstream secondary factors then regulate the distribution of energy sources and delivery of oxygen. Long-term activation can lead to a compromised hydromineral imbalance and immune system function (Mommsen et al., 1999; Schreck and Tort, 2016). Acute exposure to dilbit in the present study resulted in a classical physiological stress response in juvenile sockeye; plasma cortisol, glucose and lactate concentrations in exposed fish were significantly elevated, with a consistent reduction in liver glycogen concentration. These results that are consistent with those found in coho salmon (Thomas and Rice, 1987), flathead grey mullet (*Mugil cephalus*) (Thomas et al., 1980), European flounder (*Pleuronectes flesus*) (Alkindi et al., 1996), and Pacific herring (Kennedy and Farrell, 2005, 2006, 2008) exposed to WSF of crude oil. The stress response following acute exposure is attributed to lighter volatile aromatic hydrocarbons (e.g. naphthalenes and BTEX) (Kennedy and Farrell, 2005; Thomas et al., 1997). As WSFs are generated from unweathered dilbit at the beginning of exposures, the proportions of naphthalenes, BTEX, and naphthenic acids are highest, resulting in the activation of the HPI axis and ensuing sequelae. Plasma cortisol, glucose, and lactate concentrations remained elevated, and hepatic glycogen concentrations did not return to baseline values as the exposure continued through 96 h. In other studies, these parameters typically return to baseline values by 24 h, suggesting that activation is transient due to the reduction in the relative abundance of light-weight-hydrocarbons as WSF generation from crude oil continues (Kennedy and Farrell, 2005; 2006; Thomas et al., 1980). In the present study, these compounds only gradually reduced from initial values; their relative contributions to TPAC may still able to stimulate the HPI axis at 96 h. Exposure to crude oils or PAHs has been shown to activate this response (Gesto et al., 2008; Kennedy and Farrell, 2005, 2008; Oliveira et al., 2007; Thomas et al., 1980; Thomas and Rice, 1987; Tintos et al., 2008), or to impair this response on a long term

basis (Girard et al., 1998; Hontela et al., 1992; Hontela, 1998; Kennedy and Farrell, 2005; 2008), which is maladaptive (Schreck and Tort, 2016).

While the neuroendocrine stress response to acute dilbit WSF exposure is consistent with existing literature, the observed response to chronic exposure was different than following exposure to crude oil. By d 21 of exposure, plasma cortisol and lactate concentrations, as well as liver glycogen content were still significantly altered in the highest treatment groups, whereas plasma glucose had returned to baseline values. Kennedy and Farrell (2005, 2008), however, reported that all stress related parameters returned to baseline values when lower molecular weight compounds were exhausted from the WSF, and that a stress response was only initiated if a new WSF was generated containing LMW hydrocarbons. Herring chronically exposed to pulsed WSF of crude oil showed a muted cortisol response and decreased elevations in serum lactate and glucose levels (Kennedy and Farrell, 2005, 2008). It was suggested that the muted stress response seen in chronic exposures may be the result of known components of oil that may act as endocrine disruptors acting on the pituitary or adrenocortical tissues, interfering the HPI axis hormonal signal transduction pathway (Kennedy and Farrell, 2005; 2008; Kennedy, 2014b). This is supported by field and laboratory studies (Aluru and Vijayan, 2006; Girard et al., 1998; Hontela et al., 1992, 1995; Reddam et al., 2017; Wilson et al., 1998) suggesting that chronic stress or direct action of chemicals on endocrine tissues may inhibit the stress response. In addition, certain PAHs are believed to interact with aryl hydrocarbon receptor (AhR) and subsequently alter the transcription of several genes (e.g. cyp1a), resulting in decreased circulating cortisol concentrations (Billiard et al., 2002, 2004). Clearly, dilbit exposed fish have a functional HPI signally pathway and the ability to mount a sustained stress response with subchronic exposure.

Decreased plasma glucose and hepatic glycogen concentrations in subchronically exposed fish may be a direct consequence of continuous energy substrate mobilization (Mommsen et al., 1999) during stress resulting in depleted energy reserves. Increased metabolic rates have been reported in teleosts exposed to crude oil (Davison et al., 1992; Klinger et al., 2015) or PAH (dos Santos et al., 2006) which may be attributed to several costs including compensating for stress-induced physiological alterations (e.g. altered hydromineral balance), costs of induced and increased

biotransformation rates of hydrocarbons, as well as increased energy needed for the repair chemically-induced damage (Whitehead, 2013). In addition, there is some evidence that exposure may inhibit the lactate gluconeogenesis pathway. The elevation in plasma lactate concentration following cortisol stimulation may serve as aerobic fuel to cope with the increased energy demands under chronic stress, and the increased circulating lactate is an important substrate for subsequent gluconeogenesis when it reaches hepatic tissues (Mommsen et al., 1998). Tintos et al. (2007) reported that fish exposed to naphthalene had decreased fructose 1, 6-bisphosphatase (FPBase) and glutamate dehydrogenase (GDH) activities, indicating a reduced gluconeogenic capacity. Since gluconeogenesis plays a central role in the metabolic adjustments in response to stress, disturbance to this pathway may significantly interfere with the ability to properly regulate stress responses.

Crude oil can cause osmoregulatory disturbances in teleosts (Duarte et al., 2010; Engelhardt et al., 1981; Kennedy and Farrell, 2005, 2006; 2008; Kennedy, 2014a; Matsuo et al., 2005; Whitehead, 2013; Zbanyszek and Smith, 1984). In the current study, a significant decrease in gill NKA activity was observed during both acute and subchronic dilbit exposures, however WSF-induced alterations in plasma osmolality and ion concentrations were seen at earlier time points than significant reductions in gill NKA activity. NKA is the primary enzyme that regulates the transport of Na+ and K+ across the plasma membrane of mitochondria-rich cells (MRCs) in teleost gills playing a central role in maintaining cellular homeostasis and the intracellular ionic gradients required for secondary transport of some metabolic substrates (McCormick et al., 2009). Although not fully understood, NKA is believed to be involved in Na+ uptake through teleost gills in freshwater (McCormick, 2011). Others have shown that exposure to petroleum hydrocarbons affects osmoregulatory performance by directly inhibiting gill NKA activity (Boese et al., 1982; Engelhardt et al., 1981; Gad, 2011; McCloskey Mccloskey and Oris, 1993) or causing decreased numbers of MRCs in the gill epithelium (Goanvec et al., 2011). The continuous high circulating plasma cortisol concentrations exhibited in dilbit-exposed fish may in part counteract the decreases in NKA activity in gills and loss of ions in freshwater by stimulating and increasing the surface area of MRCs (McCormick, 2011; Kennedy and Farrell, 2005). In freshwater-stage rainbow trout exposed to oil

emulsions, iono-osmoregulatory disturbances were reduced by cortisol treatments, highlighting the physiological role that cortisol plays in compensation for osmoregulatory dysfunction (Engelhardt et al. 1981). The observed disruptions in iono-osmoregulatory performance may also be attributed to alterations in gill epithelial cell membrane permeability and morphological changes to gill structural integrity (epithelial lifting, aneurysm, proliferation of mucocytes, hyperplasia, hypertrophy of lamellar epithelium and lamellar fusion in gill tissues of various teleosts [Akaishi et al., 2004; DiMichele and Taylor, 1978; Engelhardt et al., 1981; Kennedy and Farrell, 2005; Kochhann et al., 2015; Negreiros et al., 2011; Solangi and Overstreet, 1982]) (Englehardt et al., 1981), and altering the passive movement of monovalent ions (Kennedy and Farrell, 2005; Zbanyszek and Smith, 1984).

Hematological parameters can provide useful information regarding the status of an organism related to physiological performance. Fish exposed to petroleum hydrocarbons or WSF of oil (Alkindi et al., 1996; Jahanbakhshi et al., 2014; McCloskey and Oris, 1993; Simonato et al., 2008; Zbanyszek and Smith, 1984), have exhibited alterations in the number of circulating erythrocytes and hematocrit, however, few hematological changes were seen in the present study. Hemoglobin concentration increased with 96 h and 21 d exposure, along with corresponding changes in other calculated indices. This is likely related to the potential effects on the gills and resulting iono-osmoregulatory disturbances. Increased MCH and MCHC values could be attributed to reduced oxygen transfer across damaged gills. For example, European flounder exposed to the WSF of crude oil for 3 h showed a dramatic decrease in blood PO2 and significant increases in hemoglobin content (Alkindi et al., 1996) that was attributed to WSF-induced gill damage and compensatory responses of the organism.

For fish populations, a link between environmental contamination and disease has long been discussed (Bols et al., 2001; Sindermann, 1979; Snieszko, 1974). Research indicates that a wide range of chemicals compounds including petroleum hydrocarbons can alter components of the innate and acquired immune systems in teleosts, reducing their ability to defend against infectious pathogens (Anderson et al., 1995; Arkoosh et al., 1998; Bayha et al., 2017; Danion et al., 2011; Dubansky et al., 2013; Kennedy and Farrell, 2008; Meyers et al., 1994; Song et al., 2008; Song et al.,

2012). In the present study, subchronic, but not acute, exposure to dilbit increased the susceptibility of sockeye to *V. anguillarum*. Similar results from hydrocarbon exposure were seen in other fish species, with susceptibility being dependent on the duration of exposure and concentrations (Kennedy and Farrell, 2008; Ortuño et al., 2001). Recent evidence suggests that acute stress may enhance immunological responses, instead of resulting in immunosuppression (Demers and Bayne, 1997; Kennedy and Farrell, 2008; Ruis and Bayne, 1997). Enhancements of immunological performance under acute stress have been reported: increased plasma [cortisol] and lysozyme activity were reported in rainbow trout following acute stress, which was suggested to enhance innate immune defence (Demers and Bayne, 1997). Chinook salmon (*Oncorhynchus tshawytscha*) exposed to handing stress exhibited a higher disease resistance to *V. anguillarum*, despite a decreased antibody production (Maule et al., 1989). Although the stimulatory role of acute stress on the immunological performance is not fully understood, these studies may help explain why the acute exposure in the present study did not initiate immunological suppression. Similar increases in disease susceptibility were seen in other studies following subchronic or chronic exposures to hydrocarbons. For example, juvenile Pacific herring exposed to WSF of crude oil also showed a higher disease susceptibility to *V. anguillarum* under chronic exposure regimes (Kennedy and Farrell, 2008). Adult Pacific herring subchronically exposed to weathered crude oil, showed a positive correlation between tissue TPAH concentrations, the prevalence of viral hemorrhagic septicemia virus (VHSV), mortality and increasing oil concentrations (Carls et al., 1998). Juvenile southern flounder (*Paralichthys lethostigma*) exposed to oil-contaminated sediment for 7 d exhibited a higher post-*V. anguillarum* challenge mortality compared to controls (Bayha et al., 2017). Japanese halibut (*Paralichthys olivaceus*) exposed to heavy oil showed reduced antibacterial activity in plasma (Song et al., 2012) and higher mortality following challenge to viral hemorrhagic septicemia virus (Song et al., 2011).

Multiple mechanisms are likely involved in the enhanced susceptibility of fish to pathogens following exposure to hydrocarbon mixtures. PAHs can directly interact with components of the immune system (Kennedy and Farrell, 2008; Reynaud and Deschaux, 2006). For example, juvenile Pacific herring subchronically exposed to the

WSF of crude oil exhibited significantly increased macrophage respiratory burst activity (RBA) and decreased plasma lysozyme levels (Kennedy and Farrell, 2008). Japanese medaka injected intraperitoneally with the PAH benzo(a)pyrene (B[a]P) had a significantly reduced resistance to the bacterial pathogen Yersinia ruckeri, as well as suppressed lymphocyte proliferation, decreased antibody-forming cell (AFC numbers), and phagocyte-mediated O2− production (Carlson et al., 2002). European sea bass (*Dicentrarchus labrax*) injected intraperitoneally with B[a]P expressed suppressed phagocytosis activity and RBA (Lemaire-Gony et al., 1995). Similarly, oyster toadfish (*Opsanus tau*) intraperitoneally injected with 7,12-dimethylbenz[a]anthracene demonstrated depressed macrophage phagocytosis activity and abolished non-specific cytotoxic cell activity (Seeley and Weeks-Perkins, 1997). Alderman et al. (2017b) found alterations of the abundance of proteins which are associated with immune and inflammatory responses in juvenile sockeye exposed to WSF of dilbit, indicating an impairment on the immune/inflammatory response in fish.

The observed immunosuppression may also be attributed to continued activation of HPI axis leading to stress-induced suppression of the innate and adaptive immune systems increasing the risk of infectious disease (Kennedy and Farrell, 2008; Ruis and Bayne, 1997; Tort et al., 1996). There is abundant evidence that elevated cortisol levels can suppress various aspects of immune system, including reduced capacity of antibody synthesis in lymphocytes, leukocyte production and mobilization, leukocyte mitosis, and phagocytosis (Schreck and Tort, 2016; Yada and Tort, 2016), suppression in cytotoxic activity of peritoneal leukocytes, alterations in chemiluminescence, complement protein, and phagocytic activities in head kidney leukocytes and pronephros cells (Ortuño et al., 2001; Scott and Klessius, 1981; Vazzana et al., 2002; Yin et al., 1995), and reductions in the production of leukocytes (Yada and Tort, 2016).

In addition to specific effects of dilbit constituents on immune system components, and potential stress-related immunosuppression, potential alterations of gill structure and/or mucous lining could enhance the entry of pathogens; the integrity of skin and gills of teleosts are important as external barriers in this regard (Gomez et al., 2013). Baudin-Laurencin and Germon (1987) describe the gill as an important uptake route of V. anguillarum in rainbow trout. In *Paralichthys lethostigma* exposed to crude oil

and challenged with *V. anguillarum*, 75 % of the microbial community in the lower and upper gills was *V. anguillarum*, compared to the gills of non-exposed fish where the relative abundance of *V. anguillarum* was found to be negligible (Bayha et al., 2017). Paralichthys olivaceus exposed to heavy oil for 3–5 d exhibited increased bacteria concentration in the skin mucus (Song et al., 2008).

2.5. Conclusions

While the majority of dilbit toxicity studies have been centered on embryo/larval stages of fish, the present study exposed juvenile sockeye salmon to the WSF of dilbit at environmentally relevant concentrations (µg/L TPAC concentrations). The results here provide evidence that WSF exposure caused adverse effects on a variety of critical life-supporting physiological functions, including iono-osmoregulation, the stress response, and immune defense. This data supports a model for multiple adverse outcome pathways with sublethal effects that vary depending on the exposure time course to various constituents of a complex mixture. These findings are generally consistent with previous studies with conventional crude oils and their major hydrocarbon components. These adverse effects will result in reduced fitness, particularly in 1+ year old juveniles that are expected to perform a seaward migration raising long-term conservation concerns for these populations. Continued efforts are clearly required to yield the necessary information to understand the potential risks associated with the transport and accidental release of dilbit into salmon habitat, and to appropriately regulate the production, transport and use of this mixture.

2.6. CRediT authorship contribution statement

Feng Lin: Conceptualization, Methodology, Investigation, Writing - original draft. **Heather L. Osachoff**: Writing - review & editing. **Christopher J. Kennedy**: Supervision, Funding acquisition, Writing - original draft.

2.7. 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.

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Table 2. 1. Representative polycyclic aromatic compound (PAC) composition in experimental tanks supplied with the water-soluble fraction of diluted bitumen. Water samples were collected 12 h after initiating the exposure (0 d), and again at 12 and 25 d. Values are expressed as percent of total PAC. All 0 values indicate the component was below its reporting limit. Individual PACs are grouped by the number of aromatic rings (shading) and listed in increasing order of molecular weight.

PAC	Concentration											
	Control			Low			Medium			High		
	Exposure Time (d)											
	0	12	25	0	12	25	0	12	25	0	12	25
Naphthalene	13.26	14.09	14.94	7.95	4.74	3.68	7.34	5.17	2.15	7.95	3.75	2.95
C1-Naphthalenes	7.38	7.36	7.27	14.46	14.03	15.99	15.67	14.59	14.74	17.29	20.66	19.40
(1-Methylnaphthalene)	(2.70)	(2.69)	(2.66)	(6.23)	(5.31)	(7.09)	(7.43)	(6.16)	(6.39)	(8.16)	(9.99)	(8.83)
(2-Methylnaphthalene)	(4.68)	(4.67)	(4.61)	(8.23)	(8.72)	(8.90)	(8.24)	(8.43)	(8.35)	(9.13)	(10.67)	(10.57)
C2-Naphthalenes	5.04	2.69	4.96	21.68	17.63	18.31	20	19.72	18.16	19.77	20.28	18.83
(1,2-Dimethylnaphthalene)	(0.45)	(0.37)	(0.30)	(2.43)	(2.08)	(1.75)	(1.75)	(2.12)	(2.06)	(0.92)	(0.96)	(0.78)
(2,6-Dimethylnaphthalene)	(1.30)	(0.90)	(1.25)	(5.94)	(5.06)	(4.29)	(4.26)	(5.16)	(5.02)	(2.14)	(2.23)	(2.19)
C3-Naphthalenes	5.80	5.58	5.51	18.79	16.31	16.43	13.43	13.32	13.37	18.70	22.03	18.51
(2,3,5-Trimethylnaphthalene)	(0.99)	(0.98)	(0.97)	(4.04)	(3.85)	(2.92)	(3.24)	(3.92)	(3.81)	(1.80)	(2.76)	(3.43)
(2,3,6-Trimethylnaphthalene)	(2.00)	(1.99)	(0.98)	(5.31)	(5.02)	(5.84)	(4.23)	(4.88)	(4.98)	(3.58)	(4.28)	(3.86)
C4-Naphthalenes	1.61	1.61	1.58	5.69	6.27	7.39	3.81	4.12	4.03	3.65	4.06	3.60
(1,4,6,7-Tetramethylnaphthalene)	(0.24)	(0.26)	(0.21)	(0.95)	(0.87)	(0.68)	(0.74)	(0.89)	(0.87)	(0.40)	(0.42)	(0)
Biphenyl	2.54	2.53	2.50	1.04	0.99	0.75	0.84	1.01	0.99	0.43	0.44	0.36
C1-Biphenyls	20.53	20.47	20.21	0.58	0.55	0.42	0.46	0.56	0.54	0.22	0.23	0.19
C2-Biphenyls	3.46	3.34	3.30	0.32	0.30	0.23	0.26	0.31	0.30	0.14	0.15	0.12
Acenaphthylene	0.42	0.45	0.45	0.01	0.01	0	0	0.01	0.01	0	0	0
Acenaphthene	0.49	0.66	0.66	1.35	1.33	0.98	1.12	1.21	1.31	0.53	0.55	0.45
C1-Acenaphthenes	0	0	0	0.23	0.21	0.17	0.18	0.21	0.21	0.09	0.10	0.08
Fluorene	0.58	0.55	0.54	1.55	1.52	1.12	1.28	1.55	1.51	0.66	0.69	0.56
C1-Fluorenes	7.24	9.52	7.13	2.72	2.49	1.97	2.09	2.37	2.47	1.24	1.17	0.96
(2-Methylfluorene)	(0)	(0)	(0.37)	(0.40)	(0.37)	(0.29)	(0.31)	(0.38)	(0.37)	(0.16)	(0.17)	(0.14)
C2-Fluorenes	4.83	4.56	4.75	2.37	2.18	1.71	1.83	2.22	2.16	1.07	1.00	0.82
(1,7-Dimethylfluorene)	(0)	(0)	(0)	(0.19)	(0.17)	(0.14)	(0.14)	(0.18)	(0.17)	(0.08)	(0.08)	(0.07)
C3-Fluorenes	3.33	3.41	3.28	1.19	2.39	0.86	2.01	2.26	2.37	3.48	2.71	2.83
Dibenzothiophene	0.30	0.29	0.29	1.79	1.69	1.29	1.42	1.72	1.67	0.73	0.76	0.62
C1-Dibenzothiophenes	0	0	0	3.32	3.08	2.40	2.60	2.49	3.06	1.37	1.42	1.16
(2/3-Methyldibenzothiophenes)	(0)	(0)	(0)	(1.05)	(0.98)	(0.76)	(0.83)	(1.00)	(0.97)	(0.44)	(0.45)	(0.37)
C2-Dibenzothiophenes	1.17	1.17	1.16	2.99	2.71	4.63	2.28	2.68	2.68	1.32	1.31	1.07
(2,4-Dimethyldibenzothiophene)	(0)	(0)	(0)	(0.25)	(0.22)	(0.18)	(0.19)	(0.23)	(0.22)	(0.10)	(0.10)	(0.08)
C3-Dibenzothiophenes	1.24	1.23	1.22	1.37	1.13	0.99	0.95	1.15	1.12	0.54	0.56	0.46
C4-Dibenzothiophenes	6.79	6.55	6.47	0.46	8.96	13.65	12.76	12.87	15.78	15.10	11.92	21.98
Phenanthrene	2.23	2.35	2.32	2.53	2.67	1.83	2.24	2.62	2.64	1.15	1.17	0.96
C1 Phenanthrenes/Anthracenes	1.38	1.48	1.46	3.27	3.54	2.36	2.98	2.44	3.51	1.42	1.47	1.20
(1-Methylphenanthrene)	(0.30)	(0.32)	(0.26)	(0)	(0.66)	(0)	(0.56)	(0.57)	(0.65)	(0.29)	(0.30)	(0.24)
(2-Methylphenanthrene)	(0.26)	(0.25)	(0.25)	(0)	(0.69)	(0)	(0.58)	(0.60)	(0.68)	(0.30)	(0.31)	(0.15)
(3-Methylphenanthrene)	(0.35)	(0.24)	(0.24)	(0)	(0.68)	(0)	(0.57)	(0.59)	(0.67)	(0.29)	(0.31)	(0.25)
(9/4-Methylphenanthrene)	(0.26)	(0.27)	(0.24)	(0)	(1.16)	(0)	(0.98)	(0.10)	(1.15)	(0.30)	(0.22)	(0.22)
(2-Methylanthracene)	(0.21)	(0.40)	(0.47)	(0)	(0.35)	(0)	(0.59)	(0.08)	(0.36)	(0.24)	(0.33)	(0.34)
C2 Phenanthrenes/Anthracenes	1.56	1.28	1.27	1.45	1.67	1.04	1.41	1.70	1.65	0.70	0.73	0.59
(1,7-Dimethylphenanthrene)	(0.17)	(0.17)	(0.17)	(0)	(0.18)	(0)	(0.15)	(0.19)	(0.18)	(0.07)	(0.08)	(0.06)
(1,8-Dimethylphenanthrene)	(0)	(0)	(0)	(0)	(0.06)	(0)	(0.05)	(0.06)	(0.06)	(0.03)	(0.03)	(0.02)
(2,6-Dimethylphenanthrene)	(0)	(0)	(0)	(0)	(0.09)	(0)	(0.07)	(0.09)	(0.09)	(0.04)	(0.05)	(0.04)
(3,6-Dimethylphenanthrene)	(0)	(0)	(0)	(0)	(0.11)	(0)	(0.09)	(0.11)	(0.11)	(0.05)	(0.05)	(0.04)
C3-Phenanthrenes/Anthracenes	0.73	0.73	0.72	0.75	0.61	0.54	0.52	0.63	0.61	0.29	0.30	0.25

(1,2,6-Trimethylphenanthrene)	(0)	(0)	(0)	(0)	(0.03)	(0)	(0.03)	(0.03)	(0.03)	(0.02)	(0.02)	(0.01)
C4-Phenanthrenes/Anthracenes	3.57	3.57	3.60	0.80	1.31	0.57	1.10	1.34	1.30	1.14	1.19	0.97
Anthracene	0.08	0.10	0.10	0.10	0.10	0.07	0.09	0.11	0.10	0.05	0.05	0.04
Benz[a]anthracene	0	0	0	0.01	0.01	0.01	0.01	0.01	0.01	0	0	0
Dibenz[a,h]anthracene	0	0	0	0	0	0	0	0	0	0	0	0
Fluoranthene	0.68	0.75	0.74	0.04	0.05	0.03	0.04	0.05	0.05	0.02	0.02	0.02
Pyrene	1.64	1.53	1.51	0.06	0.06	0.04	0.05	0.06	0.06	0.03	0.03	0.02
C1-Fluoranthenes/Pyrenes	0.69	0.69	0.68	0.37	0.35	0.27	0.29	0.35	0.35	0.18	0.19	0.15
(3-Methylfluoranthene/Benzo[a]fluorine)	(0)	(0)	(0)	(0)	(0.16)	(0)	(0.14)	(0.17)	(0.16)	(0.09)	(0.09)	(0.08)
C2-Fluoranthenes/Pyrenes	0.98	0.98	0.97	0.31	0.25	0.23	0.21	0.26	0.25	0.12	0.13	0.10
C3-Fluoranthenes/Pyrenes	0	0	0	0	0.09	0	0.08	0.10	0.09	0.06	0.06	0.05
C4-Fluoranthenes/Pyrenes	0	0	0	0	0.03	0	0.02	0.03	0.03	0.01	0.02	0.01
Chrysene	0.16	0.16	0.15	0.03	0.03	0.02	0.03	0.03	0.03	0.02	0.02	0.01
C1-Benzo[a]anthracenes/Chrysenes	0	0	0	0	0.04	0	0.03	0.04	0.04	0.02	0.02	0.01
(1-Methylchrysene)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
(5/6-Methylchrysene)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
C2-Benzo[a]anthracenes/Chrysenes	0	0	0	0	0.03	0	0.02	0.03	0.03	0.02	0.02	0.01
(5,9-Dimethylchrysene)	(0)	(0)	(0)	(0)	(0.01)	(0)	(0.01)	(0.01)	(0.01)	(0)	(0)	(0)
C3-Benzo[a]anthracenes/Chrysenes	0	0	0	0	0.01	0	0.01	0.01	0.01	0.01	0.01	0
C4-Benzo[a]anthracenes/Chrysenes	0	0	0	0	0	0	0	0	0	0	0	0
Benzo[b]fluoranthene	0	0	0	0	0.01	0	0.01	0.01	0.01	0	0	0
Benzo[j,k]fluoranthenes	0	0	0	0	0.01	0	0	0.01	0	0	0	0
C1-Benzofluoranthenes/Benzopyrenes	0	0	0	0	0.01	0	0.01	0.01	0.01	0.01	0.01	0
C2-Benzofluoranthenes/Benzopyrenes	0.29	0.29	0.29	0.01	0	0.01	0	0	0	0.01	0.01	0.01
Benzo[a]pyrene	0	0	0	0	0	0	0	0	0	0	0	0
Benzo[e]pyrene	0	0	0	0	0.01	0	0.01	0.01	0.01	0	0	0
7-Methylbenzo[a]pyrene	0	0	0	0	0	0	0	0	0	0	0	0
Indeno[1,2,3-cd]pyrene	0	0	0	0	0.01	0	0.01	0.01	0.01	0	0	0
Perylene	0	0	0	0.01	0	0.01	0	0	0	0	0	0
Benzo[ghi]perylene	0	0	0	0	0.01	0	0.01	0.01	0.01	0	0	0
Retene	0	0	0	0	0.59	0	0.50	0.60	0.58	0.76	0.79	0.64

Table 2. 2. Whole blood mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) per cell, and mean corpuscular hemoglobin concentration (MCHC) in control fish and fish exposed to WSF of Cold Lake Blend dilbit (winter) for 24 h, 96 h or 21 d. The initial spike of TPAHs concentrations for each WSF generated in current study were 0 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Time of exposure is given as hours (h) and days (d). Data from four replicate exposure tanks were pooled, and no tank effect was detected ($p > 0.05$). Two-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations and exposure length. Values that do not share a common letter are statistically different ($p<0.05$), n = 18 for Low-21 d exposure group, the rest of groups all had n = 20.

Treatment	MCV (pL/cell)	MCH (pg)	MCHC (g/dL)	N
[24 h]				
Control	327.3 ± 16.1 ^{a}	73.7 ± 4.0 ^{a}	23.3 ± 1.4 ^{a}	20
Low	337.4 ± 16.4 ^{a}	78.8 ± 5.9 ^{a}	23.5 ± 1.4 ^{a}	20
Medium	332.1 ± 15.6 ^{a}	79.7 ± 5.7 ^{a}	24.3 ± 1.7 ^{a}	20
High	377.7 ± 10.5 ^{a}	97.0 ± 7.3 ^{a}	25.7 ± 1.8 ^{a}	20
[96 h]				
Control	278.3 ± 12.9 ^{a}	56.4 ± 3.6 ^{a}	20.4 ± 0.9 ^{c}	20
Low	269.3 ± 16.5 ^{a}	66.6 ± 6.0 ^{a}	24.2 ± 1.2 ^{bc}	20
Medium	276.7 ± 15.6 ^{a}	73.7 ± 5.0 ^{a}	26.6 ± 0.9 ^{ab}	20
High	256.2 ± 14.5 ^{a}	74.6 ± 5.7 ^{a}	29.1 ± 1.4 ^{a}	19
[21 d]				
Control	307.0 ± 14.2 ^{a}	58.7 ± 3.6 ^{b}	19.1 ± 0.8 ^{c}	20
Low	295.0 ± 19.0 ^{ab}	67.8 ± 6.5 ^{ab}	23.3 ± 1.6 ^{bc}	18
Medium	242.1 ± 10.7 ^{bc}	63.6 ± 3.2 ^{ab}	26.5 ± 1.1 ^{ab}	20
High	239.8 ± 9.1 ^{bc}	75.2 ± 4.4 ^{a}	31.5 ± 1.5 ^{a}	20

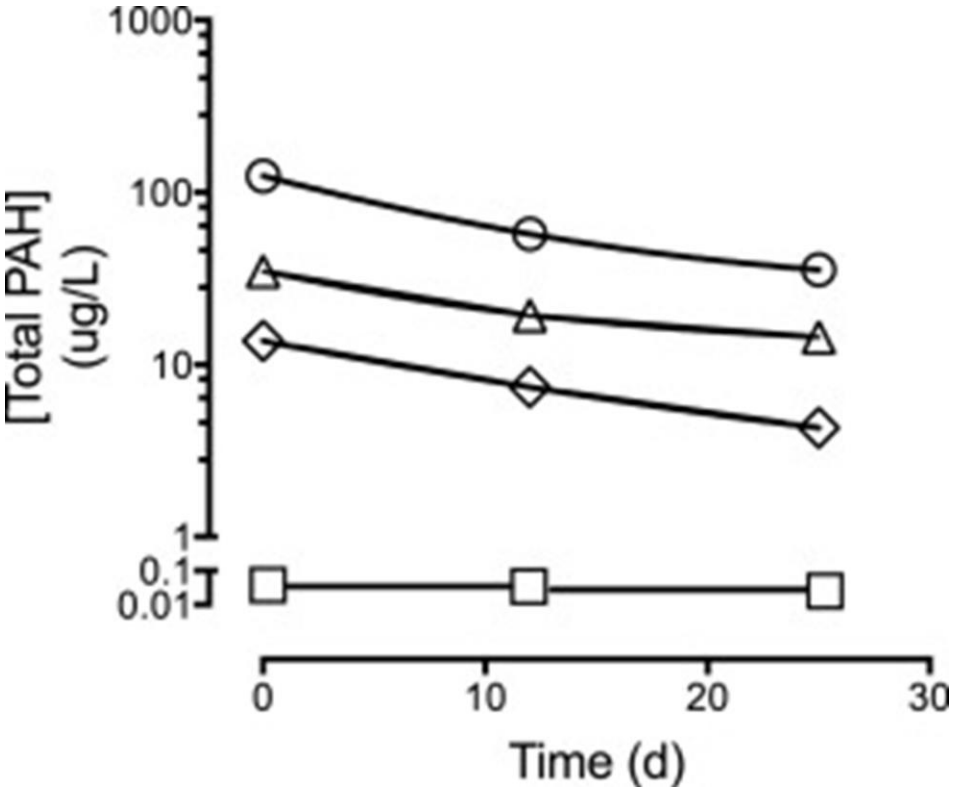

Figure 2. 1. Total polycyclic aromatic compound (TPAC) concentrations in treatment tanks as a function of time after initiation of water flow through a WSF column. Data points are single composite values for replicate tanks at each time period. Control (□), 13.7 µg/L (◇), 34.7 µg/L (△), 124 µg/L (○).

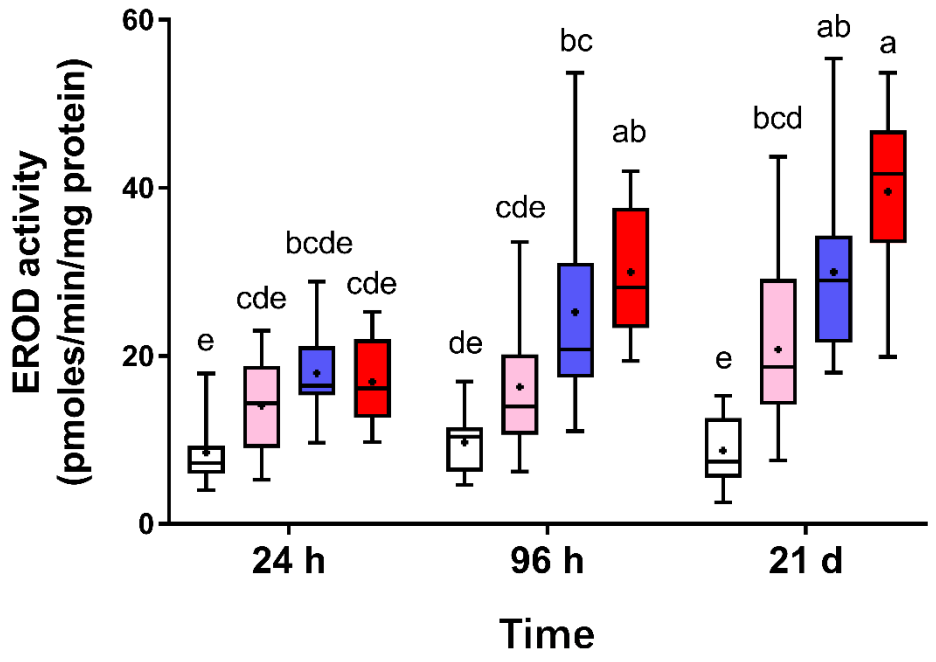

Figure 2. 2. Box-and-whisker plots of liver ethoxyresorufin-O-deethylase (EROD) activity in control fish and fish exposed to WSFs of Cold Lake Blend dilbit (winter) for 24 h, 96 h or 21 d. The initial spike of TPAHs concentrations for each WSF generated in current study were 0 (Control □), 13.7 (Low ▭), 34.7 (Medium ▭) and 124.5 (High ▭) µg/L. Time of exposure is given as hours (h) and days (d). Data from four replicate exposure tanks were pooled, and no tank effect was detected ($p > 0.05$). Two-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations and exposure length. Within each plot, ✚ indicates means, boxes that do not share a common letter are statistically different ($p<0.05$), n = 18 for Low-21 d exposure group, the rest of groups all had n = 20.

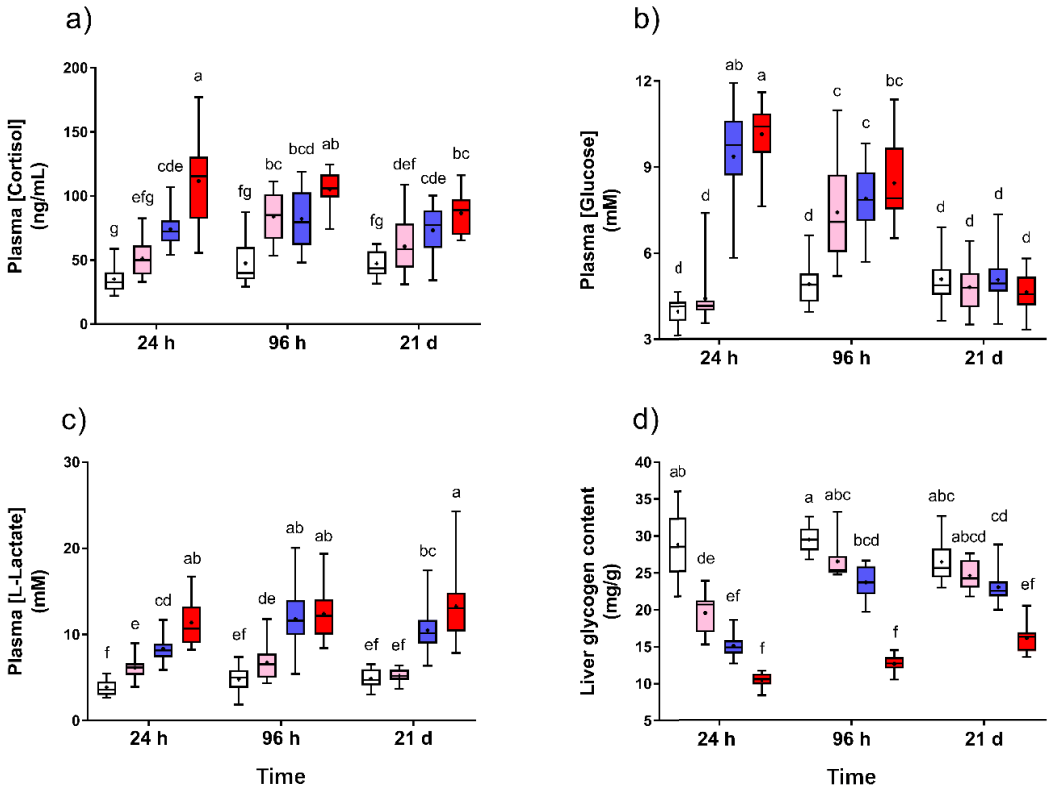

Figure 2. 3. Box-and-whisker plots of plasma cortisol (a), glucose (b), lactate concentrations (c) and liver glycogen content (d) in control fish and fish exposed to WSF of Cold Lake Blend dilbit (winter) for 24 h, 96 h or 21 d. The initial spike of TPAHs concentrations for each WSFs generated in current study were 0 (Control □), 13.7 (Low ▢), 34.7 (Medium ▢) and 124.5 (High ▢) µg/L. Time of exposure is given as hours (h) and days (d). Data from four replicate exposure tanks were pooled, and no tank effect was detected ($p > 0.05$). Two-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations and exposure length. Within each plot, ✚ indicates means, boxes that do not share a common letter are statistically different ($p<0.05$), n = 18 for Low-21 d exposure group, the rest of groups all had n = 20. n = 10 for liver glycogen content in all groups.

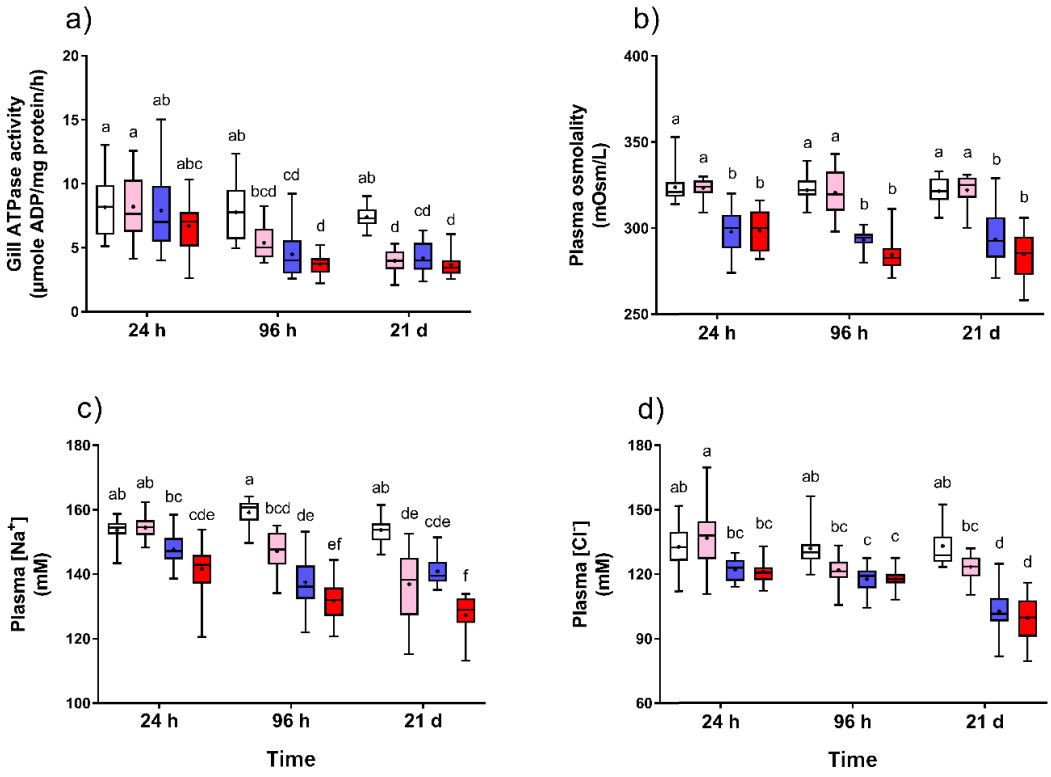

Figure 2. 4. Box-and-whisker plots of gill Na^+-K^+ ATPase activity (a), plasma osmolality (b), plasma Na^+ (c) and Cl^- (d) concentrations in control fish and fish exposed to WSF of Cold Lake Blend dilbit (winter) for 24 h, 96 h or 21 d. The initial spike of TPAHs concentrations for each WSFs generated in current study were 0 (Control □), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Time of exposure is given as hours (h) and days (d). Data from four replicate exposure tanks were pooled, and no tank effect was detected ($p > 0.05$). Two-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations and exposure length. Within each plot, ✚ indicates means, boxes that do not share a common letter are statistically different ($p<0.05$), n = 18 for Low-21 d exposure group, the rest of groups all had n = 20.

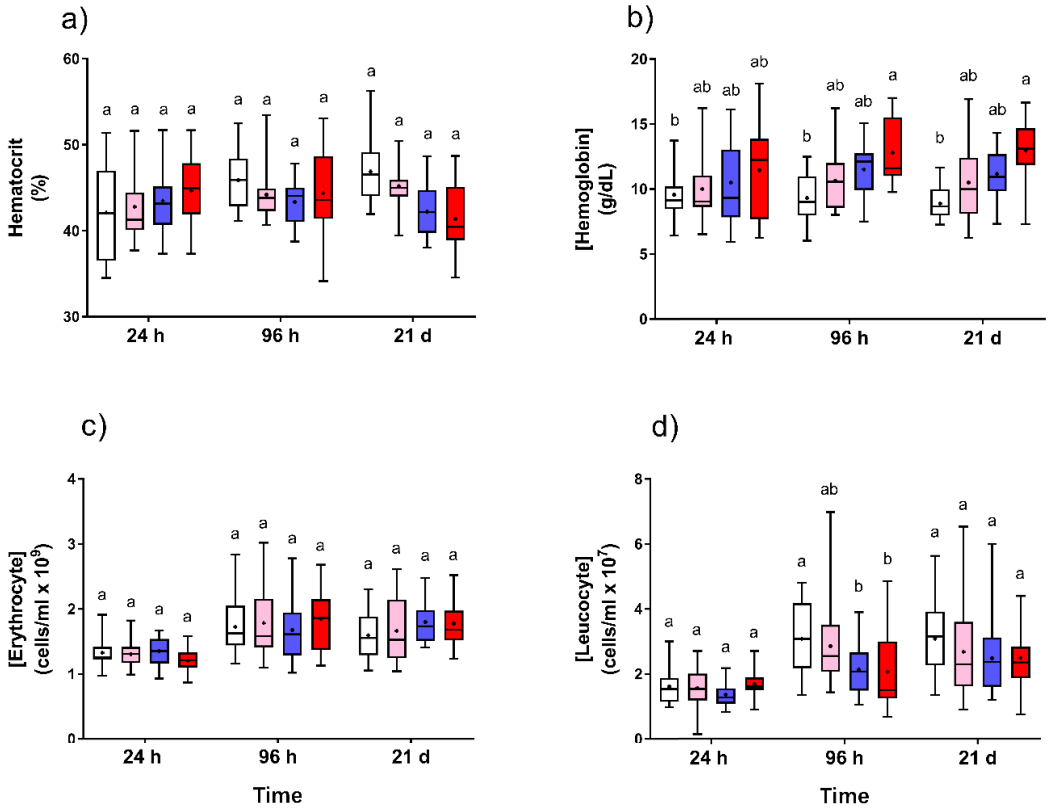

Figure 2. 5. Box-and-whisker plots of hematocrit (a), whole blood hemoglobin (b), erythrocyte (c), and leucocyte (d) concentrations in control fish and fish exposed to WSF of Cold Lake Blend dilbit (winter) for 24 h, 96 h or 21 d. The initial spike of TPAHs concentrations for each WSFs generated in current study were 0 (Control □), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Time of exposure is given as hours (h) and days (d). Data from four replicate exposure tanks were pooled, and no tank effect was detected ($p > 0.05$). Two-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations and exposure length. Within each plot, ✚ indicates means, boxes that do not share a common letter are statistically different ($p<0.05$), n = 18 for Low-21 d exposure group, the rest of groups all had n = 20.

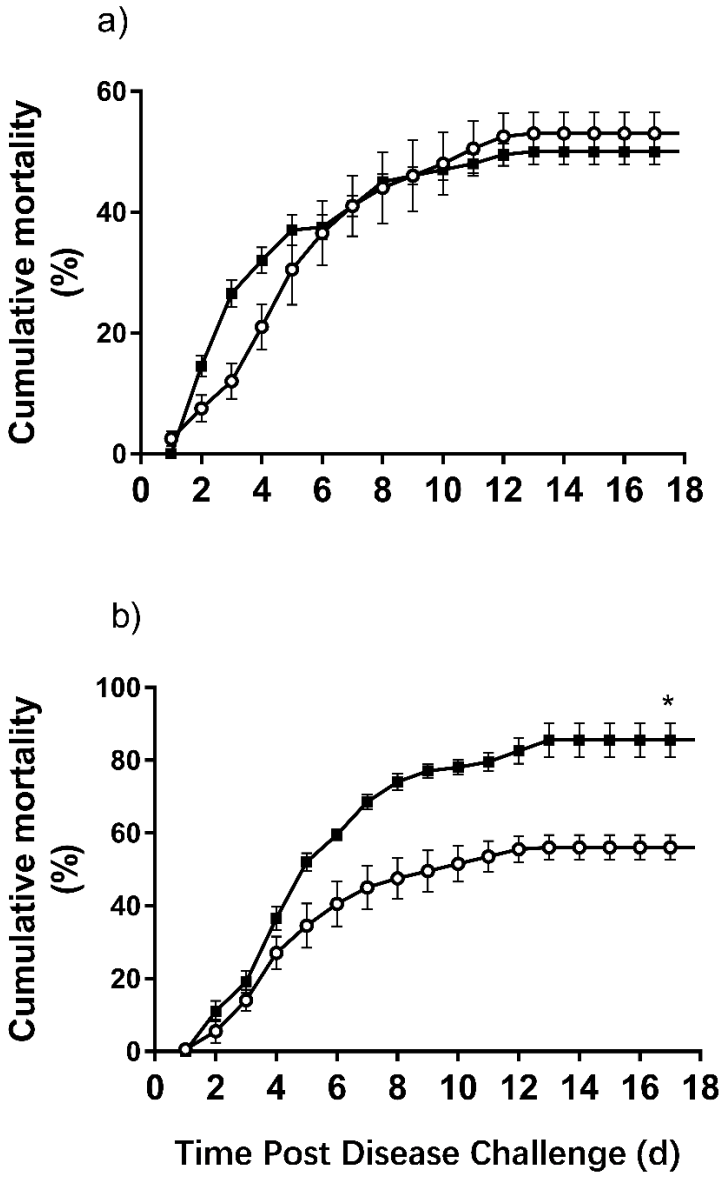

Figure 2. 6. Cumulative mortality post disease challenge with *Vibrio* (*Listonella*) *anguillarum* in control fish and fish exposed to WSF of Cold Lake Blend dilbit (winter) for 24 h (a) or 42 d (b). The initial spike of TPAHs concentrations for each WSF generated in current study were 0 (Control ○) and 124.5 (Exposed ■) µg/L. Data from four replicate communal tanks were pooled, and no tank effect was detected ($p > 0.05$). Values are means ± S.E. of four replicate communal tanks. Each communal tank contained n = 50 control fish and n = 50 exposed fish during the disease challenge and at the beginning of post-challenge monitoring period. Chi-squared test was used to exam the effect of WSF exposure, an asterisk indicates a statistical difference from the control ($p < 0.05$).

Chapter 3. Developmental and latent effects of diluted bitumen exposure on early life stages of sockeye salmon (*Oncorhynchus nerka*)

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Abstract

The early life stages of Pacific salmon are at risk of environmental exposure to diluted bitumen (dilbit) as Canada's oil sands industry continues to expand. The toxicity and latent effects of dilbit exposure were assessed in sockeye salmon (*Oncorhynchus nerka*) exposed to water-soluble fractions of dilbit (WSFd) from fertilization to the swim-up stage, and then reared in clean water for 8 months. Mortality was significantly higher in WSFd exposed embryos, with cumulative mortality up to 4.6-fold higher in exposed relative to unexposed embryos. The sublethal effects of WSF exposure included transcriptional up-regulation of CYP1A, a concentration-dependent delay in the onset and progression of hatching, as well as increased prevalence of developmental deformities at total polycyclic aromatic hydrocarbon (TPAH) concentrations ≥35 μg L^{-1}. Growth and body composition were negatively affected by WSFd exposure, including a concentration-specific decrease in soluble protein concentration and increases in total body lipid and triglyceride concentrations. Mortality continued during the first 2 months after transferring fish to clean water, reaching 53% in fish exposed to 100 μg L^{-1} TPAH; but there was no latent impact on swimming performance, heart mass, or heart morphology in surviving fish after 8 months. A latent effect of WSFd exposure on brain morphology was observed, with fish exposed to 4 μg L^{-1} TPAH having significantly larger brains compared to other treatment groups after 8 months in clean water. This study provides comprehensive data on the acute, subchronic, and latent impacts of dilbit exposure in early life stage sockeye, information that is critical for a proper risk analysis of the impact of a dilbit spill on this socioeconomically important fish species.

Keywords: Bitumen; crude oil; fish development; toxicity; morphogenesis

3.1. Introduction

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

Recent laboratory studies in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias Latipes*) reported a range of concentration-dependent biological effects in embryonic fish exposed to water-accommodated fractions (WAF) of dilbit. These effects included malformations of the heart and skeleton; failure of swim bladder inflation; genomic responses in pathways related to upregulation of phase I and II biotransformation, oxidative and cellular stress responses, and tumorigenesis; as well as increased mortality and altered behavior (Madison et al., 2017, 2015; Philibert et al., 2016). These responses parallel biological effects observed in ELS fathead minnow (*Pimephales promelas*) and white sucker (*Catostomus commersoni*) exposed to natural bituminous sediments (Colavecchia et al., 2006, 2004), and are generally consistent with

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

Pacific salmon hold considerable socioeconomic and environmental value to Canadians (Fisheries and Oceans Canada, 2018), and many populations are at risk of dilbit exposure due to pipeline routes that bring bitumen products to sea terminals on the Pacific coast of North America (Levy, 2009). For example, the expansion of the existing Trans Mountain pipeline will triple the volume of dilbit moving between Edmonton, AB and Burnaby, BC to about 900,000 b/d. This pipeline corridor extends through the lower section of Canada's largest salmon-bearing river system, the Fraser River Watershed (FRW), which supports millions of early life stage (ELS) salmon including sockeye (*Oncorhynchus nerka*). Importantly, these ELS are especially sensitive to anthropogenic pollution (Cohen, 2012) and ELS survival prior to seawater migration is critical to overall population viability (Henderson and Graham, 1991). Some 40% of sockeye populations currently monitored by the Canadian government are listed as stocks of conservation concern (Fisheries and Oceans Canada, 2018), including those that rely on habitat in the

lower FRW. Recently, we showed that juvenile sockeye exposed to low, environmentally relevant concentrations of the water soluble fraction of dilbit (WSFd) for 4 wk experience considerable changes in the serum proteome consistent with WSFd-induced tissue damage (Alderman et al., 2017a), as well as altered aerobic swimming performance, cardiac gene expression and cardiac histology (Alderman et al., 2017b). However, the sensitivity of developing sockeye to WSFd remains unknown, as does the nature and persistence of biological effects. Therefore, the present study was conducted to determine the immediate and latent impact of dilbit exposure on ELS sockeye.

3.2. Materials and Methods

3.2.1. Fish

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

3.2.2. Diluted bitumen exposure

Water-soluble fractions of dilbit (WSFd) were generated as previously described (Alderman et al., 2017b; Kennedy and Farrell, 2005) using Cold Lake Summer Blend dilbit. Four concentrations (in duplicate) of total polycyclic aromatic hydrocarbon (TPAH)

were achieved by varying the quantity of dilbit-soaked Siproax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) in PVC generator columns (15 cm diameter, 80 cm length). Ceramic beads were re-coated with dilbit every 14 d, and control columns contained only clean beads. Target TPAH concentrations were chosen to span a nominal range of 3 orders of magnitude, with a maximum concentration reflecting reported values at shoreline sites in the Gulf of Mexico after the Deepwater Horizon oil spill in 2010 (Allan et al., 2012). Dechlorinated municipal water flowed up through the columns and into one of 4 header tanks, and then was piped to Heath trays using submersible pumps. This set-up enabled duplicate exposures, which were applied from 2 d post fertilization (2 dpf) through to swim-up (Figure 3.1). Water samples were collected 6 h after initiating the exposure (0 d), and again at 7 d and 14 d for quantification of individual PAH concentrations by GC–MS (SGS Axys Analytical Services Ltd., Sidney, BC) as previously described (Alderman et al., 2017b).

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

Dead embryos were counted and carefully removed daily under red lighting. Hatching was quantified daily from 45 to 73 dpf, when hatch was considered 100% complete (no further eggs hatched for 5 d). Morphometric and deformity analyses were carried out at 76 dpf. A random selection of viable alevins (n=50 per Heath tray) was euthanized with MS-222, blotted dry, then weights and lengths were recorded. Development was scored based on staging series for developing salmonids (Velsen, 1980; Vernier, 1969). Teratogenesis was scored for frequency and severity on a scale of 0 (normal) to 3 (severely deformed) as previously described (Rudolph et al., 2008); deformities included craniofacial, skeletal, and fin deformities, as well as edema of the yolk sac and/or pericardial space. The mean frequency and severity was calculated for each tank and treatment by averaging incidence and scores, respectively.

3.2.4. Body composition analysis

Following deformity analysis, a subset of randomly selected alevins was weighed, snap frozen whole on dry ice and stored at −80 °C until body composition analysis (n=60 per treatment). For protein quantification, alevins were thawed on ice

(n=20 per treatment) and homogenized in 1 mL assay buffer (500mM Tris−HCl, 100mM DTT, 5.5mM EDT; pH 7.4) containing 1x protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO) using a Mixer Mill homogenizer. The crude homogenates were sonicated, centrifuged at 12,000 g for 15 min at 4 °C, and then protein content of the supernatant was measured in duplicate using a Bradford Protein Assay Kit and bovine serum albumin standards (Catalog# 5000002; Bio-Rad, Mississauga, ON). Whole body lipid content was quantified after drying alevins to constant weight at 65 °C (n=20 per treatment), using a standard chloroform/methanol extraction protocol exactly as previously described (Johnston et al., 2013). To quantify whole body triglyceride content, alevins were thawed on ice (n=20 per treatment) and homogenized in 1 mL of assay reagent (Catalog# 70024; Cayman Chemical Company, Ann Arbor, MI) containing 1mM EDTA as above. The crude homogenates were sonicated, centrifuged at 10,000 *g* for 10 min at 4 °C, and then triglyceride content of the supernatant was quantified in duplicate using a commercial colorimetric assay kit (Catalog# 10010303; Cayman). Data are expressed relative to original wet weight of the alevin.

3.2.5. Gene expression

A subset of randomly selected alevins was bisected into head and tail regions at the rostral boundary of the yolk sac and perpendicular to the body axis. The head regions were snap frozen on dry ice (n=8 per treatment group) and stored at −80 °C. Total RNA was extracted with Trizol (Life Technologies, Grand Island, NY) following manufacturer's instructions. RNA integrity was verified by gel electrophoresis then purity and quantity were measured using a NanoDrop 2000. After DNase I treatment (Life Technologies), 500 ng total RNA was used to synthesize cDNA (High Capacity cDNA Kit, Life Technologies) according to manufacturer's instructions. Transcript abundances of *cyp1a*, *ahr*, and ribosomal protein L8 (r*pL8*) were quantified in duplicate RTqPCR reactions, exactly as previously described and including all appropriate control reactions (Alderman et al., 2017b). Data were normalized to the abundance of the stably expressed reference gene, *rpL8*, and are shown as fold-change from control.

3.2.6. Swimming tests

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

3.2.7. Brain morphometrics

Brain morphometric analyses were conducted in surviving juveniles after rearing for 8 months in clean water (average mass 47.9 ± 0.9 g; average fork length 16.0 ± 0.1 cm; n=10–21 per treatment, 73 fish total). Following euthanasia, decapitated fish heads were affixed with plastic numbered tags and preserved in a large volume of neutral buffered formalin for long-term storage. Brains were removed, blotted dry and weighed, then digitally photographed using a Nikon D90 equipped with a Tamron 90mm Macro lens. To orient the brains and ensure symmetrical imaging, brains were gently placed in

depressions made in agarose-filled petri dishes. A ruler was included in each photo for calibration, and each brain was imaged in dorsal, ventral, and left lateral views. Regional volumes (V) were estimated using previously described methods (Edmunds et al., 2016a, 2016b; Gonzalez-Voyer and Kolm, 2010). Briefly, maximum length (l), width (w), and height (h) to the nearest tenth mm were measured in ImageJ for each of 5 discrete brain regions (olfactory bulbs, telencephalon, optic tectum, cerebellum, hypothalamus) and used in the formula for ellipsoid volume:

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

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

3.2.8. Heart morphometrics

Excised hearts from exhausted fish were passively cleared of blood in physiological saline, and digitally photographed as described above (Section 3.2.7). Hearts were then blotted dry and weighed whole and with the ventricle isolated to determine relative ventricular mass (RVM). Digital images were analyzed in ImageJ to determine maximal length and width (Incardona et al., 2015), and normalized to individual fish fork length. Aspect ratios for each heart were calculated as length/ width.

3.2.9. Statistics

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

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

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

3.3. Results

3.3.1. Water chemistry

Aqueous total PAH concentrations (sum of 50 PAH) for each concentration at the beginning of the exposures (0 d) were: Control =<0.1 μg L^{-1}, Low=4 μg L^{-1}, Medium=35 μg L^{-1}, and High=100 μg L^{-1}. With the exception of Control, TPAH declined with time and at a similar rate across concentrations during the 14 d between column recharges, so distinct treatments were maintained throughout the exposure period (see Alderman et al., 2017b).

3.3.2. Developmental effects

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

Hatching was initiated at 47 dpf in controls, but was delayed 1 to 3 d in Heath trays supplied with water containing WSFd (Figure 3.2B). Although hatching progressed in all treatments (significant main effect hatching level, $p < 0.001$; Figure 3.2C), the concentration-dependent effect of WSFd on hatching time persisted throughout the hatching period (significant main effect concentration, $p < 0.001$; Figure 3.2C). For example, time to 50% hatch (54 dpf in controls) was 3 to 4 d later in WSFd-exposed embryos. By 27 d after hatching began, 97–98% of eggs had hatched in the 0, 4, and 35 μg L^{-1} treatment groups, whereas only 92% of eggs had hatched in the 100 μg L^{-1} TPAH treatment (Figure 3.2B); unhatched eggs at this time were deemed non-viable. Thus, the combined influences of mortality and failed hatching increased overall embryo loss from 4.7% in Control (0 μg L^{-1} TPAH) to 15.2% in the 100 μg L^{-1} TPAH treatment. There was no effect of WSFd concentration on developmental stage of alevins at 76 dpf, with average stage across treatments being 33 (Table 3.1).

Teratogenesis was quantified as the frequency and severity of deformities in viable alevins at the end of the WSFd exposure, but not in embryos prior to hatch; the contribution of WSFd-induced malformations to observed mortalities was not evaluated. The frequency of developmental deformities was higher in WSFd-exposed alevins compared to controls, reaching approximately 10-fold increased frequency over controls in the highest concentration groups (17–20% incidence in 35 μg L^{-1} and 100 μg L^{-1}; Table 3.1). The most common deformity observed in all treatments was edema, primarily around the yolk sac, followed by craniofacial defects. Skeletal and finfold deformities were rare, with only one observation with WSFd (Table 3.1). With few exceptions, observed deformities were scored as 1 (minor), resulting in average severity scores of<1 across all treatments (Table 3.1).

3.3.3. Biochemical effects

At the end of the hatching period, differences in alevin mass and length between WSFd concentrations were observed (Table 3.1). Alevins exposed to 35 μg L^{-1} TPAH during embryogenesis were significantly heavier than all other treatments, and alevins exposed to 100 μg L^{-1} TPAH were significantly shorter in length than controls. There was a concentration-dependent effect of WSFd on the body composition of alevins

measured at the end of the exposure period (76 dpf). Soluble body protein content was negatively impacted by WSFd exposure, with the two highest exposure groups having 1.4-fold lower protein concentrations than control fish (Figure 3.3A; n=20; $p < 0.05$). The effect of WSFd on total body lipid was positively correlated to concentration, with 1.5-, 1.9-, and 2.5-fold higher lipid contents in fish from the 4 μg L^{-1}, 35 μg L^{-1}, and 100 μg L^{-1} treatments, respectively, relative to controls (Figure 3.3B; n=20; $p < 0.05$). The threshold concentration for an effect of WSFd on whole body triglyceride content of alevins was 35 μg L^{-1}, but there was no increase in the effect at 100 μg L^{-1}. Relative to controls, average alevin triglyceride contents were 1.6- and 2.1-fold higher in the 35 μg L^{-1} and 100 μg L^{-1} exposure groups, respectively (Figure 3.3C; n=20; $p < 0.05$).

3.3.4. Molecular effects

The expression of *cyp1a* in alevin head regions was significantly elevated above controls in all exposure groups in a concentration-dependent manner, ranging from 17- to 20-fold higher in fish exposed to 4 μg L^{-1} and 35 μg L^{-1}, respectively, and 37-fold higher in the 100 μg L^{-1} exposure group (Figure 3.4; n=8, $p < 0.001$). The expression of *ahr* did not significantly change with WSFd exposure (Figure 3.4; n=8, p=0.07).

3.3.5. Latent effects

Mortality in all treatment groups was followed daily for 8 months after the exposures, during which fish were held in clean water. Final cumulative mortality during this period was similar for Control, Low and Medium treatments (11–15% mortality). In contrast, on-going mortality was appreciably higher in fish exposed to 100 μg L^{-1} TPAH, especially during the first 60 days (40% mortality), with final mortality of 53% (Figure 3.5).

There was a concentration-dependent impact of early life stage exposure to WSFd on brain morphology across all brain regions (Figure 3.6; n=10–21; $p < 0.05$) with the exception of the olfactory bulbs (Figure 3.6B). There was a 9–16% increase in relative volume of the telencephalon (Figure 3.6C), hypothalamus (Figure 3.6D), optic tectum (Figure 3.6E), and cerebellum (Figure 3.6F) of fish exposed to 4 μg L^{-1} TPAH

during development compared to control fish. The effect at other concentrations was regionally specific. There was a 15% increase in telencephalon volume in fish exposed as embryos to 35 μg L^{-1} TPAH relative to controls (Figure 3.6C), while fish exposed as embryos to 100 μg L^{-1} TPAH showed increases in the volumes of the hypothalamus (Figure 3.6D) and optic tectum (Figure 3.6E), relative to controls.

There were no differences in relative ventricular mass between any WSFd-exposed and control fish, regardless of whether ventricle mass was expressed relative to whole heart mass or body mass (n=24–61, $p > 0.05$; Table 3.2). Heart shape was assessed from digital images of isolated hearts taken from a subset of fish in each treatment. There were no significant differences in ventricle length or width, normalized to fish length, among the treatment groups (n=4–9, $p > 0.05$; Table 3.2). The aspect ratio was significantly lower only in fish that were exposed to 35 μg L^{-1} WSFd during development relative to controls ($p < 0.05$; Table 3.2).

There were no differences in absolute (cm s^{-1}) or standardized (BL s^{-1}) critical swimming speed (U_{crit}) of juvenile fish exposed as embryos to WSFd (Table 3; n=5–15). Similarly, absolute and standardized maximum swimming speeds (U_{max}) did not differ in juvenile sockeye exposed as embryos to control or 100 μg L^{-1} TPAH (Table 3.3; n=5).

3.4. Discussion

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

The immediate effects of WSFd exposure on sockeye ELS were examined by measuring mortality and sub-lethal effects on development. A concentration-dependent

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

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

and teratogenesis in rainbow trout larvae (Hodson et al., 2007). In addition, CYP1A catalytic measurement (EROD activity) is considered a sensitive biomarker for xenobiotic exposure (Whyte et al., 2000), and EROD activity is reliably increased in fish gill (e.g., Blanc et al., 2010) and liver (e.g., Kennedy and Farrell, 2005) tissues when exposed to low levels of petrogenic chemicals. Here we show that *cyp1a* is increased as much as 35-fold in the head region of exposed ELS sockeye and at concentrations as low as 4 μg L^{-1} TPAH, supporting the sensitivity of this parameter for confirming contaminant uptake and biological response in ELS sockeye. By contrast, we did not detect a significant difference in *ahr* abundance, supporting our previous finding of a modest and transient transcriptional response in this receptor to dilbit exposure (Alderman et al., 2017b).

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

The latent effects of WSFd exposure were examined by measuring mortality, brain and cardiac morphology, and swimming performance of juvenile fish that were held in uncontaminated water for 8 months after the ELS exposure. Immediately following transfer to clean water, fry that had been exposed to 100 μg L^{-1} TPAH experienced considerable mortality compared to unexposed control fry, with 40% mortality occurring

during the first 60 d and final mortality surpassing 50%. Similarly, Johansen and colleagues report increased latent mortality in several species of coral reef fish exposed to weathered HEWAF of Deepwater Horizon crude oil during development (Johansen et al., 2017), and Brown and colleagues found a higher post-exposure incidence of mortality in Atlantic killifish (*Fundulus heteroclitus*) exposed during development to a complex PAH mixture (Brown et al., 2016). This suggests that delayed mortality is a common outcome of ELS crude oil exposure, and when combined with acute mortality and the numerous early sub-lethal effects of crude oil on fish development, warns of exacerbated population-level impacts of an oil spill in the aquatic environment.

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

mortality observed during and following WSFd exposure occurred in individuals with oil-induced cardiac defects, precluding any apparent latent impact on cardiac form and function.

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

3.5. Conclusions

This study presents data on adverse effects of WSFd exposure to developing sockeye embryos, including increased mortality, delayed hatching, teratogenesis, decreased utilization of energy stores, and reduced growth. In addition, high post-

exposure mortality of fry and lasting changes in brain morphology could extend the impact of ELS dilbit exposure by further reducing population size and impairing the physiology and behavior of surviving fish. Continued efforts to understand the nature and consequences of latent effects will deepen our understanding of the impacts of petrochemical exposure.

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Table 3. 1. Comparison of alevin development across treatments. Alevins from duplicate exposure tanks were analyzed, and data is presented pooled for each concentration. Wet weight, length, and developmental stage are presented a mean ± SEM. Results from the deformity analysis are presented as both frequency of occurrence (%) and severity (mean ± SEM). For each parameter, differences between exposure concentrations were determined by a one-way ANOVA and Bonferroni test for multiple comparisons. For each metric, values that do not share a common letter across concentrations are statistically different (n=100; $p < 0.05$).

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

Table 3. 2. Heart biometrics of juvenile sockeye exposed during early development to WSFd (in total polycyclic aromatic hydrocarbons, TPAH: Control=0 µg L^{-1}, Low=4 µg L^{-1}, Medium=35 µg L^{-1}, High=100 µg L^{-1}), and then raised in clean water for 8 months. Relative ventricular mass (RVM) is standardized to both whole heart mass and body mass (n=24–61 per treatment). Ventricular dimensions (length, width) were determined from digital images of the excised hearts of a subset of fish that also underwent a swimming trial (n=4–9), and are normalized to individual fork length. The aspect ratio of the ventricle was calculated as length:width for each heart. All data is expressed as mean ± SEM. Significance was determined using a one-way ANOVA and Holm-Sidak test.

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

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

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

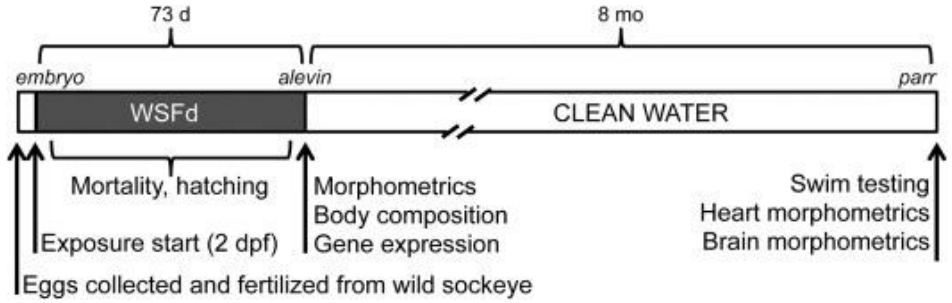

Figure 3. 1. Schematic of experimental design. Fertilized sockeye eggs were exposed to 1 of 3 concentrations of the water-soluble fraction of diluted bitumen (WSFd) or to clean water (control) from 2 d post fertilization (dpf) to swim-up (76 d total exposure length). A subset of fry were then transferred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

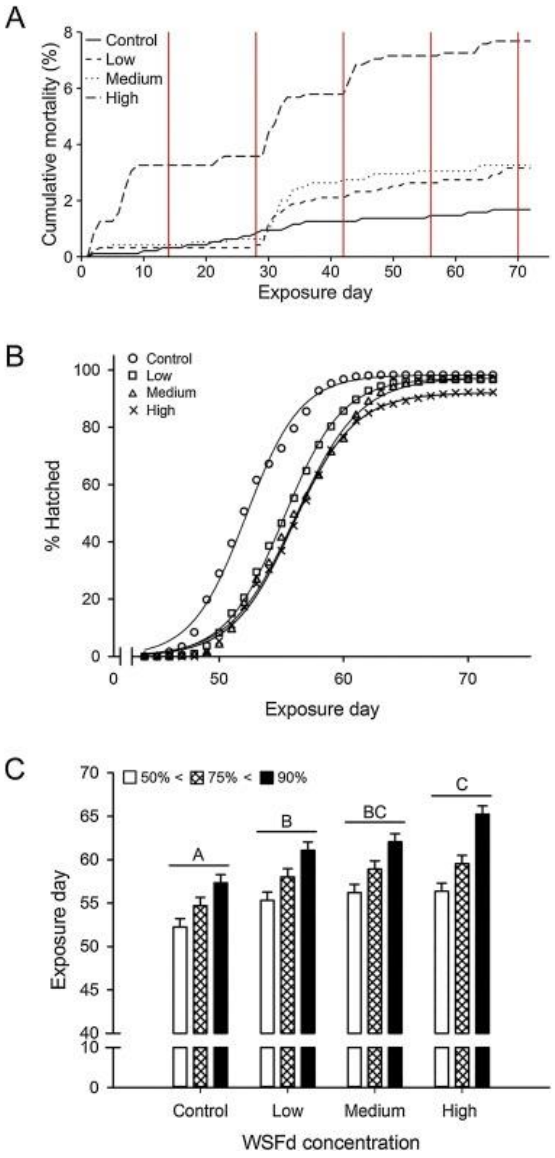

Figure 3. 2. Mortality and hatching. Sockeye embryos were exposed in duplicate to WSFd (in TPAH: Control=0 µg L^{-1}, Low=4 µg L^{-1}, Medium=35 µg L^{-1}, High=100 µg L^{-1}) from 2 d post fertilization until the end of hatching. (A) Cumulative mortality (%) was monitored daily, and is shown combined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

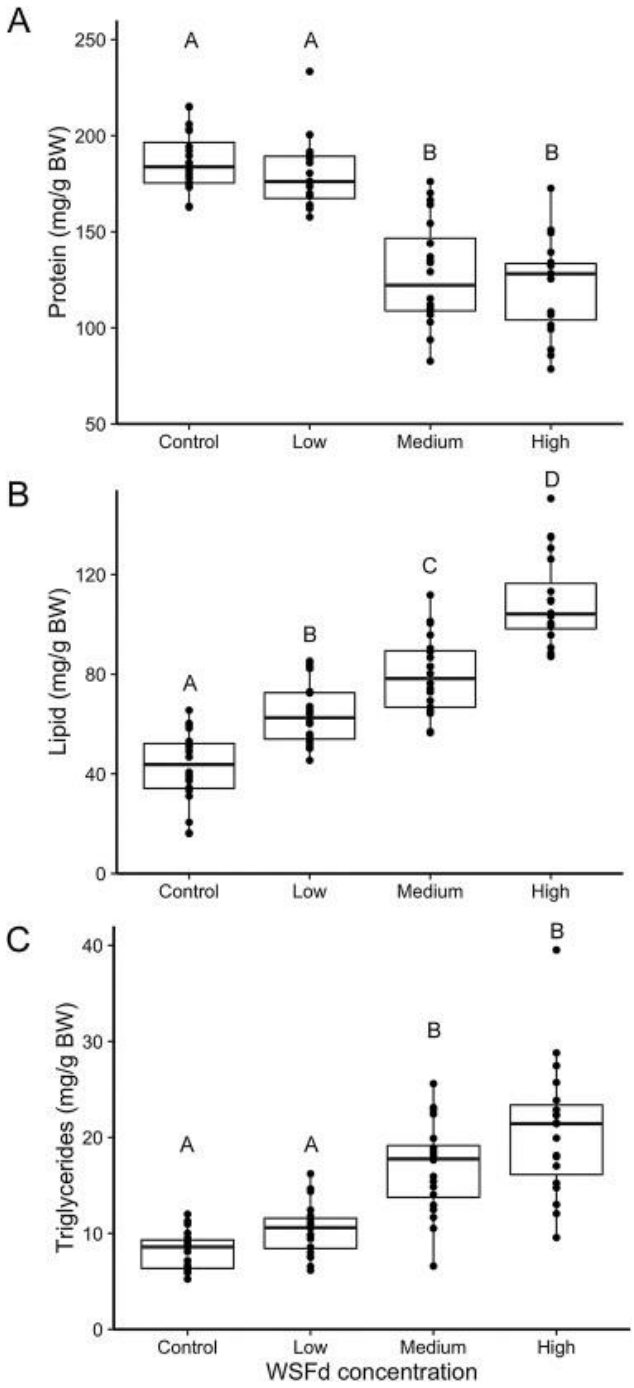

Figure 3. 3. Body composition. Soluble protein (A), total lipid (B), and total triglyceride (C) content was quantified in alevins following 76 d exposure to WSFd (in TPAH: Control=0 µg L^{-1}, Low=4 µg L^{-1}, Medium=35 µg L^{-1}, High=100 µg L^{-1}). Data were standardized to individual wet weight (BW).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

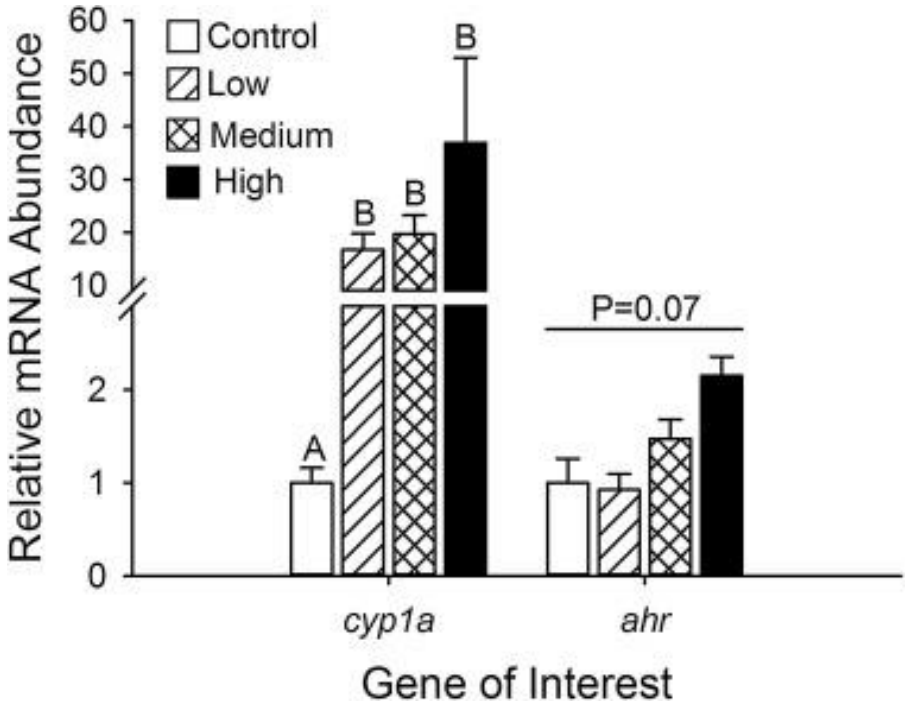

Figure 3. 4. Gene expression. Relative mRNA abundance of cytochrome P4501a (*cyp1a*) and aryl hydrocarbon receptor (*ahr*) in alevin heads following chronic exposure to WSFd during early development. Gene expression was quantified by RT-qPCR and standardized to a stable housekeeping gene (*rpL8*). Data are mean ± SEM and is presented as fold-change from the normalized control fish values. Significant differences for each gene were determined with a one-way ANOVA and Holm-Sidak test. Bars that do not share a common letter are statistically different (n=8; $p < 0.05$).

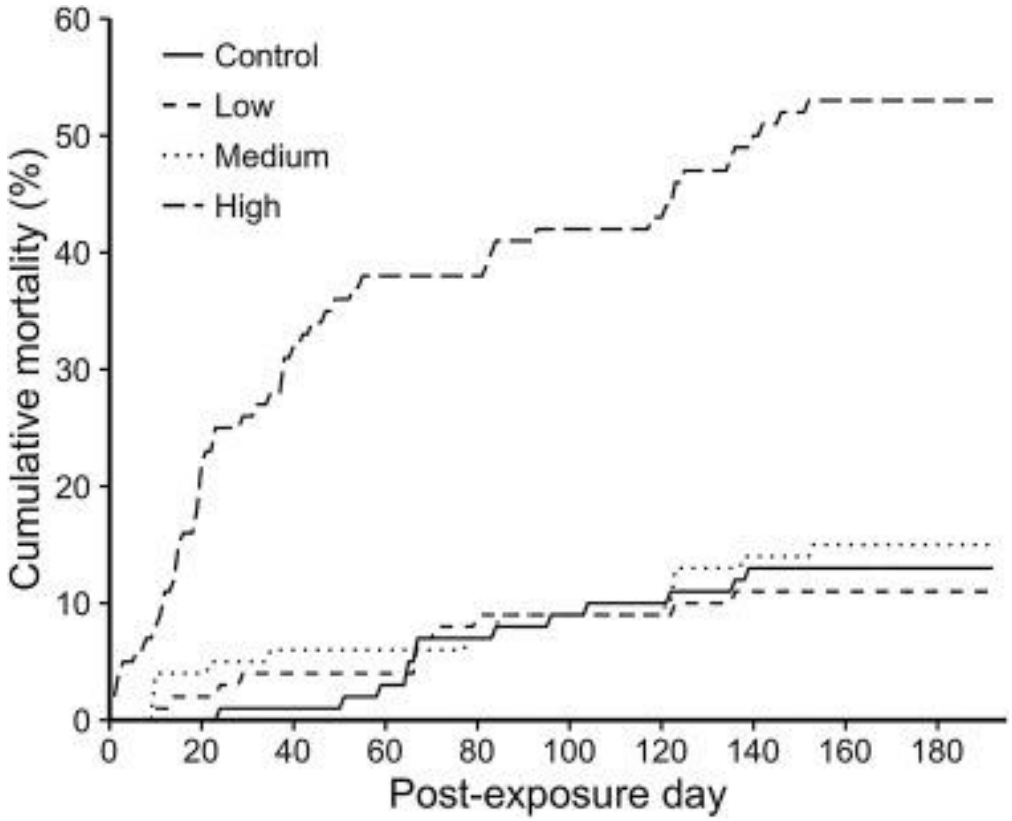

Figure 3. 5. Post-exposure mortality. Cumulative mortality of sockeye salmon during an 8-month period in clean water following embryonic exposure to WSFd (in TPAH: Control=0 µg L^{-1}, Low=4 µg L^{-1}, Medium=35 µg L^{-1}, High=100 µg L^{-1}).

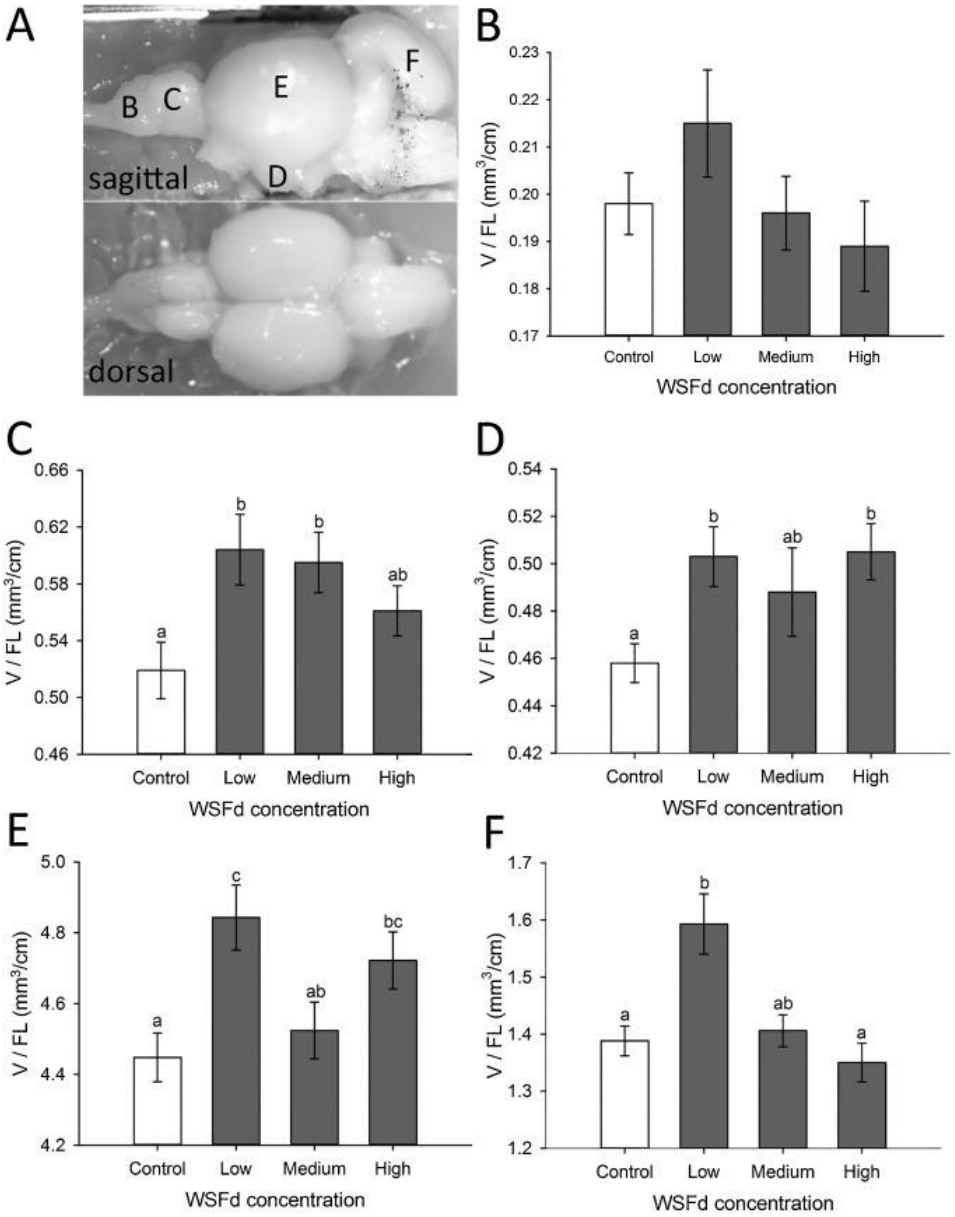

Figure 3. 6. Brain morphometrics. Comparisons of brain region volumes (V) in sockeye salmon that were exposed as embryos to WSFd (in TPAH: Control=0 µg L^{-1}, Low=4 µg L^{-1}, Medium=35 µg L^{-1}, High=100 µg L^{-1}) and then raised in clean water for 8 months. (A) Representative brain, photographed in sagittal and dorsal planes, with 5 quantified regions indicated: (B) olfactory bulbs, (C) telencephalon, (D) hypothalamus, (E) optic tectum, and (F) cerebellum. The maximum length, width, and height of each region were used to calculate volume (Eq. (1)), doubled if the region contained paired lobes (olfactory bulbs, telencephalon, optic tectum), and standardized to individual fork length (FL). Data are shown as mean ± SEM, and differences were determined for each region using one-way ANOVA and Holm-Sidak tests (n=10–21; $p < 0.05$).

Chapter 4. Environmental modulators of diluted bitumen effects in juvenile pink salmon (*Oncorhynchus gorbuscha*)

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Abstract

Recent and potential expansions in the transportation of diluted bitumen (dilbit) through marine terminals in coastal regions of British Columbia require the examination of potential risks to estuarine species such as Pacific salmon. The estuarine habitat of out-migrated pink salmon (*Oncorhynchus gorbuscha*) exhibits dynamic temperature and salinity regimes, possibly modifying dilbit exposure, bioavailability and/or its effects. To examine dilbit toxicity and its modification by environmental stressors, juvenile pinks were subchronically exposed for 3 months to the water-accommodated fraction (WAF) of Cold Lake Blend dilbit (winter) in seawater at three salinities (7, 14, and 28 ‰ [temperature 12.5 °C]) and three temperatures (8.5, 12.5, and 16.5 °C [salinity of 28 ‰]). Temperature and salinity alone did not affect any measured endpoints in control fish. Dilbit exposure induced higher mortality in the high (16.5 °C) and low temperatures (8.5 °C) as well as higher salinity (28 ‰) in fish exposed to the highest dilution of WAF [total polycyclic aromatic compounds (TPAC) = 128.9 µg/L]. A concentration-dependent reduction of growth was evident in fish exposed to the medium (TPAC = 97.3 µg/L) and high dilution of WAF at higher temperatures (12.5 and 16.5 °C) and high salinity (28 ‰). At 28 ‰, swimming performance (U_{burst}) was decreased in fish exposed to the highest concentration of dilbit at all exposure temperatures. Gill Na^+-K^+-ATPase activity, white muscle lactate, glycogen, and triglyceride concentrations were altered by dilbit exposure and modified by temperature and salinity. In addition, gene expression associated with phase I biotransformation, energy metabolism, mitochondrial activity, and inflammation showed significant upregulation with exposure and temperature stress. Dilbit exposure at PAC concentrations in the ppb range, affected pink salmon at the molecular, biochemical, and whole organism level; effects that were exacerbated by environmental temperature and salinity.

Keywords: Oil sands; diluted bitumen (dilbit); crude oil; toxicity; fish; pink salmon; swimming performance; stress; osmoregulation; molecular response; gene expression; physiology

4.1. Introduction

Canada is currently the 4th largest crude oil producer and 4th largest oil exporter in the world; its total oil production volume reached 4.2 million barrels per d in 2017 (NRC, 2019) with over 96% of its oil deposits in oil sands (CAPP, 2018a, b). Petroleum extracted from this source is primarily high viscosity/density bitumen (King et al., 2014) that requires dilution with either natural gas condensate or synthetic oil to form diluted bitumen (dilbit) that is more easily transported *via* pipeline (Dew et al., 2015; NRC, 2019). Several proposed pipeline projects (e.g., Trans Mountain expansion project [Kinder Morgan] and Northern Gateway project [Enbridge]) provide convenient export routes to tidewater for shipment to foreign markets (NEBC, 2018) raising environmental concerns for estuarine and marine inhabitants, including pink salmon outmigrants.

The Lower Fraser River estuary and Salish Sea in southwestern British Columbia (BC) serve as important habitat, rearing ground, and migratory routes for Pacific salmon (*Oncorhynochus spp.*) (Godin, 1981; Healey, 1982). Fraser River pink salmon (*Oncorhynchus gorbuscha*) hatch and immediately migrate downriver to reside in nearshore estuarine areas for several months. Following two years at sea as adults, they return to freshwater natal spawning streams. Pinks are particularly vulnerable ecological receptors with respect to potential dilbit exposure for several reasons: 1) embryo and alevin stages are relatively immobile and are unable to avoid contaminant exposure (reviewed in Alsaadi et al., 2018a), 2) they are dependent on the near shore estuarine habitat as an early nursery before their transition to a completely marine existence (Godin, 1981), and 3) migration through the Lower Fraser River estuaries and the Salish Sea occurs twice in their lifecycle (Heard, 1991).

Dilbit can cause sublethal effects in several fish species including early life stage (ELS) salmonids. For example, dilbit exposure induces cardiac remodeling in juvenile sockeye (*Oncorhynchus nerka*), resulting in impaired cardiac function and swimming performance (Alderman et al., 2017a). Exposed sockeye also exhibits an altered serum proteome, with significant changes in protein expression related to immune and inflammatory responses, blood coagulation, and iron homeostasis (Alderman et al., 2017b). Dilbit exposure negatively affects the survival, hatching success, time of

hatching, growth, and body composition in sockeye during embryonic development, as well as latent effects on the brain morphology in survivors following transfer into uncontaminated water (Alderman et al., 2018). Recent studies report an increase in the incidence of blue sac disease and post-hatching malformations, transcript abundance changes associated with genes for xenobiotic biotransformation, oxidative stress, cell cycling and mutagenesis in Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), and yellow perch (*Perca flavescens*); reduced survival during embryonic development, altered patterns of both shelter-seeking, and continuous swimming behavior have also been reported in zebrafish (*Danio rerio*) exposed to dilbit (Madison et al., 2015, 2017; Alsaadi et al., 2018b; McDonnell et al., 2019; Philibert et al., 2016). These toxic outcomes are generally consistent with those seen with exposure to conventional crude oil and their constituents (including benzene, toluene, ethylbenzene and xylene [BTEX], naphthenic acids, and polycyclic aromatic hydrocarbons [PAHs]) (e.g., Incardona et al., 2013; Kennedy, 2014; NASEM, 2016).

Estuaries are biologically, chemically, and physically dynamic environments; dominant environmental stressors for fish include salinity and temperature, parameters that can potentially alter the magnitude of toxic effects elicited by contaminants. Few studies have directly addressed the interactive effects between natural and anthropogenic stressors in this regard. Here, juvenile pink salmon were chronically exposed to the WAF of a winter mixture of Cold Lake Blend (CLB) dilbit under different salinity and temperature conditions; toxic outcomes were evaluated through a series of endpoints relevant to the ecological and physiological performance of pinks at several levels of biological organization, including growth and swimming performance, as well as both biochemical and molecular responses. Complementary global DNA methylation analysis was also performed to examine potential epigenetic effects previously described in ELS fish exposed to dilbit (McDonnell et al., 2019) or PAH (Fang et al., 2013a, b).

4.2. Materials and Methods

4.2.1. Fish

Fertilized pink salmon embryos were obtained from the Tenderfoot Creek hatchery (Brackendale, BC) and raised in heath trays supplied with dechlorinated municipal water at ambient temperature (average 11.8 °C) in the dark until the swim-up fry stage. All phenotypically normal swim-up fry were transferred into 200-L fiberglass tanks supplied with flow-through water for 2 additional weeks. A salinity acclimation regime was conducted to gradually introduce seawater into rearing tanks. Starting from freshwater, salinity was increased by 5 ‰ every 2 d until a target salinity of 28 ‰ was attained. All fry were reared in full strength seawater (28 ‰) at 12.5 °C until fish were 7 months old. Fish were fed twice daily *ad libitum* with commercial salmonid fry feed (Skretting, Vancouver, BC) under a 12 h light:12 h dark photoperiod.

Salt-water acclimated fry (mass 0.67 ± 0.01 g [X±SE]) were randomly distributed into glass aquaria (40 L) in water at 28 ‰ and 12.5 °C (n = 8 fish per tank, 2 replicate tanks for each treatment group). Prior to WAF exposure, fish in temperature treatments were acclimated to target temperatures by either gradually decreasing water temperature to 8.5 °C or increasing water temperature to 16.5 °C at a rate of approximately 0.25 °C/d. Fish in the varying temperature treatment groups (8.5, 12.5, and 16.5 °C) were maintained at a constant salinity of 28 ‰. Temperature manipulation in the present study was fulfilled by holding exposure tanks in temperature-controlled water baths.

Prior to WAF exposure, fish in salinity treatments were acclimated to target salinities by slowly decreasing the water salinity by 1.3 ‰ per day to either 7 or 14 ‰. Fish in the final salinity treatments (7, 14, and 28 ‰) were then maintained at a constant water temperature of 12.5 °C. Final salinity levels in tanks were achieved by dilution of full strength seawater with municipal dechlorinated freshwater (6.12 mg/L $CaCO_3$; DOC < 1 mg/L; pH 7.0). The entire temperature / salinity acclimation period was 21 d; 80% of the rearing water was renewed every 48 h, and no mortality during acclimation. The care

and experimental use of fish were approved by the University Animal Care Committee according to Canadian Council on Animal Care guidelines.

4.2.2. Dilbit Exposures

Dilbit WAFs were prepared according to Singer et al. (2000). In brief, 10 mL of CLB dilbit (winter) was mixed into 32 L of saltwater in a glass carboy using low energy stirring for 18 h with a 25% vortex depth. The solution was allowed to settle for 1 h, and the bottom clear aqueous layer used for WAF exposures. Fish were sub-chronically exposed to WAF dilutions (100, 50, 25 and 0% [control]) under the different salinity and temperature regimes for 90 d. A 75% volume (30 L) WAF replacement was performed every 48 h. Fish were fed twice daily with fry feed at a ration of 3.5% bw/d. The quantity of food supplied to each tank was calculated using the initial average wet weight of fish, and was increased weekly using a growth equation that presumes a food conversion efficiency of 20% (Meador et al., 2006). Uneaten food was removed by siphoning from the tanks during water changes. The salinity and temperature of each individual tank was monitored daily using a conductivity meter (YSI, Yellow Springs, OH). Ammonia concentration in each tank was measured daily using a water quality kit (A7867; Fluval, Baie d'Urfé, QC) to ensure water quality (≤ 0.02 ppm). All experimental tanks were lightly aerated, and DO levels were maintained at > 90 %.

4.2.3. Swim Performance Test

Following exposure, burst-swimming performance was assessed using a mini swim tunnel system (Loligo® Systems, Tjele, Den). The apparatus consisted of a 1.5 L cylindrical glass chamber equipped with an electric propeller submerged inside a water reservoir. The temperature in the reservoir was regulated at a fish's exposure temperature and salinity; DO was maintained at > 95%. Water velocity was calibrated using slow-motion video and dye test following the manufacturer's protocol.

The swim test consisted of a 20-min acclimation period (flow 5 cm/s; approx. 1 body length [BL]/s]) followed by a ramp protocol in which water velocity is rapidly increased to 2 BL/s for 1 min and subsequent increases of 0.5 BL/s each min until fish

are exhausted (inactively resting on rear baffle for > 2 s). Fish were removed, euthanized with MS 222 and wet weight (g) and fork length (cm) measured. Burst-swimming speed (U_{burst}) was calculated as the maximum speed attained by each fish normalized to fork length (BL/s [Farrell, 2008; Osachoff et al., 2014]). The cross-sectional area of all swim-tested fish were less than 10% of swim tunnel cross-sectional area and fish density were under 0.2 g/L, therefore no correction for a solid blocking effect was needed (Smit et al., 1971; Webb, 1971).

4.2.4. Tissue Preparation

Following euthanasia, livers were removed and preserved in 1 mL RNAlater™ stabilization solution (Invitrogen™, Carlsbad, CA) and kept at room temperature for 24 h, and then stored at -80 °C. Dorsal white muscle tissue was dissected from fish, flash-frozen in liquid N_2, and stored at -80 °C. Gill filaments (20-30) were removed and placed in 125 µL ice-cold SEI buffer containing 150 mM sucrose (Sigma-Aldrich, Oakville, ON), 10 mM EDTA (ACP Chemicals Inc., Saint Leonard, QC), 50 mM imidazole (Sigma-Aldrich) at pH 7.3. Gill samples were snap frozen on dry ice and then transferred to -80 °C.

4.2.5. Biochemical Analysis

Gill samples were homogenized in SEID buffer using a Mixer Mill homogenizer (Model MM300; Qiagen, Mississauga, ON) at 25 Hz for 30 s; homogenates were centrifuged (5000 *x g*) at 4 °C for 30 s to remove insoluble debris. Gill Na^+-K^+ (NKA) activity was measured in homogenates using a microplate spectrophotometric method (BioTek Epoch™ 2, Winooski, VT) following a standard protocol (McCormick, 1993). The protein content of each gill sample was quantified via a Bradford protein assay kit using bovine serum albumin as a standard (Catalog# 23200; Thermo Fisher Scientific, Mississauga, ON).

A subset of frozen muscle tissue was severed into smaller pieces and homogenized in liquid nitrogen using a pestle. Lyophilized samples were then mixed with 80% ethanol at a tissue/ethanol ratio of 1:8; the crude homogenates were kept at 4 °C

for 1 h for glucose and lactate extraction, followed by a centrifugation (10,000 *x g*) at 4 °C for 10 min to remove insoluble content. The top clear supernatant was used to measure the amount of glucose and lactate in each muscle sample using commercial colorimetric assay kits (Catalog# 120003400P; 120001400P; Eton Bioscience, San Diego, CA).

For glycogen content measurement, a subset of pre-weighed frozen muscle was thawed on ice and severed into smaller pieces and homogenized using a pestle and mixed with distilled water at a ratio of 10 mg tissue/200 µL diH20. Crude homogenates were immediately boiled for 10 min, followed a centrifugation (18,000 *x g*) at 4 °C for 10 min to remove insoluble material. The clear top supernatant was used to measure the amount of glycogen in each muscle sample using a commercial colorimetric assay kit (Catalog# K646-100; BioVision, Milpitas, CA).

To measure triglyceride content, a subset of pre-weighed frozen muscle tissue was severed into smaller pieces and homogenized using the Mixer Mill in diluted standard diluent assay reagent containing 1mM EDTA (Catalog# 70024; Cayman Chemical Company, Arbor, MI) at a ratio of 175-200 mg tissue/ mL reagent at 30 Hz followed by sonication on ice bath for 60 s to ensure complete homogenization. The homogenate was centrifuged (10,000 *x g*) for 10 min at 4 °C, and the clear top supernatant was used to measure the muscle triglyceride content using a commercial colorimetric assay kit (Catalog# 10010303; Cayman Chemical Company).

4.2.6. RNA Extraction

Total RNA was extracted from 20-25 mg liver using Trizol® Reagent (Ambion™ RNA, Life Technologies, Carlsbad, CA) using the methodology described in McDonnell et al. (2019). Total RNA was quantified with a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific) and used as a template to synthesize complementary DNA (cDNA). A total of 1 µg of DNAse treated RNA was reverse transcribed in cDNA with the Promega, GoScript Reverse Transcription System kit. All qPCRs were run on a Stratagene MX3000P. Genes of interest included the cyclooxygenase, *cox*; citrate synthase, *cs*; cytochrome P450, *cyp1a*; heat shock protein 70KDa, *hsp70*; heat shock

protein 90KDa, *hsp90*; glutathione-S-transferase-pi, *gst-π*; superoxide dismutase 1, *sod1*; peroxisome proliferator-activated receptor alpha, *pparα*; hypoxia-inducible factor 1-alpha, *hif1α*; and interleukin 1 beta, *Il1β* (Supplemental Information Table S1). Samples without reverse-transcriptase and no-template-controls were also included. Samples were run in duplicate, including samples without reverse-transcriptase, no-template controls, and positive water controls. qPCR methodology was done following the MIQE guidelines (Bustin et al., 2009) and accordingly to Madison et al. (2017). All genes of interest were assessed using relative change to the reference gene, the elongation factor 1 *α* (*ef1α*).

4.2.7. Global CpG Methylation Assay

Genomic DNA from liver tissue was extracted following RNA extraction. To the bottom phase of the Trizol/Chlorophorm lysate, 300 µl of 100% EtOH was added to precipitate DNA. The DNA pellet was then washed with 0.1M sodium citrate/10% EtOH and incubated for 30 min at room temperature. Then, two washing steps of the pellet with 75% EtOH were performed. The pellet was then dried at room temperature for 6 min and re-suspended in 50 µl of RNAse/DNAse-free water. The quantity and quality of DNA were checked by using a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific).

The methylation protocol was modified from Pierron et al. (2014) and is fully described in Supplemental Information. Briefly, 200 µL of DNA samples containing 400 ng of DNA were heated at 94 °C for 2 min and immediately cooled on ice. 50 µL of heat-treated DNA was added to microplate wells and incubated for 1 h at 37 °C. After DNA attachment, wells were washed five times with 200 µL PBS (NaCl 0.14 M, Na_2HPO_4 0.01M, pH 7.3). Primary antibodies (anti-5-methylcytosine monoclonal), were diluted with PTB-BSA solution (PBS solution with BSA 2% and Tween-20 0.02%) at a final concentration of 0.5 ng/µL. Secondary antibodies (goat anti-mouse IgG1 antibody HRP conjugated) were diluted with PTB-BSA solution to a final concentration of 0.2 ng/µL. The reaction was stopped by the addition of 50 µL of 2 N H_2SO_4. Absorbance was read at 450 nm in a plate reader (Model Infinite M1000 Pro; Tecan, Morrisville, NC).

4.2.8. Water Sample Analysis

The hydrocarbon composition of the WAF generated from CLB dilbit (winter) was characterized by measuring the concentrations of 50 representative polycyclic aromatic compounds (PACs). In brief, water samples from 2 replicated 100% WAF stock solutions generated in each different water salinity (7, 14, and 28 ‰) were pooled immediately after the 1 h settling period, and analyzed for individual PAC concentrations (Axys Analytical Services Ltd., Sidney, BC) by gas chromatography-mass spectrometry (GCMS) as described in Alderman et al. (2017a).

4.2.9. Statistical analysis

Prior to statistical analysis, tank effect was tested using restricted maximum likelihood (REML) method by treating it as a random factor. The tank effect was determined not to be statistically significant between the duplicate tanks for all endpoints assessed in the present study. Survival, body size comparison, U_{burst}, gill NKA activity, and all post-exercise body biochemical composition data collected from fish were then pooled. The individual effect of WAF exposure and environmental factors (temperature or salinity), as well as the interaction of the co-exposures were determined using a two-way analysis of variance (ANOVA) and Tukey's multiple comparisons test ($p < 0.05$ or $p < 0.01$). Gene expression (mean ± SE) was normalized to the mean of the reference genes, ef1a. One gene expression group was missing due to a lack of data in the temperature treatment. One-way ANOVA analyses Holm-Sidak post hoc analysis ($p < 0.05$) was used and all groups were compared with their respective control.

4.3. Results

4.3.1. WAF Analysis

Total polyaromatic compound (TPAC) concentrations for each of the three 100% WAF stock solutions generated using 7, 14, and 28 ‰ water were 128.9, 97.3, and 29.2 µg/L, respectively. Individual PACs were identified and quantified in each 100% WAF, detailed analysis results are provided in Supplemental Information (Table S2). The

concentrations of all PACs detected in WAF solutions decreased with increasing water salinity. Individual PAC proportions did not change consistently across different water salinities.

4.3.2. Effects on Survival and Growth

Temperature altered the responses of fish to the effects of WAF; survival was significantly lower in fish exposed to 100% WAF exposed at 8.5 °C and 16.5 °C compared to controls (Figure 4.1). No difference was seen between the growth of control fish under the three rearing temperatures (Table 4.2). At 8.5 °C, final body weight (g) and fork length (cm) of fish exposed to 50% WAF were significantly lower than the control group. At 12.5 °C, growth in 50% and 100% WAF-treated fish were significantly lower than controls. A concentration-dependent reduction in body weight was seen in WAF-exposed fish at 16.5 °C.

At 7 ‰ and 14 ‰ salinities, no mortality occurred in any treatment group, however, at 28 ‰, survival of fish exposed to 100% WAF was < 50% (Figure 4.1). No differences in final body weight and fork length of control fish were seen among salinity levels (Table 4.2). At 7 ‰, exposure to WAF did not affect fish growth, however the body weight and fork length of fish exposed to 50% and 100% WAF were significantly lower than controls in the 14 and 28 ‰ treatments. Maximum reductions of growth were seen in 50% and 100% WAF treatment groups at 28 ‰, where mean body weights were 48.6% and 68.8% lower than controls, respectively.

4.3.3. Swimming Performance

U_{burst} values for control fish ranged between 6.0 (± 0.1) and 7.0 (± 0.2) BL/s; no difference in U_{burst} was seen between control fish reared at 8.5, 12.5, and 16.5 °C (Figure 4.2). Exposed fish generally exhibited lower U_{burst} values compared to controls at all exposure temperatures. At 8.5 °C, exposure to 25% WAF did not affect U_{burst}; however, it was reduced in exposed fish at the higher temperatures of 12.5 °C and 16.5 °C. The highest reductions of burst swimming speed were observed in all 100% WAF groups across all temperatures; mean percent reductions ranged from 35.4 to 45.2%.

Salinity did not affect the U_{burst} values for control fish which ranged between 6.7 (± 0.2) and 7.1 (± 0.2) BL/s (Fig. 2). Exposure to all three dilutions of CLB dilbit did not result in a decrease in U_{burst} speed at 7 ‰ and 14 ‰. Fish exposed to 50% and 100% WAF at 28 ‰ had significantly reduced U_{burst} which were 33.9% and 32.2% lower than controls, respectively.

4.3.4. Biochemistry

Temperature had no effect on gill NKA activity in controls, and ranged from 13.1 (± 1.2) to 17.9 (± 1.3) µmole ADP/mg protein/h (Figure 4.3). At 8.5 °C, exposure to WAF did not affect gill NKA activity, however, significant reductions in gill NKA activities were observed in fish exposed to 100% WAF at 12.5 °C, as well as the 50% and 100% WAF groups at 16.5 °C. Maximum decreases were seen in fish exposed to 100% WAF at 12.5 °C and 16.5 °C (42.1% and 52.6% lower than their respective controls). Gill NKA activity of controls was positively correlated with rearing salinity; the highest activity level reached 14.3 ± 1.3 at 28 ‰ (Figure 4.3). There was no difference between the NKA activities of control fish reared 7 ‰ and 14 ‰, mean baseline activities were between 7.5 ± 0.8 and 7.9 ± 0.7 µmole ADP/mg protein/h. Exposure to WAF did not alter gill NKA activity at the two lower salinity levels, while a significant decline (43.4% lower than control) was seen in fish exposed to 100% WAF at 28 ‰.

Exposure temperature had no effect on baseline muscle glycogen, glucose, lactate or triglyceride concentrations in fish following exhaustive exercise (Figure 4.4, 4.5, 4.6, 4.7). Exposure to WAF did not affect muscle glycogen content in fish at 8.5 °C; however, as exposure temperature increased to 12.5 °C and 16.5 °C, fish exposed to 100% WAF exhibited significantly lower muscle glycogen content (< 50% of controls) after the swim test (Figure 4.4). Glucose levels in white muscle were not altered by WAF exposure at 8.5 °C and 12.5 °C, while exposure to 100% WAF at 16.5 °C significantly increased muscle glucose content by 1.8-fold (Figure 4.5). Muscle lactate content was not affected by WAF exposure at 8.5 °C (Figure 4.6), however at higher exposure temperatures, fish exposed to 50% and 100% WAF exhibited significantly elevated muscle lactate levels post-swim. The highest increase in muscle lactate was 2-fold, seen in the 100% WAF-exposed group at 16.5 °C. Muscle triglyceride contents were not

altered by WAF exposure at 12.5°C, however individuals exposed at 8.5 °C and 16.5 °C exhibited reduced levels of triglyceride (Figure 4.7). At 8.5 °C, only the 100% WAF groups showed a decrease (by 42.7%); exposure to all concentrations of WAF at 16.5 °C resulted in significant reductions ranging from 26.2 to 40.5%.

Following the swim test, baseline glycogen, lactate and triglyceride concentrations in muscle were not different across the three salinity levels in control fish (Figure 4.4, 4.5, 4.6, 4.7). The 100% WAF groups at 7 ‰, 14 ‰, 28 ‰ had significantly reduced muscle glycogen levels (Figure 4.4) compared to controls. Maximum reductions in glycogen content was seen at 28 ‰ (60.5% lower than controls). No effect on muscle glucose and lactate content was seen in fish exposed to any WAF dilutions at 7 ‰ and 14 ‰ (Figure 4.5, 4.6). At 28 ‰, exposure to 100% WAF caused a moderate but significant increase in muscle lactate (1.6-fold). The muscle triglyceride content was not affected by WAF exposures at any salinity levels (Figure 4.7).

4.3.5. Gene Transcripts and Global Methylation

No statistical differences in gene expression level were observed between controls and treatments for reference gene (*ef1α*; $p < 0.05$; Supplementary Information Figure S1). Gene expression is presented as mean ± SEM. Molecular transcripts for biotransformation, cellular stress, and immune system genes were altered by WAF exposure when combined with a gradient of salinity or temperature. Both salinity and temperature increased the relative mRNA levels of *cyp1a* (Figure 4.8A and 4.8G). For fish treated in a range of salinities, only the highest WAF concentrations showed a significant and consistent increase in *cyp1a* mRNA level ranging (between a 2- and 4-fold change) in all 3 salinity treatments (Figure 4.8A). At both 8.5 °C and 12.5 °C significant increases in *cyp1a* transcript levels were seen (Figure 4.8G). Fish exposed to different dilutions of WAF at 12.5 °C showed increases in *gst-π* mRNA levels (by 1.8- to 3-fold) (Figure 4.8H). In addition, fish exposed to WAF at the higher temperatures showed a significant difference in mRNA levels for *hsp70* compared to controls (Figure 4.8J). WAF exposure at 12.5 °C showed an increase in mRNA level (ranging from 2.6- to 3.3-fold) for *il1β* (Figure 4.8L), while a significant 60% decrease in *il1β* transcript levels were observed in fish exposed to the highest WAF dilution at 16.5 °C. In addition, the

expression profile of *cs* (involved in energy metabolism through the Krebs' cycle) showed increases at 8.5 °C and 16.5 °C (Figure 4.8K). The other genes tested in this study (*hsp90*; *cox*; *pparα*; and *hif1α*) did not show significant changes compared to their respective controls (data shown in Supplemental Information Figure S2). Of note, one treatment (8.5 °C at 100% WAF) could not be included in analysis due to the low sample size (n = 2). Furthermore, global CpG methylation of DNA did not show any significant differences compared to with their respective controls (Figure 4.9).

4.4. Discussion

Coastal estuaries are dynamic habitats, challenging species with environmental stressors alone and in conjunction with anthropogenic contamination. In this study, the impacts of temperature and salinity on the effects of a chronic dilbit WAF exposure were examined in early life stage (ELS) pink salmon, under realistic estuarine conditions. In the present study, reduced survival, decreases in burst swimming performance, alterations in body biochemical composition, and changes in the expression of specific hepatic genes were seen with dilbit exposure and were modified by both temperature and salinity.

4.4.1. Temperature

In the present study, temperature did not alter growth in control fish. The lack of thermal modulation was not expected; in general, growth rates of juvenile salmonids increase linearly when temperature rises from 5 °C to between 12-17 °C, and previous studies have shown that pink growth is positively correlated within this thermal range (Mortensen and Savikko, 1993; reviewed in Brett, 1979). The optimum ration for maximal growth is temperature-dependant (Brett et al., 1969). For fingerling sockeye salmon, the optimal ration at 8.5 °C is approximately 3% bw/d, 4% bw/d at 12.5 °C and 5% bw/d at 16.5 °C (Brett et al., 1969). There is also a complex interaction between maximal food conversion efficiency (FCE) and temperature (reviewed in Brett, 1979). In the present study, it is possible that the growth potential of fish at the two higher

temperatures was not fully realized due to suboptimal rations and/or decreased FCE, accompanied by an escalated maintenance energy cost.

Temperature also affects the aerobic locomotory performance of fish (Griffiths and Alderdice, 1972; Sisson and Sidell, 1987), while anaerobically-powered burst swimming ability is often reported to be temperature-independent (Beamish, 1978). In the present study, no difference was detected in the U_{burst} of fish reared across 3 temperatures. Results in other species show varying responses: increased temperature has been shown to increase (Claireaux et al., 2007; Guderley et al., 2001), decrease, or exert no effect (Carey and Franklin, 2009) on the burst swimming speed of fish.

Temperature can also result in biochemical alterations that affect burst speed, including changes in glycolytic metabolism that directly fuels anaerobic locomotion (McKenzie, 2011). In the present study, temperature did not affect post-exercise glycogen, glucose, lactate, or triglyceride concentrations in white muscle which is primarily responsible for burst swimming behaviour (Webb, 1998), results that support the lack of temperature effects on U_{burst}. It is possible that the glycolytic enzymes or their activities were not thermally modulated. The limited information that exists shows contrary responses. For example, Guderley et al. (2001) report that temperature altered the activity and sensitivity of enzymes involved in glycolytic metabolism in muscle of threespine stickleback (*Gasterosteus aculeatus*). Conversely, activities of phosphofructokinase and lactate dehydrogenase were not different between flounder (*Platichthys flesus* L.) reared at 5° C or 23 °C (Johnston and Wokoma, 1986). It is possible that juvenile pink salmon are evolved to maintain similar levels of burst swimming performance over a range of thermal conditions that are relevant to their naturally dynamic thermal habitat (DFO, 2018), which overlaps with the temperatures used in the present study.

4.4.2. Salinity

NKA plays a central role in teleost ion-water homeostasis and has been adopted as an indicator of osmoregulatory ability (McCormick et al., 1998, 2009 and seawater readiness in anadromous salmonids (Aarestrup et al., 2000). Here, gill NKA activity in

pinks was significantly greater at the high salinity compared to lower salinities; it is well reported that salmonids significantly upregulate branchial NKA activity within several d when acclimating to seawater (Madsen and Naamansen, 1989; Berge et al., 1995; Morgan and Iwama, 1998)

The lack of difference observed in the growth of juvenile pinks reared among the three salinity levels may have been partly attributed to osmoregulatory costs which are considered to be significant (Brett and Grove, 1979; Jobling, 1994). It has been hypothesized that rearing at isosmotic salinities should result in lower metabolic costs increasing the potential for growth (Imsland et al, 2001; Morgan and Iwama, 1991), however, while studies report growth enhancement at salinities under isotonic condition (Canagaratnam, 1959; Otto, 1971), others report no growth advantage (McCormick et al., 1989; Morgan and Iwama, 1991). In the present study, growth enhancement at isosmotic salinities or reductions at high salinity were not seen, suggesting that the difference in overall energetic costs of osmoregulation in salmonids may be negligible between salinity conditions (Eddy, 1982; Kirschner, 1993, 1995), however, other factors including interspecies difference, developmental stage, and most importantly ration, complicate this interpretation (McCormick et al., 1989; Morgan and Iwama, 1991).

In the present study, there was no effect of salinity alone on either Uburst or post-exercise biochemistry in white muscle. A recent study reported a similar finding in juvenile coho which exhibited no difference in maximum swimming speed (U_{max}) or repeated U_{max} values following rearing at 2.5, 10, and 30 ‰ (Fang et al., 2019).

4.4.3. WAF Exposure

Other than physical effects that could occur from insoluble dilbit matrices, toxicity results from the bioavailable fraction of the dilbit components dissolved in the water phase. A concentration-dependent decrease in TPAC concentrations occurred with increasing salinity, which is consistent with previous studies which found lower PAH solubility and bioavailability in seawater *v.* freshwater (Ramachandran et al., 2006; Shukla et al., 2007). The exposure concentrations, ranged from 7.3 to 128.9 µg/L TPACs, and likely represent environmentally realistic exposure concentrations in a

potential dilbit spill. Previous studies demonstrated comparable ranges of TPAC concentrations in water-soluble fractions (WSF; initial TPACs [4 to 124.5 µg/L] (Alderman et al., 2017a; Alderman et al., 2018; Lin et al., 2020) and WAFs (up to 100 µg/L TPAH) (Alsaadi et al., 2018b; Barron et al., 2018; Madison et al., 2015, 2017; McDonnell et al., 2019; Philibert et al., 2016) using various dilbit blends. Additionally, studies that analysed water samples collected from the active spill sties during the Deepwater Horizon (DWH) have documented TPAH concentrations ranging from <0.01 to 85 µg/L at the water's surface and up to 10 m below (Bejarano et al., 2013; Diercks et al., 2010).

Pink fry exposed chronically to dilbit WAFs exhibited reduced survival. Limited data currently exists in this regard for dilbit; 1+ y old juvenile sockeye showed no mortality exposed to the WSF of CLB dilbit (initial TPAH [4 to 124 µg/L]) for up to 4 weeks (Alderman et al., 2017a; Lin et al., 2020). Dilbit studies generally report no acute necrotic effects to developing embryos and larvae of several freshwater species at TPAC concentrations up to 100 µg/L (Alsaadi et al., 2018b; Madison et al., 2015, 2017; McDonnell et al., 2019). However, lethality (24 h or 96 h LC50) was reported for rainbow trout fingerlings (*Oncorhynchus mykiss*) exposed to WAF of unweathered CLB dilbit and larval fathead minnow exposed to WAF of unweathered Access Western Blend (AWB) dilbit, the corresponding TPAH concentrations for these acute effects were estimated to range between 0.5 and 2.0 µg/L (Robidoux et al., 2018). Exposure to WAF of fresh Western Canadian Select (WCS) dilbit and CLB dilbit also resulted in lethal effects (96 h LC20 or LC50) in larval fathead minnow (7-12 d old) and inland silverside (*Menidia beryllina*: 10-14 d old) with TPAH concentrations at 8-40 µg/L (Barron et al., 2018). Kennedy (2014) reviewed the acute toxicity of crude oil, chemically dispersed oil, WAF and WSF of oil or its components to various fish species and reported that the acute lethal toxicity of conventional crude oils to fish is generally reported at concentrations above the water solubility values for most of the oil components.

Growth impairment following crude oil exposure has been reported in various species of salmonids (Lockhart et al., 1996; Vignier et al., 1992; Wang et al., 1993; Woodward et al., 1983) and several flatfish (Dey et al., 1983; Moles and Norcross, 1998) and is likely through multiple mechanisms: suppression of feeding behavior and

reductions in food conversion rates (Moles and Rice, 1983; Vignier et al., 1992), alterations in energy allocation, and increases in energy expenditures. For example, oil-induced impairment of branchial function and the resulting iono-osmoregulatory dysfunction can elevate the costs for osmoregulation (Whitehead, 2013). Here, pinks exposed to 100% WAF exhibited reduced branchial NKA activity (consistent with previous studies in fish exposed to crude oil [Boese et al., 1982; Engelhardt et al., 1981; Gad, 2011]), suggesting a possible elevated osmotic stress, supporting the energy conflict theory between osmoregulatory disturbance and growth (Whitehead, 2013). As well, the upregulation of enzymes (e.g. CYP1A-mediated ethoxyresorufin O-deethylase [Alderman et al., 2017a; Lin et al., 2020]) associated with hydrocarbon biotransformation or contaminant induced damage or stress (Lin et al., 2009; Van Scoy et al., 2010) could result elevated metabolic demands. Increased routine metabolic demands were previously reported in chub mackerel (*Scomber japonicus*) exposed to the WAF of DWH crude oil (Klinger et al., 2015), bald rockcod (*Pagothenia borchgrevinki*) exposed to the WSF of diesel fuel (Davison et al., 1992), and Florida pompano (*Trachinotus carolinus*) exposed to naphthalene (dos Santos et al., 2006). The additional metabolic expenditure due to oil exposure may be seen as a loss in the fish's energetic flexibility or versatility, causing prolonged taxation over energy budgeted for growth and development.

Burst swimming has relevance for fish in foraging, migration, and predator avoidance (Marras et al., 2010; Reidy et al., 2000) and has direct bearing on early survival in estuaries and seaward migration success in salmonids (Eliason et al., 2011; Kieffer, 2000). Burst swimming performance was negatively affected by exposure to dilbit WAFs, a result similar to other studies reporting reductions in Ucrit (critical swimming speed; an endurance measurement fueled aerobically) following crude oil exposure in salmonids (Alderman et al., 2017a; Thomas and Rice, 1987) and other species (Johansen and Esbaugh, 2017; Kennedy and Farrell, 2006; Mager et al., 2014; Stieglitz et al., 2016). Mechanisms underlying impaired swimming performance in has been directly linked to diminished maximum metabolic rate and aerobic scope (Johansen and Esbaugh, 2017; Stieglitz et al., 2016), PAH cardiotoxicity (Incardona et al., 2014; Jung et al., 2013; Khursigara et al., 2017) including decreased stroke volume and cardiac output (Nelson et al., 2017), disruptions in excitation-contraction, prolonged

action potentials, inhibition of calcium current and calcium cycling in cardiomyocytes (Brette et al., 2014), and alterations in compact myocardium composition (increased collagen content and decreased proportion of myocytes) (Alderman et al., 2017a).

Dilbit may also alter anaerobic metabolism in the white skeletal muscle, affecting anaerobic exercise performance. Pinks exposed to dilbit consistently showed a higher accumulation of lactate and lower glycogen content in white muscle post-swim. During an exhaustive anaerobic exercise, muscle glycogen is utilized, leading to lactate accumulation (Hammer, 1995; Moyes and West, 1995) possibly limiting the anaerobic capacity of white muscle (Moyes and West, 1995), and lower Uburst values. Oil-exposed juvenile mahi-mahi exhibit lower mitochondrial respiration rates in cardiac muscle, possibly suppressing cellular ATP supply, and increasing the reliance on anaerobic respiration (Kirby et al., 2019). An increased reliance on anaerobic metabolism white muscle may lead to the greater depression of glycogen storage seen here. It is also possible that dilbit-exposed fish had lower stored glycogen and higher lactate levels before the swim test due to a higher basal glycolytic activity (e.g., phosphofructokinase, lactate dehydrogenase, creatine phosphokinase activities) or stress response. Exposure to crude oil and PAHs can initiate a physiological stress response and elevate of circulating catecholamine and cortisol levels (Hontela et al., 1992; Kennedy and Farrell, 2005), increasing the metabolism of glycogen and accumulation of lactate (George et al., 2013). Stress under WAF exposure may alter energy relocation towards fuel-intensive activities in fish, thereby negatively impacting anaerobic performance.

Post-exercise muscle triglyceride concentrations were significantly lower in fish exposed to WAF. Hepatic triglyceride levels were also depleted in Atlantic cod (*Gadus morhua*) and winter flounder (*Pseudopleuronectes americanus*) chronically (24-week) exposed to crude petroleum (Dey et al., 1983). Triglyceride is another major energy substrate utilized for sustained and prolonged swimming through aerobic oxidation in red muscle (Hammer, 1995; Moyes and West, 1995). Liver and skeletal muscle generally have similar levels of triglycerides (Moyes and West, 1995), however the diminished post-exercise triglyceride content is unlikely to have contributed to impaired exercise performance as burst swimming is fueled via anaerobic pathways (Moyes and West, 1995). Alterations in lipid metabolism are more likely a consequence of enhanced basal

free fatty acid catabolism in WAF-exposed fish. Recent transcriptomic responses in larval red drum and mahi-mahi exposed to DWH crude oil suggest that pathways related to sterol biosynthesis, the metabolism of fatty acids, and lipid homeostasis may be the potential targets of oil toxicity (Xu et al., 2016; Xu et al., 2017).

4.4.4. Effects of Environmental Temperature and Salinity on WAF Effects

Both high and low temperature modulated the effects of dilbit on the survival and growth of pinks. In another study, pink fry had lower 96 h median tolerance limits to the WAF of Cook Inlet crude oil and toluene at 4 °C compared to 12 °C, possibly due to the higher persistence of aromatic hydrocarbons, monoaromatics and other lower molecular weight (LMW) compounds at the lower temperature (Korn et al., 1979). LMW compounds (< C12) are relatively smaller and more bioavailable to fish, and are believed to be the primary compounds responsible for acute toxicity following crude oil exposure (Lee et al., 2015). It has been generally reported that fish residing in colder waters tend to have lower LC50 values compared to species from warmer environments (Rice et al., 1977). Contrary data exists in this regard; for example, the survival of Arctic cod (*Boreogadus saida*) and fourhorn sculpin (*Myoxocephalus quadricornis*) following an 8 d exposure to the WSF of Cook Inlet crude oil or naphthalene were not altered by temperature (Carls and Korn, 1985). The inconsistent pattern of the effects of temperature are not surprising as modulating effects on adverse organism responses to contaminants involve temperatures effects on environmental (e.g., water solubility), exposure (e.g. bioavailability), toxicokinetic (e.g., biotransformation), and toxicodynamic factors (e.g., target site).

The sublethal toxicity of dilbit is also modulated by environmental temperature, with the magnitude of adverse effect modulation more evident at higher temperatures. Specifically, reductions in U_{burst} was only significantly in 100% WAF group at 8.5 °C, while all three WAFs had exerted negative impacts on the bursting speeds of fish at 12.5 °C and 16.5 °C. In contrast, Mager et al. (2018) exposed juvenile mahi-mahi to WAF of DWH crude oil at sublethal concentrations, the oil-induced impairment to critic swimming performance was found to be possibly ameliorated when exposure temperature was

increased from 27 °C to 30 °C. Alterations of gill NKA activity, post-exercise muscle lactate, triglyceride contents were consistently seen in WAF-exposed fish at the two higher temperatures. Studies used isolated PAH species have demonstrated that the uptake, distribution, and metabolism of petrogenic hydrocarbons as well as the excretion of metabolites can be remarkedly altered by water temperature, and rates of these biological processes generally are generally accelerated as temperature rises (Collier et al., 1978; Jimenez et al., 1987; Kennedy and Walsh, 1997; Varanasi et al., 1981). The higher energy expenditure posed by increased hydrocarbon metabolism could also result in a diminished energy available for maintaining routine metabolic activities, leading to lower energy stores, reduced metabolic capacity, and performance. However, another important energy substrate in white muscle, glycogen, did not exhibit much change that varied with temperature.

4.4.5. Combined Effect of Salinity & WAF Exposure

Significant modulation of dilbit WAF effects were seen in pink survival, growth, Uburst, and some post-exercise muscle biochemical parameters in fish at higher salinities. Interestingly, analysis of water samples in the present study show that the concentrations of all individual PAC molecules were over 4-fold higher at 7 ‰ compared to 28 ‰, results consistent with studies showing that the solubility of crude oil-derived PAH is higher at lower salinities and may lead to increased bioavailability (Ramachandran et al., 2006; Shukla et al., 2007). Previous studies have reported that juvenile salmonids are more sensitive to crude oil or their components in seawater compared to fresh or brackish water (Moles et al., 1979; Moles et al., 1987; Stickle et al., 1982). For example, seawater-acclimated mummichog (*Fundulus heteroclitus*) exposed in hyperosmotic salinities demonstrated higher uptake of naphthalene compared to fish in brackish or freshwater, which correlated with increasing osmotic disturbances, metabolic stress, and morality in seawater (Levitan and Taylor, 1979). The results of these studies and the present study indicate that general osmoregulatory stress, coupled with morphological and functional alterations in teleost gills (e.g., enhanced passive loss/gain of ions, decreased chloride cells [e.g., Claireaux et al., 2004; Goanvec et al., 2011; Kennedy and Farrell, 2005]) thereby enhancing PAH-induced toxicity may

be aggravated under hyperosmotic conditions. Toxicokinetics may be influenced by salinity; high salinity can increase drinking rates of fish, potentially increasing exposure to dissolved chemicals, (Whitehead, 2013). No salinity-induced modulation of growth effects was seen, however, at lower salinities (7 ‰ and 14 %) reduced effects on survival and swimming performance were seen despite higher TPAC concentrations. These results suggest that lower PAC concentrations in coastal water or estuaries at higher salinities may result in greater adverse outcomes *v.* those in freshwater.

4.4.6. Molecular Responses

Juvenile pink salmon exposed to WAF showed stronger effects in fish conjunction with varying temperature compared to salinity. The expression of *cyp1a* was significantly modulated by temperature with the highest expression measured at 12.5 °C and with the most reduced expression at 16.5 °C, suggesting that warmer conditions may prevent pinks from efficiently metabolizing some dilbit constituents compared to colder temperatures. In Atlantic halibut, warmer temperatures significantly decreased EROD activity (Almroth et al., 2019), which is consistent with data obtained in this study. In addition, the synthesis of gene transcripts of biomarkers associated with the phase II biotransformation, oxidative stress and energy metabolism (e.g., *gst*, *sod1*, and *cs*) showed moderate increases with dilbit exposure. These results suggest the induction of xenobiotic defenses and are in accordance with previous studies (Alsaadi et al., 2018b; Madison et al., 2015, 2017). For example, embryos of fathead minnow exposed to CLB (winter blend) showed an increase in *gst* correlating with an increase in TPAH (Alsaadi et al., 2018b), similar to the significant increase observed in the 12.5 °C treatment in the present study. In addition, the decrease of survival rate measured in the present study observed at 8.5 °C and 16.5 °C, corresponds to an increased expression of *cs*, suggesting an enhanced energy demand in these fish.

PAHs are known to alter DNA methylation in fish. Benzo[a]pyrene decreased the rate of global DNA methylation in zebrafish embryos (Corrales et al., 2014; Fang et al. 2013a, b), while CLB dilbit altered global CpG methylation in yellow perch (McDonnell et al., 2019). Results of the present study did not indicate observable effects in global DNA methylation and interference with the epigenetic status in juvenile pink salmon.

4.5. Conclusions

Juvenile salmon residing in estuarine and marine nurseries are constantly challenged with natural environmental stressors which may potentiate the effects of a dilbit following a spill event. Chronically exposure pink salmon fry exhibited multiple, possibly unrelated toxic outcomes in several physiological systems at several levels of biological organization, including reduced survival and growth, decreased swimming performance, alterations in body biochemical composition, and changes in the expression of various hepatic genes. Both lethal and sublethal toxicity were modified by salinity and temperature, generally with increasing temperature or salinity enhancing the magnitude of affected endpoints. These findings are particularly relevant to Pacific salmon species that utilize coastal estuaries and nurseries, resulting in lower fitness levels with implications to the conservation of at risk populations.

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Table 4. 1. Custom designed primer pairs for quantitative real-time PCR analysis in this study.

Associate Biological function	Gene of Interest	Forward Primer (5' - 3')	Reverse Primer (5' - 3')
Housekeeping gene	*ef1a*	CTGTTGCCTTTGTGCCCATC	TTCCATCCCTTGAACCAGCC
Xenobiotic metabolism Phase I	*ahr*	GGGGCTGTTACGTTTTGCAC	GGTGGCTGGTTAGAGTGGAC
Xenobiotic metabolism Phase I	*hsp90*	GCTGAACAAGACCAAGCCCA	AGCCAGGTGTTCCTCCCA
Xenobiotic metabolism Phase I	*cyp1a*	GATGTCAGTGGCAGCTTTGA	TCCTGGTCATCATGGCTGTA
Xenobiotic metabolism Phase II	*gst-π*	CTACTTTGGAGTCCGAGGGC	CGTCCAGTCAGCAAAGTCCA
Xenobiotic metabolism Phase II	*sod1*	TTACTGGGAGCCCTGGTACA	ACAGAGCCTTCTATGGGGGT
Oxidative metabolism	*cs*	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG
Oxidative metabolism	*cox4*	TACGTGGGGGACATGGTGTT	CCCAGGAGCCCTTCTCCTTC
Stress temperature	*hsp70*	GATGTGGTGTTGGGGCAGTA	GGACAGTGGGGTCATCAAGG
Stress hypoxia	*hif1α*	GACTCCGCCCCAGATTTCC	GGTGCTGGTGATGAGGTGAG
Immune system	*il1β*	TAAAGGGTGGCGAGGGGGT	ACCTTGCTCCCTACCTTCCA
Lipid metabolism	*pparα*	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT

Table 4. 2. Growth indices of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Fish were exposed to 3 dilutions of CLWB-WAFs at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰. Another set of fish were exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Weight and fork length of fish before and after 90-day exposure were presented as mean ± SE. For each index, two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments, values that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, bold data indicate a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$).

	Initial body weight (g)	Initial fork length (cm)	Final body weight (g)	Final fork length (cm)
8.5 °C				
Control	0.76 ± 0.06 ^{a}	3.81 ± 0.09 ^{a}	3.08 ± 0.25 ^{ab}	7.21 ± 0.14 ^{abc}
WAF-25%	0.79 ± 0.06 ^{a}	3.67 ± 0.10 ^{a}	2.39 ± 0.15 ^{bc}	6.73 ± 0.13 ^{cde}
WAF-50%	0.78 ± 0.05 ^{a}	3.77 ± 0.10 ^{a}	1.82 ± 0.13 ^{c}	6.28 ± 0.14 ^{e}
CLWB-100%	0.73 ± 0.07 ^{a}	3.57 ± 0.10 ^{a}	1.76 ± 0.37 ^{bc}	6.40 ± 0.30 ^{abcde}
12.5 °C				
Control	0.76 ± 0.06 ^{a}	3.73 ± 0.09 ^{a}	3.75 ± 0.19 ^{a}	7.71 ± 0.13 ^{ab}
CLWB-25%	0.74 ± 0.04 ^{a}	3.59 ± 0.06 ^{a}	2.98 ± 0.25 ^{ab}	7.26 ± 0.18 ^{abc}
CLWB-50%	0.66 ± 0.07 ^{a}	3.59 ± 0.09 ^{a}	2.70 ± 0.21 ^{bc}	7.09 ± 0.17 ^{abcd}
CLWB-100%	0.64 ± 0.05 ^{a}	3.59 ± 0.07 ^{a}	1.96 ± 0.20 ^{c}	6.54 ± 0.21 ^{cde}
16.5 °C				
Control	0.71 ± 0.06 ^{a}	3.74 ± 0.08 ^{a}	3.86 ± 0.21^{a}	7.80 ± 0.17 ^{a}
CLWB-25%	0.79 ± 0.06 ^{a}	3.71 ± 0.08 ^{a}	2.74 ±0.21 ^{bc}	7.02 ± 0.19 ^{bcde}
CLWB-50%	0.70 ± 0.05 ^{a}	3.68 ± 0.07 ^{a}	2.52 ± 0.15 ^{bc}	7.08 ± 0.13^{abcde}

CLWB-100%	0.72 ± 0.05 [a]	3.70 ± 0.07 [a]	1.66 ± 0.26 [c]	6.18 ± 0.27 [de]
7 ‰				
Control	0.65 ± 0.04 [a]	3.70 ± 0.07 [a]	4.18 ± 0.25 [ab]	8.04 ± 0.17 [abc]
CLWB-25%	0.59 ± 0.06 [a]	3.58 ± 0.10 [a]	3.85 ± 0.19 [ab]	7.94 ± 0.22 [abc]
CLWB-50%	0.61 ± 0.06 [a]	3.60 ± 0.11 [a]	3.80 ± 0.18 [ab]	7.91 ± 0.16 [abc]
CLWB-100%	0.62 ± 0.05 [a]	3.61 ± 0.10 [a]	3.71 ± 0.19 [ab]	7.87 ± 0.22 [abc]
14 ‰				
Control	0.60 ± 0.05 [a]	3.54 ± 0.11 [a]	4.54 ± 0.22 [a]	8.23 ± 0.18 [a]
CLWB-25%	0.57 ± 0.05 [a]	3.72 ± 0.10 [a]	3.95 ± 0.23 [ab]	8.08 ± 0.16 [ab]
CLWB-50%	0.59 ± 0.05 [a]	3.67 ± 0.14 [a]	3.04 ± 0.23 [bc]	7.32 ± 0.16 [bc]
CLWB-100%	0.63 ± 0.05 [a]	3.73 ± 0.07 [a]	3.06 ± 0.30 [bc]	7.22 ± 0.20 [c]
28 ‰				
Control	0.56 ± 0.04 [a]	3.70 ± 0.08 [a]	3.68 ± 0.15 [ab]	7.70 ± 0.10 [abc]
CLWB-25%	0.51 ± 0.06 [a]	3.43 ± 0.09 [a]	3.28 ± 0.32 [abc]	7.29 ± 0.18 [bc]
CLWB-50%	0.71 ± 0.05 [a]	3.74 ± 0.09 [a]	1.89 ± 0.13 [cd]	6.35 ± 0.07 [d]
CLWB-100%	0.67 ± 0.04 [a]	3.68 ± 0.06 [a]	1.15 ± 0.13 [d]	5.57 ± 0.16 [d]

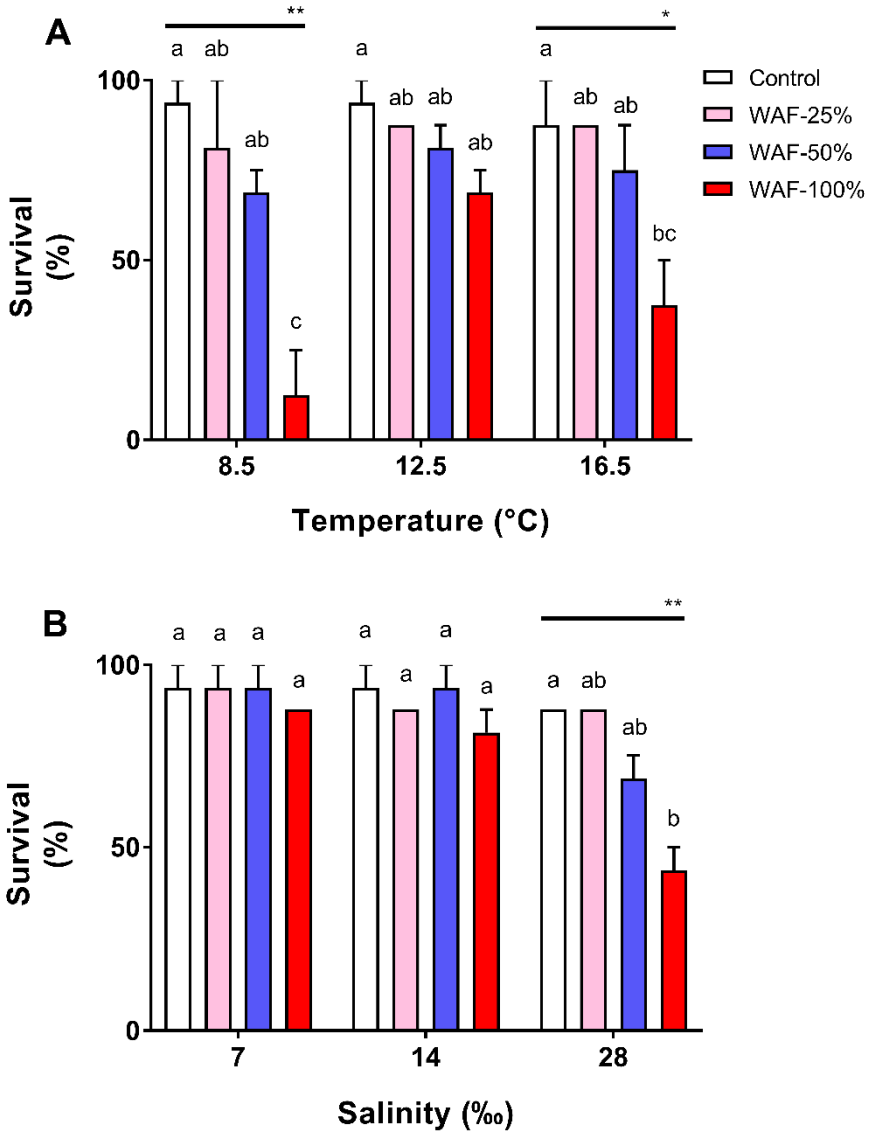

Figure 4. 1. Survival of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows survival of fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows survival of fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Data are mean ± SE, bars that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$).

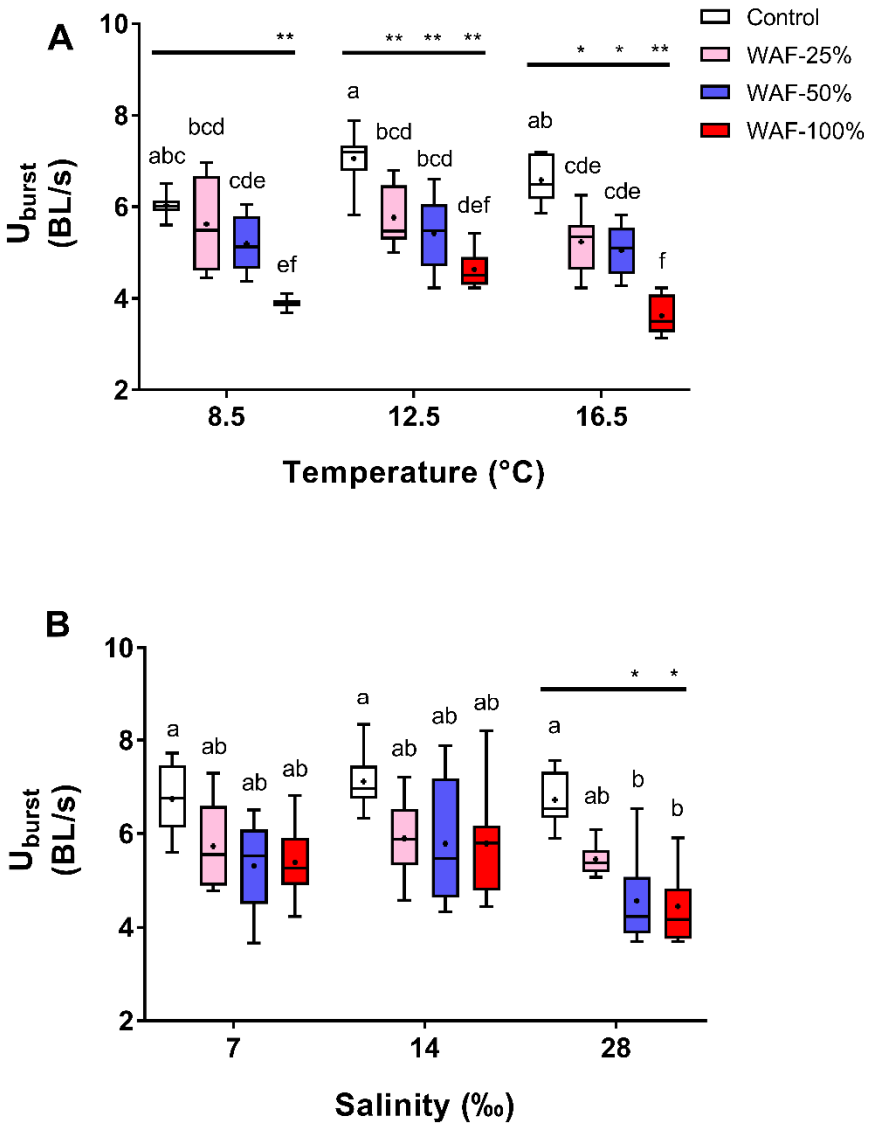

Figure 4. 2. U_{burst} of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows U_{burst} of fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows U_{burst} of fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

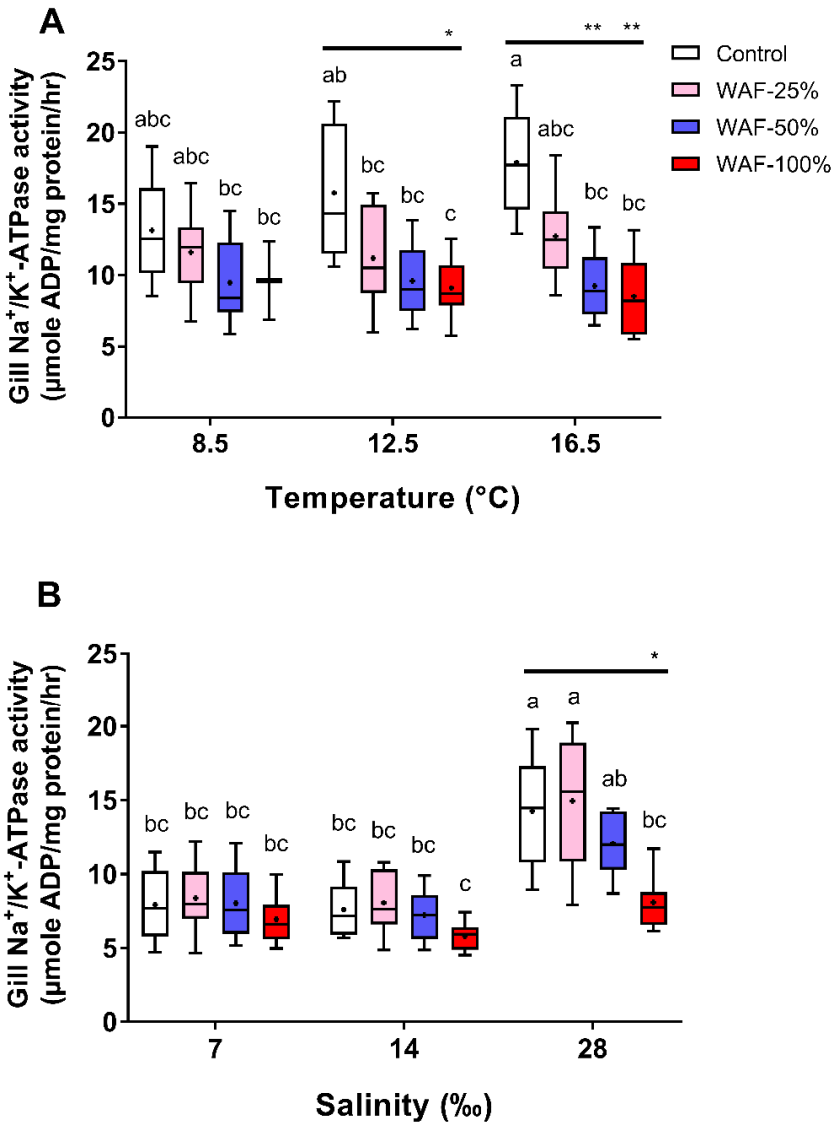

Figure 4. 3. Gill Na^+-K^+ ATPase (NKA) activity of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows NKA activity of fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows NKA activity of fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

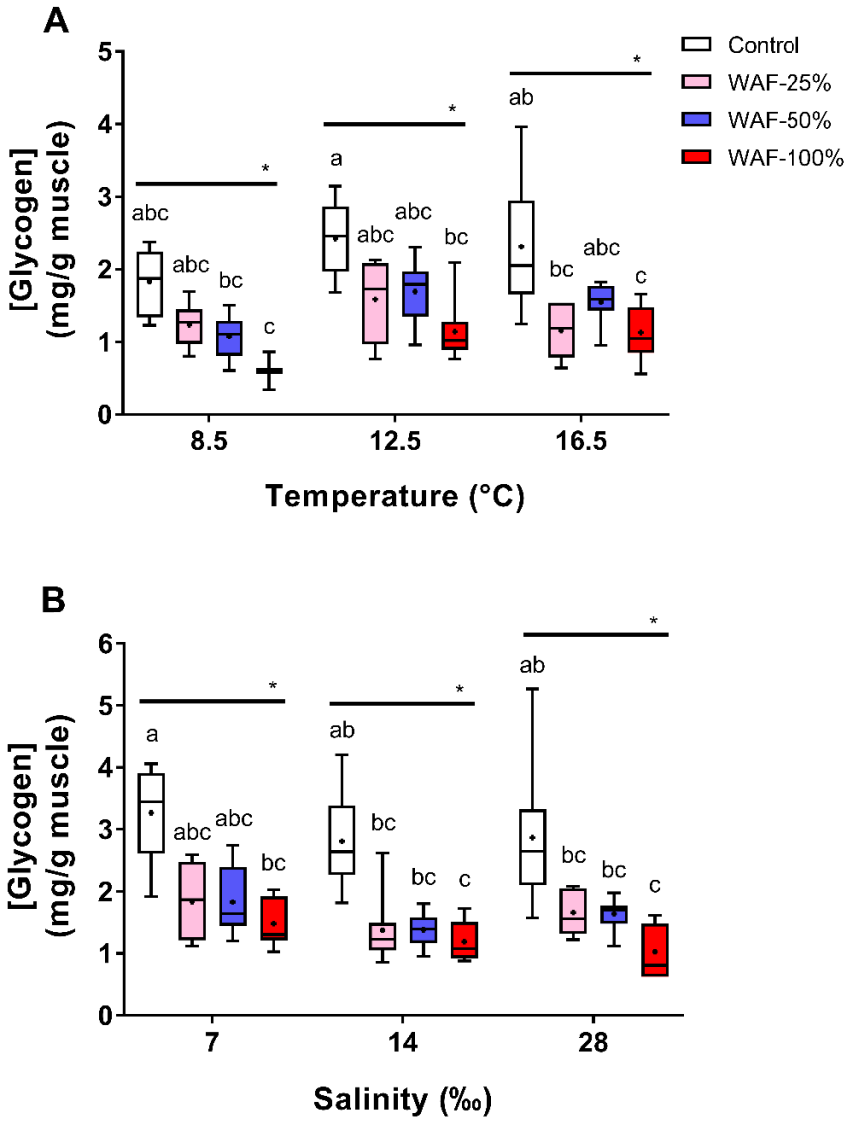

Figure 4. 4. White muscle glycogen content of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

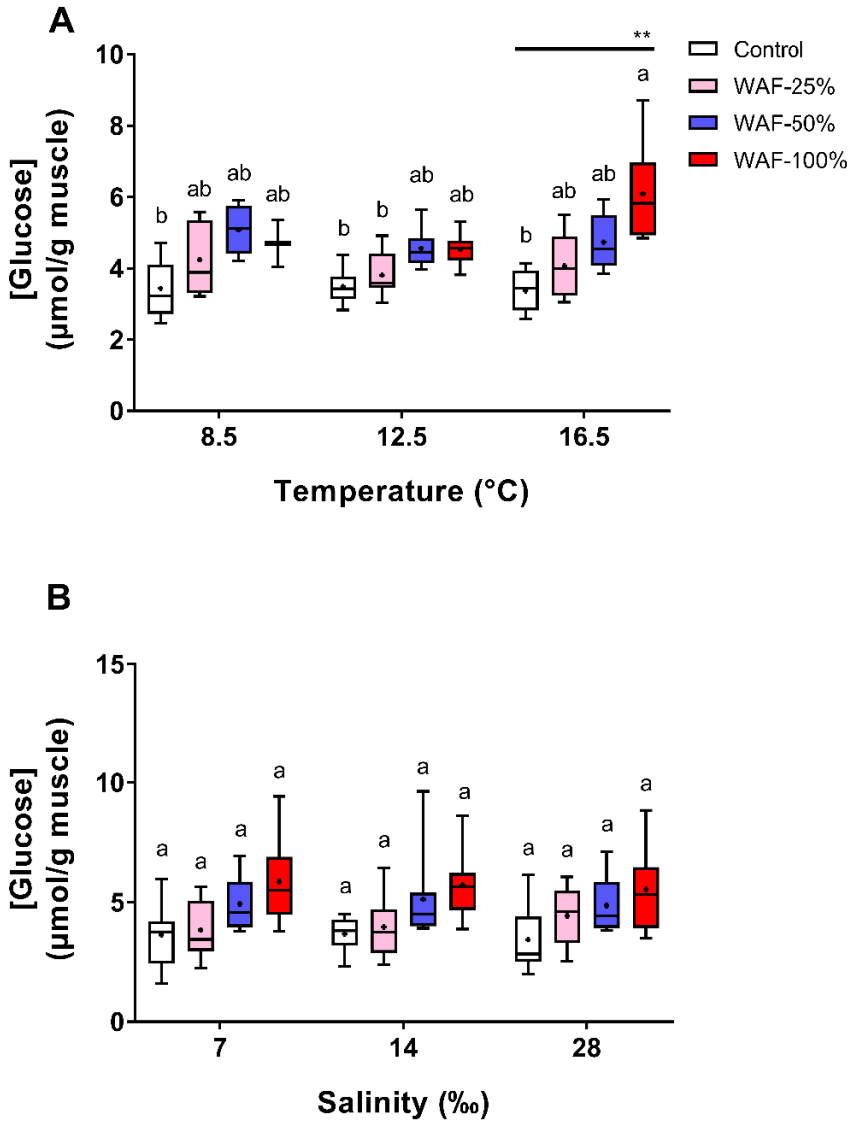

Figure 4. 5. White muscle glucose content of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

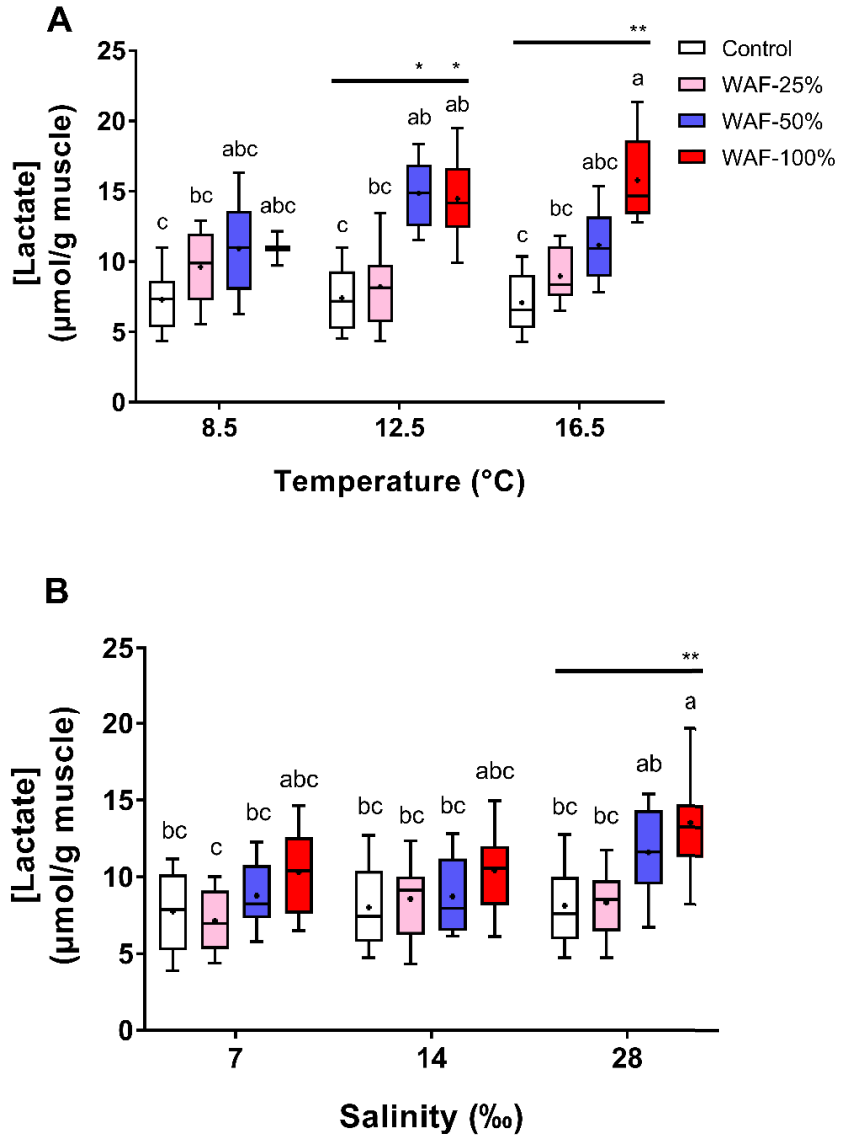

Figure 4. 6. White muscle lactate content of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows data from fish exposed to 3 dilutions of CLWB-WAF at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

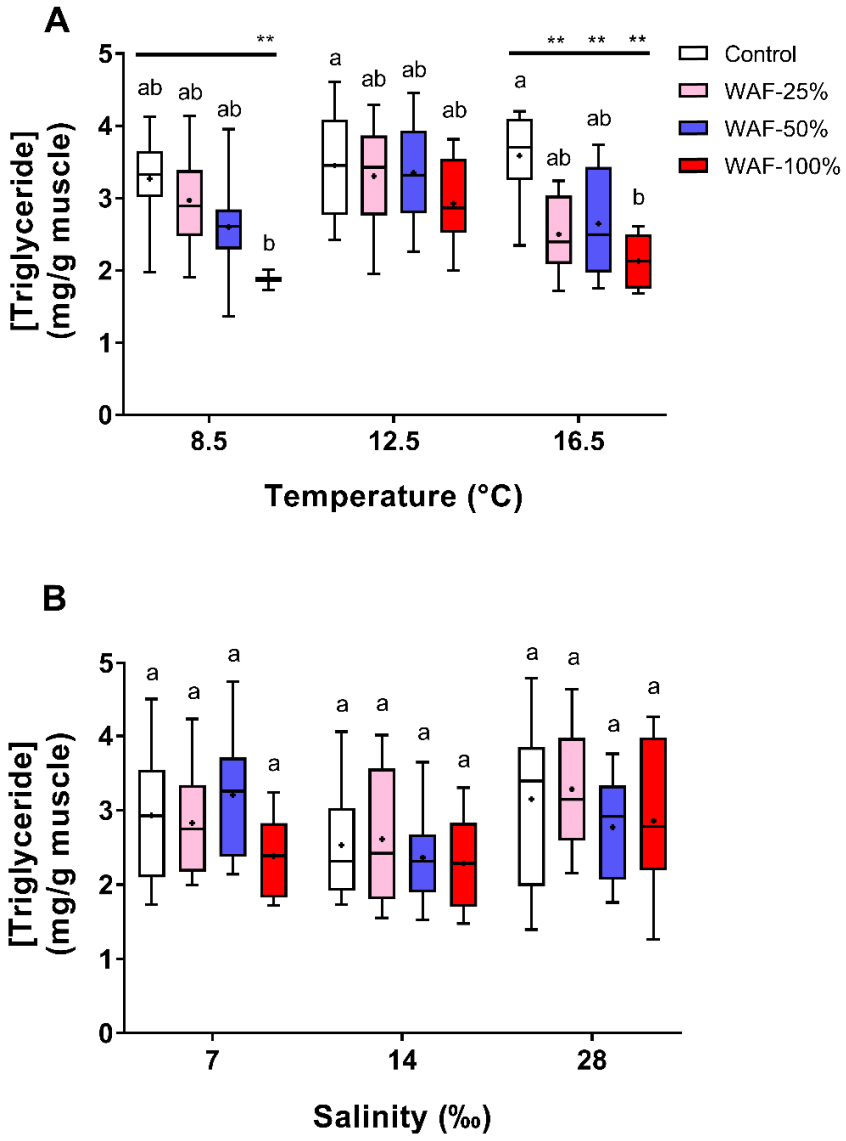

Figure 4. 7. White muscle triglyceride content of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. Panel A shows data from fish exposed to 3 dilutions of CLWB-WAFs at 3 different temperature conditions (8.5, 12.5, and 16.5 °C), while water salinity was kept at 28 ‰; panel B shows data from fish exposed to 3 dilutions of CLWB-WAFs at 3 different salinity conditions (7, 14, and 28 ‰), while water temperature was kept at 12.5 °C. Two-way ANOVA and Tukey's multiple comparison test were used to compare across treatments. Within each plot, ✚ indicates mean, boxes that do not share a common letter are statistically different ($p < 0.05$). Within a salinity or temperature group, an asterisk indicates a difference with their respective control (*, $P < 0.05$; **, $P < 0.01$). Note: n = 2 for 8.5 °C CLWB-100% group; n = 6 for 16.5 °C CLWB-100% group; n = 7 for 28 ‰ CLWB-100% group; the rest of groups all had n = 8.

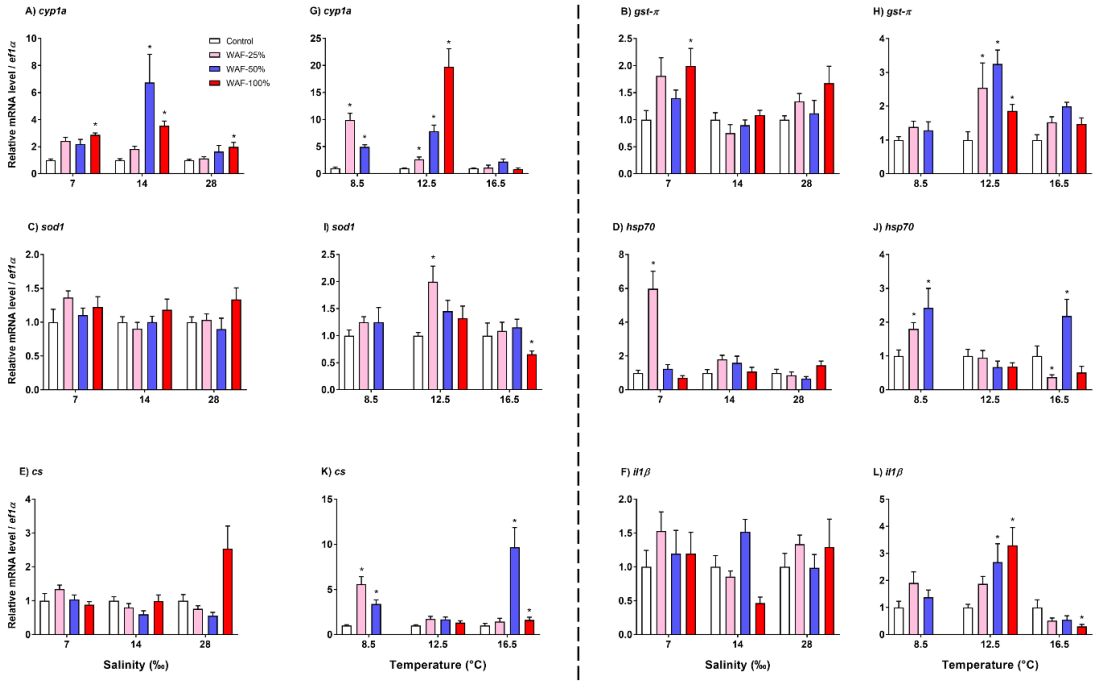

Figure 4. 8. The effect of WAF of CLB dilbit and salinity treatment on the regulation of A) *cyp1α*, B) *gst-π*, C) *sod1*, D) *hsp70* and E) *cs* and F) *il1β* and WAF and temperature treatment of G) *cyp1α*, H) *gst-π*, I) *sod1*, J) *hsp70* and K) *cs* and L) *il1β* mRNA level on juvenile pink salmon fish. As in 8.5 °C condition, one group was missing due to the lack of individual (n = 2), a one-way ANOVA was used between each condition with their respective control, in each salinity and temperature condition groups ($p < 0.05$). Data are mean ± SE for n = 5-8 fish. Within a salinity or temperature group, an asterisk indicates a difference with their respective control.

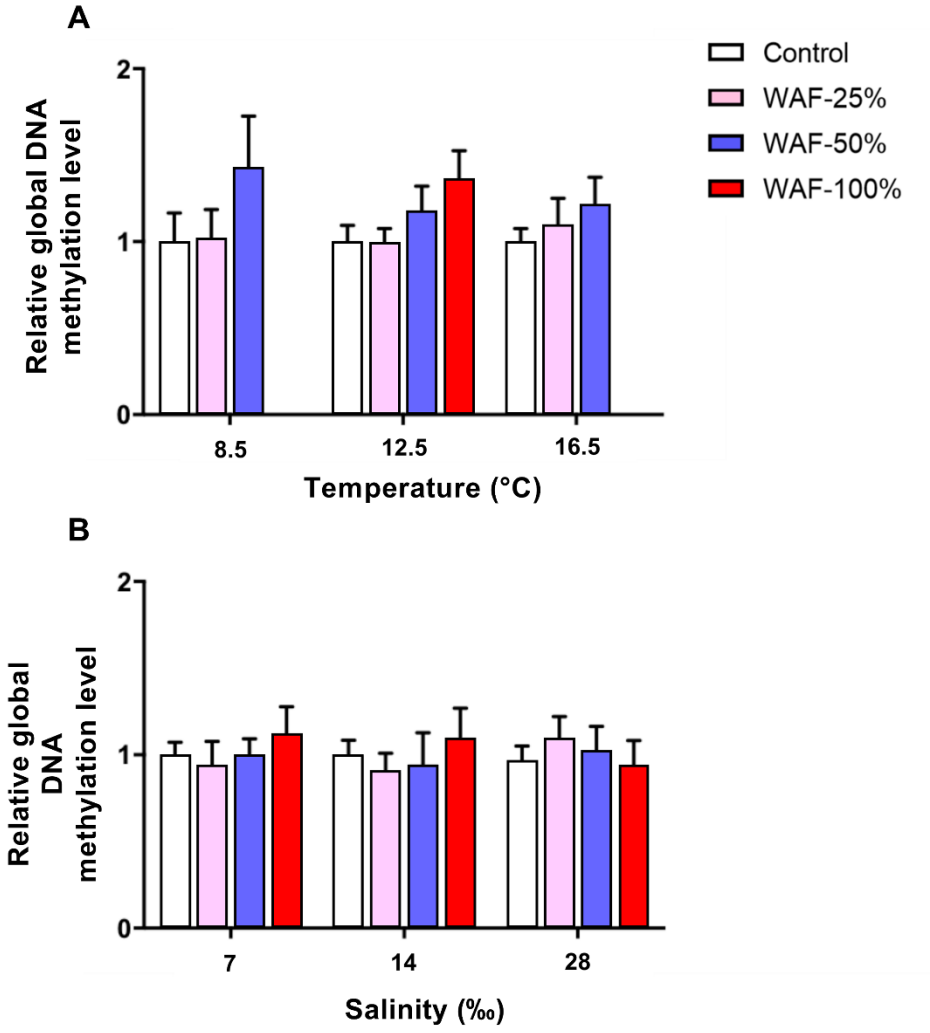

Figure 4. 9. Change in relative global CpG methylation level in fish exposed to WAF of CLB dilbit at varying A) temperatures or B) salinities. Data are expressed as mean ± SE for n = 5-8 fish. No statistical changes were measured between treatments.

Chapter 5. The effects of diluted bitumen on sockeye salmon (*Oncorhynchus nerka*) following an embryo to juvenile-stage exposure

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Effects of diluted bitumen exposure on juvenile sockeye salmon: From cells to performance. Environmental Toxicology and Chemistry, 36 (2017): 354-360. https://doi.org/10.1002/etc.3533

Abstract

The existing and recently proposed expansions in the transportation of diluted bitumen (dilbit) products *via* pipeline, railway, and marine terminals in coastal regions of British Columbia increase the risks of exposure to early life stage (ELS) Pacific salmon. To increase the understanding of the hazard dilbit poses to salmon ELS, fertilized embryos were exposed to 4 concentrations (initial total polycyclic aromatic compounds [TPAC] concentrations of 0, 13.7, 34.7, and 124.5 µg/L) of the water-soluble fraction (WSF) of Cold Lake Blend dilbit (winter) through embryonic development and was terminated when fish reached 8 months of age. Transcript abundances of *edn1a* and *cyp1a* were quantified in alevin head regions (containing the heart), swim-up fry, and ELS fish. There was a concentration-dependent increase in the expression of *cyp1a* at all life stages, with the most pronounced increase occurring in the hearts of ELS fry, and the smallest response occurring in swim-up fry. In contrast, *edn1a* was modestly increased in the hearts of swim-up fry exposed to the higher concentrations of dilbit. Fish exposed to the two higher WSFs exhibited increased mortality, impaired growth, as well as reductions in both critical (U_{crit}) and burst swimming speed (U_{burst}) in free-swimming fry. These effects correlated with alterations in energy substrate reserves at all stages, including significant reductions in soluble protein and glycogen content, as well as significant elevations in whole body lipid, triglyceride, and cortisol concentrations. Post-exercise body composition data suggest that exposure to the higher WSF concentrations result in an interference in the utilization of lipid energy sources in fish resting or exercising fish, as well as a potential impairment in the ability of these fish to mount a physiological stress response.

Keywords: Oil sands; diluted bitumen (dilbit); crude oil; toxicity; early life stages (ELS); sockeye salmon; swimming performance; gene expression; biochemistry

5.1. Introduction

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

Dilbit is a mixture composed of various petrogenic hydrocarbons (e.g., benzene, ethylbenzene toluene xylenes [BETX], polycyclic aromatic compounds [PACs], naphthenic acids) with demonstrated toxicity to fish; acute and sublethal effects following exposure to crude oils or their constituents include developmental defects at early life stages (ELS), impaired growth, reductions in reproductive capacity, changes in behavior, alterations in biochemistry and gene expression, suppressed immune function, genetic damage, and endocrine disruption (Dupuis and Ucan-Marin, 2015; Kennedy, 2014; NASEM, 2016). The unique environmental fate and behavior of dilbit results in expensive and challenging post-spill habitat recovery and clean-up (Dew et al., 2015; Alsaadi et al., 2018a) and have the potential to result in substantial impacts on the

aquatic ecosystems. For example, it is estimated that up to 30% of the residual oils (approximately 0.96 million L) from the Kalamazoo River dilbit spill (Marshall, MI) still remain in the adjacent river system and are associated with sediments, following years of clean-up efforts (U.S. EPA, 2013). The tendency for dilbit to become entrained in freshwater environments, make it necessary to investigate the sublethal / chronic effects on aquatic biota under prolonged exposure conditions (Alderman et al., 2018).

Limited studies exist on the toxicity of dilbit to fish species in general (Dupuis and Ucan-Marin, 2015; NASEM, 2016). Compounding uncertainties regarding potential dilbit hazards, hydrocarbon profiles vary greatly between petroleum products or blends, making it difficult to predict dilbit toxicity from existing data from studies on other crude oils (NASEM, 2016). The majority of existing literature has been focused on the developmental and molecular responses following exposure in embryo and larval fish (Alsaadi et al., 2018b; Madison et al., 2015a, 2017; McDonnell et al., 2019; Philibert et al., 2016) with few studies directly investigating effects on older life stages (e.g., fry and juveniles).

Proposed and existing pipeline and rail routes transects hundreds of salmon-bearing streams and rivers in the Fraser River Watershed, BC, and oil tankers will traverse the lower Fraser River estuary (Levy, 2009; RCF, 2018). As one of the most productive salmon migration routes in the world, the Fraser River watershed and its estuary also serves as vital spawning and nursery habitat for all five species of Pacific salmon (Henderson and Graham, 1998; Labelle, 2009). There is increasing body of evidence suggesting that dilbit exposure can negatively affect the survival, early development, and critical physiological systems of Pacific salmon. These effects include delayed hatching time, mortality during embryonic development, deformities, impairment of growth and alteration of body composition (Alderman et al., 2018). Older ELS are also affected by dilbit exposure. For example, exposed 1+ year-old sockeye exhibited altered gene expression decreased swimming ability, alterations in cardiac tissues, and the plasma proteome (Alderman et al., 2017a; Alderman et al., 2017b). The objective of this study was to investigate the effects of a chronic dilbit exposure on developing sockeye (survival, growth, and biochemical endpoints). In addition, the immediate and latent

effects on locomotory performance was examined, including measures of pre- and post-exercise biochemistry.

5.2. Materials and Methods

5.2.1. Fish

Sockeye gametes were obtained from Upper Pitt River Hatchery (Fisheries and Oceans Canada) and fertilization performed at Simon Fraser University according to standard procedures (OMNR, 2009). Fertilized embryos were immediately distributed to, and incubated in, Heath trays (MariSource, Fife, WA) (372 embryos per tray; mean mass 0.24 g ± 0.11 SD). Embryos were supplied with dechlorinated municipal water (flow rate 6 L/min; dissolved O_2 > 95% saturation, hardness 6.12 mg/L $CaCO_3$, DOC < 1 mg/L, pH 7.0) at 11.3 °C without light until the swim-up fry stage (no visible external yolk sac). Mortality was recorded daily under red light, and dead embryos were immediately removed. Phenotypically normal swim-up fry were collected from rearing trays and transferred to 250 L fibreglass tanks supplied with water as above at 13 °C (flow rate of 7.5 L/min and 12h light:12h dark photoperiod). Fry were fed 5% body weight/day commercial salmonid feed (Skretting Canada, Vancouver, BC) and was increased weekly according to a growth equation that included a feed conversion efficiency of 20% (Meador et al., 2006) until they were 8 months of age (8M). The care and use of all fish were approved by the University Animal Care Committee at Simon Fraser University following Canadian Council on Animal Care (CCAC) guidelines.

5.2.2. Diluted Bitumen Exposure

The WSF of dilbit was generated using an apparatus previously described (Alderman et al., 2017b; Kennedy and Farrell, 2005). In brief, Siproax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) were soaked (controls were not) in unweathered Cold Lake blend (CLB) summer dilbit (COOGER, Fisheries and Oceans Canada) for 24 h (‘charged’), and placed into PVC columns (16 cm diameter x 80 cm length) supplied with continuous flow of dechlorinated municipal water (6 L/min). By varying the amounts of dilbit-coated ceramic beads in generator columns, 4 WSF

concentrations were achieved in duplicate; columns were recharged every 14 d. Water containing the WSF of dilbit was pumped into 500 L fiberglass header tanks, and then distributed into Heath trays. Embryos were exposed to dilbit in duplicate Heath stacks immediately post-fertilization until fish reached the swim-up stage. At this stage, phenotypically normal fish were transferred into 200 L fiberglass tanks (n = 200 fish in each duplicate tank) supplied with water containing dilbit, and exposed for a further 90 d until fish were 8 months of age (an 8 month total exposure). The detailed experimental design is depicted in Figure 1. Water samples were collected from duplicate header tanks at 0, 7, and 14 d after initiation of WSF generation and was analyzed for individual PACs using GC–MS (SGS Axys Analytical Services Ltd., Sidney, BC) as previously described in Alderman et al. (2017b).

5.2.3. Tissue Collection

At 94, 147 and 237 d post-fertilization (dpf), developing alevins (n = 35), swim up fry (n = 44), and ELS fry (n = 44) were randomly sampled from each exposure group, and euthanized using buffered MS222 solution (1 g/L) and weighed and lengthed. This sampling was repeated at 147 d and 237 d following the swim test. For one subset of fish, whole bodies were immediately snap-frozen in liquid nitrogen, transferred to -80 °C for body lipid and triglyceride content measurement. From another subset of fish, the head region of alevins (bisected at the rostral boundary of the yolk sac and perpendicular to the body axis) or the isolated hearts (fry) were individually snap-frozen in liquid nitrogen, and stored at -80 °C until RT-qPCR analysis (n = 8 per developmental stage).

5.2.4. Swim Tests

Swim performance (critical swimming speed [U_{crit}] and burst swimming speed [U_{burst}] tests [Farrell, 2008; Oschoff et al., 2014]) was determined in swim-up and 8M fry (n = 10 from each treatment) using a mini swim tunnel system (Loligo® Systems, Tjele, Denmark). The swim tunnel system (1.5 L cylindrical glass chamber submerged inside a reservoir) was temperature regulated by a custom chilled bath circulator, and DO was maintained at > 95% by constant aeration. Water velocity was calibrated daily using

slow-motion video and dye injection. The swim tunnel was isolated to avoid disturbance; monitoring and the velocity ramp protocol was conducted remotely.

For the U_{crit} test, fish were transferred to the swim tunnel and acclimated for 20 min at a water velocity of 1.5 body length per second (BL/s). Water velocity was then increased by 1.5 BL/s every 20 min until fish were exhausted (Farrell, 2008). The U_{burst} test (Farrell, 2008; Nendick et al., 2009) began with a 20 min acclimation period in the tunnel at a water velocity of 1.0 BL/s. Water velocity was rapidly increased to 2 BL/s over a 1 min interval, and was subsequently increased by 0.5 BL/s every 1 min. Both tests were complete when exhausted fish were inactive on the rear baffle for over 2 s and would not resume swimming after a brief decrease in water velocity. Fish were immediately removed from the tunnel, euthanized in buffered MS 222 and wet weight (g) and fork length (cm) recorded. Euthanized U_{crit}-tested fish were snap-frozen in liquid nitrogen, and immediately transferred to -80 °C for post-exercise body composition analysis as above. U_{crit} and U_{burst} were calculated according to Farrell (2008). The cross-sectional area of all swim-tested fish were found to be less than 10% of swim tunnel cross-sectional area and fish density was under 0.2 g/L, therefore U_{crit} and U_{burst} values were not corrected for solid blocking effects (Bell and Terhune, 1970).

5.2.5. Biochemical Measurements

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

Total soluble protein content was quantified in pre-weighed whole fish that were thawed on ice and homogenized in 9 volumes of lysis buffer (0.5 M Tris-HCl and 0.1 mM EDTA at pH = 8) using a Mixer Mill homogenizer (Model MM 300; Qiagen, Mississauga, ON) (Cassidy et al., 2016). Crude homogenates were centrifuged at 13,000 *x g* at 4 °C

for 60 min, and the soluble protein content in supernatants measured using a Bradford protein assay kit with bovine serum albumin as the standard (Catalog# 5000002; Bio-Rad, Mississauga, ON).

For whole body pre- and post-exercise cortisol, glycogen, triglyceride, lactate concentrations, pre-weighed alevin or fry were thawed on ice and homogenized in 0.2 M sodium citrate buffer at pH = 5 (Catalog# 200-675-3; EMD Chemicals Inc., Gibbstown, NJ) using a Tissue Tearor (Fisher Scientific, Houston, TX). Each crude homogenate sample was aliquoted into separate microcentrifuge tubes and stored at -80 °C until subsequent analysis. Glycogen concentration was determined following a standard protocol (Weber et al., 2008) using Type IX bovine liver glycogen as a standard (C940M53; Sigma-Aldrich, Oakville, ON). Whole body triglyceride concentration was determined following the microplate spectrophotometric assay protocol as described in Weber et al. (2003). Whole body lactate content of each fish was determined using a commercial colorimetric assay kit (Catalog# 120001400P; Eton Bioscience, San Diego, CA) performed according to the manufacturer's protocol. Total cortisol concentration was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Catalog# EA65; Oxford Biomedical Research, Oxford, MI) (McPhee and Janz, 2014). All colorimetric assays were performed in duplicate using an Epoch™ 2 microplate spectrophotometer (Bio-Tek, Winooski, VT) and Corning® 96-well microplate (Greiner Bio-One International, Monroe, NC).

5.2.6. RT-qPCR Analysis

Transcript abundances of *edn1a*, *cyp1a*, and *rpL8* were quantified in alevin head regions (containing the heart), swim-up fry, and ELS fry following standard quality control guidelines (Bustin et al., 2009), and using total RNA extraction, cDNA synthesis, and RT-qPCR methods exactly as previously described (Alderman et al., 2018). Primer sequences were: *edn1a* (F, R, efficiency); *cyp1a* (F: tcatcaacgacggcaaga, R: gttcaccaagcccaacag, 110% efficiency); *rpL8* (F: ttggtaatgttctgcctgtg, R: gggttgtgggagatgactg, 103% efficiency). Data were normalized to the abundance of the stably expressed reference gene, *rpL8*.

5.2.7. Statistical Analysis

No statistically significant tank effect was found (using either one-factor or two-factor ANOVA) in which tank was included as a random factor), therefore data from replicate tanks were pooled for all analysis. Mortality, body composition measures, and swim test data were analyzed using one-factor ANOVA and Tukey's multiple comparison test ($\alpha = 0.05$). The pre-exercise and post-exercise biochemical data from different treatment groups were combined and compared using two-factor ANOVA followed by a Tukey's multiple comparison test ($\alpha = 0.05$). Differences in transcript abundances were determined using one-factor (concentration) or two-factor (concentration x stage) ANOVA and Tukey's or Holm-Sidak multiple comparisons tests, respectively.

5.3. Results

5.3.1. Water Chemistry

Initial TPAC concentrations were 0.2 (control), 13.7 (low), 34.7 (medium) and 124.5 (high) µg/L. TPAC concentrations decreased over time, while concentration gradients were maintained across different WSF treatments. Individual PACs found in water samples and their concentrations are provided Lin et al. (2020).

5.3.2. Mortality and Growth

Cumulative mortality from the embryo to swim-up fry exposed to the low WSF concentration was not statistically different from controls (Figure 5.2), however the medium and high WSF groups exhibited significantly higher ($p < 0.05$) mortality during embryogenesis (13.0 ± 0.9% and 24.4 ±1.3%, respectively), compared to controls (6.3 ± 0.9%). At the alevin stage, fish from the high treatment group had significantly lower body mass compared to controls (Table 5.1). When fish reached the swim-up fry stage, significant decreases were observed in the body mass of fish in the medium and high treatment groups (reductions of 13.4% and 15.2%, respectively). Reduced fork length was consistently seen in WSF-exposed fish in a concentration-dependent manner. No difference was seen in mortality the post swim-up stage. Up to 8M, the body mass of fry

in the medium and high group remained significantly lower than controls. These reductions were similar to those seen in swim-up fry (16.7% and 13.4%, respectively).

5.3.3. Body Composition

Developing alevins exposed to dilbit exhibited increased total body lipid and triglyceride concentrations and a reduced total soluble protein content (Figure 5.3). The whole body lipid content of alevins in the medium and high treatment groups were 1.9- and 2.2-fold higher than controls ($p = 0.048$, $p = 0.015$). Triglyceride levels in these two groups were also elevated by 1.6- and 2.0-fold, respectively ($p = 0.034$, $p < 0.01$). Fish in these two exposure groups had lower total soluble protein levels (35% and 27%, respectively) ($p < 0.01$, $p = 0.025$).

Swim-up fry in the two higher treatment groups also exhibited increased total lipid content, which were 2.0- and 2.2-fold higher than in controls (Figure 5.4; $p < 0.01$, $p < 0.01$). In 8M old fry, only fish in the high exposure group had a higher lipid content (1.7-fold) compared to controls ($p < 0.01$). Whole body soluble protein levels were significantly decreased (by 42%) in swim-up (Figure 5.4; $p < 0.01$) and 8M old fry (by 27%) in the high treatment group (Figure 5.5; $p = 0.048$) compared to controls.

5.3.4. Swim Performance

Dilbit exposure affected the U_{crit} and U_{burst} of both swim-up and 8M fry. The U_{crit} value for swim-up fry in the medium and high treatments were approximately 20% lower than controls (Figure 5.6; $p < 0.01$, $p < 0.01$); for 8M fry, exposure to the highest dilbit concentration reduced U_{crit} by 22.8% compared to controls ($p < 0.01$). Decreases in U_{burst} were seen in swim-up fry in the medium and high groups (26% and 39%, respectively) compared to controls (Figure 5.7; $p < 0.01$, $p < 0.01$); for the 8M fry, exposure to medium and high WSF caused decreases in U_{burst} by 16.3% and 22.2%, respectively, compared to controls ($p < 0.01$, $p < 0.01$).

5.3.5. Pre- and Post- Exercise Biochemistry

Swim-up and 8M fry exposed to the medium and high concentrations of dilbit had higher pre-exercise baseline whole body cortisol concentrations compared to controls (range 1.7- to 2.7-fold) (Figure 5.8; $p < 0.01$, $p = 0.037$). In swim-up fry, the U_{crit} test caused a significant elevation in whole body cortisol concentration in controls (2.1-fold, $p = 0.049$) and fish in the low treatment group (2.2-fold, $p = 0.023$). In contrast, the post-exercise body cortisol concentrations in fish from the medium and high treatments were not different from pre-exercise baseline values. This lack of an exercise-induced cortisol increase was consistently observed in 8M fry exposed to all 3 dilbit concentrations.

In swim-up and 8M fry, the exhaustive U_{crit} test induced significant depletions in total body glycogen content in control and WSF-exposed fish (Figure 5.9). However, pre-exercise body glycogen reserves were consistently lower in those fish exposed to medium and high WSF of dilbit compared to controls. In 8M fry, exposure to the highest concentration of dilbit significantly reduced glycogen stores (2.0 ± 0.2 mg/g bw) compared to controls (3.4 ± 0.4 mg/g bw) before swimming ($p < 0.01$), but exhibited a marked decline in post-exercise glycogen (by 65.6%) compared to controls.

Significant increases in whole body lactate levels were observed in swim-up and 8M fry following the U_{crit} trial in control and exposed fish (Figure 5.10). Pre-exercise lactate levels of exposed fish were not different than controls, however, fish exposed to the highest concentration of dilbit exhibited higher accumulations of lactate than controls at both life stages ($p = 0.023$, $p = 0.019$).

Swim-up fry showed significant decreases in whole body triglyceride concentrations following exhaustive exercise in control fish and those from the low exposure treatment, however no difference was seen in the higher treatment groups post-exercise (Figure 5.11). Pre-exercised fish in the medium high treatment groups had significantly elevated body triglyceride levels (2.3- and 2.0-fold higher, respectively) compared to controls ($p < 0.01$, $p < 0.01$). At 8M, exposure to the high concentration resulted in a significant increase in the pre-exercise level of triglycerides ($p < 0.01$); the U_{crit} test did not result in a depletion of whole body triglyceride level in this group.

5.3.6. Molecular Responses

There was a concentration-dependent increase in transcript abundance of *cyp1a* at all life stages, with the most pronounced increase occurring in the hearts of ELS fry, and the smallest response occurring in swim-up fry (Figure 5.12A). However, when comparing *cyp1a* expression in the hearts of swim-up and 8M fry, baseline expression in unexposed control fish was 32-fold greater and the dilbit-induced increase at the highest exposure concentration was double at swim-up relative to 8 months old fry (Figure 5.12B).

The effect of dilbit exposure on the expression of *edn1a* was concentration and developmental stage dependent (Figure 5.13). There was no specific response to dilbit in alevin head regions, whereas edn1a was modestly increased in the hearts of swim-up fry exposed to the higher concentrations of dilbit. In contrast, *edn1a* was greatest in the hearts of ELS fry exposed to the lowest concentration of dilbit.

5.4. Discussion

The effects of dilbit exposure on developing sockeye survival was stage-dependent; the embryo to swim-up stage was more sensitive compared to that of emerged fry (full absorption of external yolk sac). Sensitivity was positively correlated with increasing dilbit concentration; the lowest TPAC concentration that resulted in mortality was 34.7 µg/L. Alderman et al. (2018) reported mortality in sockeye embryos at TPAC concentrations as low as 4 ug/L, however at similar concentrations (35 µg/L) mortality was much lower (8%) than was seen in the present study (13.0% and 24.4% at [TPAC]s of 34.7 µg/L and 124.5 µg/L, respectively). The exposure in Alderman et al. (2018) ended at 76 dpf, and thus the higher mortality in the present study may be due to the longer exposure of 147 d. These findings also suggest that a delayed response may occur at a later stage; this hypothesis is further supported by Alderman et al. (2018), in which fry exposed to [TPAC]s at 100.4 µg/L exhibited 40% mortality during the first 2 months of depuration. Similarly, developing pink salmon (*Oncorhynchus gorbuscha*) embryos exhibited approximately 20% higher mortality than a control group at initial aqueous [TPAH]s of 18.0 µg/L using a similar dosing method, and over 40% reduction in

survival in fish exposed to WSF containing initial [TPAH]s of 48.0 µg/L (Heintz et al., 1999). In contrast, dilbit WAF-exposed larval fathead minnow (*Pimephales promelas*, 7 to 12 d old) and inland silverside (*Menidia beryllina*, 10 to 14 d old) exhibited no acute lethal effects at [TPAH]s between 8 and 40 µg/L (Barron et al., 2018). Philibert et al. (2016) reported that the survival of zebrafish (*Danio rerio*) embryos was not affected by exposure to WAF of dilbit at TPAHs = 27.7 µg/L, and the estimated LC50 of dilbit WAF was lower than that for conventional crude oil. Low lethal toxicity has also been reported in fathead minnow, Japanese medaka (*Oryzias latipes*), and yellow perch (*Perca flavescens*) following exposure to dilbit WAF during embryonic development at [TPAH]s ≤ 100 µg/L (Alsaadi et al., 2018b; Madison et al., 2015a; Madison et al., 2017; McDonnell et al., 2019). These results indicate that Pacific salmon ELS, particularly developing embryos and alevin, are extremely sensitive to dilbit compared to other species, with increasing tolerance in fry and juveniles, results consistent with dilbit-exposed 1+ year sockeye parr at initial TPAHs concentration of 3.5 to 66.7 µg/L (for 1 week and 4 weeks) that did not result in mortality (Alderman et al., 2017a).

Alterations in body indices were observed in alevins following exposure to dilbit; the effects on growth persisted through exposure. Exposure to petroleum products impairs growth in salmonids in both freshwater and marine stages (Atlantic salmon, *Salmo salar* [Vignier et al., 1992], cutthroat trout, Oncorhynchus clarkia [Woodward et al., 1983], rainbow trout, *Oncorhynchus mykiss* [Lockhart et al., 1996], pink and chinook salmon (*Oncorhynchus tshawytscha*) [Meador et al., 2006; Wang et al., 1993]). Multiple mechanisms may directly or indirectly contribute to observed growth reductions including the suppression of feeding behaviour and decreases in food conversion efficiency (Moles and Rice 1983; Vignier et al., 1992), physiological stress (Kennedy and Farrell, 2005, 2006), compromised iono-osmoregulatory performance (Kennedy and Farrell, 2005; Kochhann et al., 2015), and elevations in metabolic rate (e.g., dos Santos et al., 2006; Klinger et al., 2015). The energetic expenditure required to compensate for oil-induced effects could result in reductions in energetic pliability, and reduce the total energy budgeted for growth.

Growth impairment correlates with stage-dependant changes in body composition suggesting that dilbit exposure alters energy metabolism and the allocation

of bioenergetic substrates negatively affecting growth. The availability of critical energy substrates (e.g., glycogen phosphoproteins, lipoproteins, triglycerides, fatty acids) can directly affect the success of embryonic development, survival, and early growth of alevins (Srivastava et al., 1991). Here, developing alevins and swim-up fry exposed to dilbit had significantly increased body lipid and triglyceride levels, and lower soluble protein and glycogen levels; providing indirect evidence that exposed fish experienced altered lipid metabolism capacity and/or an enhanced lipid synthesis, increased protein catabolism, and carbohydrate utilization. Similar changes in body energetic substrates were consistently seen in 8M fry. Disrupted catabolism of lipid storage and reductions in free protein levels have been previously documented in salmon alevins exposed to dilbit, alterations that have been linked to delayed development and shortened body lengths (Alderman et al., 2018). Transient exposure to North Slope crude oil (NSCO) during embryogenesis caused significant elevations lipid content (e.g. triacylglycerols, free fatty acids, sterols), reductions post-hatching body size, and survival of polar cod (*Boreogadus saida*) (Laurel et al., 2019). In developing Atlantic haddock (*Melanogrammus aeglefinus*), crude oil exposure may disrupt yolk lipid utilization through the downregulation of genes involved in the mobilization of lipoprotein-cholesterol in the yolk syncytial layer, and the upregulation of genes that control the biosynthesis of intrinsic cholesterol (Sørhus et al., 2017). It has been suggested that the dysfunction of lipid metabolism from yolk may be a consequence of the heart and circulatory system failing to deliver lipoproteins from the yolk in embryos and the intestine in larvae (Sørhus et al., 2017). Increased biotransformation demands in conjunction with compensatory requirements for repairing chemical damage or other physiological effects (e.g., stress) can be energetically costly (Whitehead, 2013). Disruptions of lipid utilization may result in the adoption of protein and carbohydrate as alterative substrates. In a recent study, developing mahi-mahi exposed to high-energy-WAF (HEWAF) of Deepwater Horizon crude oil (DWHCO) exhibited significantly increased oxygen consumption (MO_2) and excretion of nitrogenous wastes (Pasparakis et al., 2016) providing supporting evidence that elevated metabolic demands and increased energy requirements occur, and are possibly fueled via enhanced endogenous protein catabolism (Pasparakis et al., 2016). In addition, transcriptomic studies in larval mahi-mahi and red drum (*Sciaenops ocellatus*) have also revealed that

pathways involved in amino acids metabolism, protein digestion, and steroid biosynthesis are significantly altered following exposure to DWHCO (Xu et al., 2016, 2017). The possible effects on energy substrate metabolism following dilbit treatment may also explain the higher mortality that occurred in embryo to non-fed swim-up fry stages. ELS salmon solely rely on the nutrients stored in yolk sac until the swim-up stage where exogenous feeding begins (Heming and Buddington, 1988). Impaired lipid utilization and increased protein catabolism during this phase could directly result in supply deficiencies of these two critical nutritive groups. In comparison, 8M fry were fed since swim-up stage; the exogenous supply of these key nutrients may have mitigated the disturbance to nutrient allocation induced by dilbit exposure, and thereby causing increased mortality among WSF exposure groups and the control group, while the growth reductions were still evident in the medium and high WSF groups at this age.

Swimming impairment is considered a reduction in physiological fitness as it can directly determine salmonid early survival in freshwater or estuarine habitats, as well as the success of their future seaward migration (Eliason et al., 2011; Kieffer, 2000). Molecular responses, functional deficits, and morphological and histopathological alterations in the cardiorespiratory system during cardiogenesis have been well-described following dilbit exposure (Alsaadi et al., 2018b; Madison et al., 2015a, 2017; McDonnell et al., 2019) and crude oil (Brette et al., 2014; Edmunds et al., 2015; Incardona, 2017; Incardona et al., 2014; Jung et al., 2013; Khursigara et al., 2017) and correspond to decreased swimming capacity (Hicken et al., 2011; Incardona et al., 2015). Effects on swimming performance are also attributed to the molecular and histopathological alterations in cardiac tissues (Alderman et al., 2017b) and disruptions to cardiovascular capacity (Johansen and Esbaugh, 2017; Nelson et al., 2017; Stieglitz et al., 2016) following petroleum exposure in juvenile teleosts. While a clear link between cardiotoxicity and the impairment of swimming ability is widely accepted (e.g., Brett et al., 2014; Incardona et al., 2015), petroleum toxicity *via* interference in other physiological systems is less well understood. Oil- or PAH-induced adverse effects on other major physiological systems may also potentially contribute to the immediate and latent impacts on exercise capacity, for instance, altered internal stress responses and changes in the metabolism of energy stores (Kennedy and Farrell, 2006).

In the present study, exposure to dilbit resulted in decreased Ucrit in immediately emerged swim-up fry and in juveniles at higher concentrations. Similar results were seen in juvenile sockeye subchronically exposed to dilbit for 4 weeks; the suggested underlying mechanism was dilbit-induced remodelling of compact myocardium composition (Alderman et al., 2017a). Juvenile Pacific herring exposed to crude oil exhibited decreased Ucrit at concentrations of 40 and 100 µg/L TPAHs (Kennedy and Farrell, 2006). Philibert et al. (2016) compared the toxicity of CLB dilbit with mixed sweet blend (MSB) crude oil to developing zebrafish embryos. Alterations in shelter-seeking behaviour and continuous swimming were reported in hatched zebrafish exposed to dilbit containing 46.1 µg/L TPAH, whereas the threshold for those effects was much higher for the WAF of MSB at 206 µg/L TPAHs (Philibert et al., 2016). For conventional crude oil studies, reductions in immediate or latent swimming performance in various fish species were found at comparable TPAH concentrations (0.23 to 45 µg/L [Hicken et al., 2011; Incardona et al., 2015; Johansen and Esbaugh, 2017; Mager et al., 2014; Stieglitz et al., 2016]). In teleosts, routine, sustained, and prolonged swimming are primarily fueled by slow oxidative metabolism of triglycerides in the slow-twitch red skeletal muscles, whereas sprint and burst swimming are associated with glycolytic metabolism of glycogen in fast-twitch white muscle (Hammer, 1995; Moyes and West, 1995). The significant elevations in body triglyceride in exposed fish did not appear to provide advantages for swimming performance. The unchanged whole body triglyceride content in fish post-U_{crit} trial may reflect a decreased lipolytic capacity in exposed fish. It is plausible that a poorer utilization of lipidic energy sources during the aerobic portion of U_{crit} and diminished carbohydrate availability for final anaerobic bursting may have impaired U_{crit}. In comparison, a concentration-dependent reduction in U_{burst} was observed in emerged exposed fry, an effect which continued until fish were 8 months old. Since U_{burst} measures the maximal swimming speed maintained by fish within a short period of time, and is almost exclusively fueled anaerobically through the utilization of muscle glycogen, reductions in U_{burst} may be directly attributed to the lowered body glycogen stores prior to the swim trial. The greater accumulation of lactate and depletion of glycogen post-exercise in fish exposed to dilbit suggests a potentially enhanced anerobic metabolism during burst swimming.

Exposure to dilbit induced elevations in cortisol in free-swimming fry along with the other multiple effects seen in this study. These multiple adverse outcomes may be linked to the activation of a physiological stress response following exposure (Mommsen et al., 1999; Barcellos et al., 2010; Ramsay et al., 2006). Acute exposure to dilbit or crude oil results in increases in plasma cortisol concentrations, generally followed by a short-term hyperglycemic response (DiMichele and Taylor, 1978; Kennedy and Farrell, 2005, 2006; Lin et al., 2020), results consistent with the present findings. The stress response induced by crude oil exposure has been attributed to the irritant properties of the lighter, more volatile and acutely toxic components of oil, such as naphthalenes, BTEX, and naphthenic acids (Kennedy and Farrell, 2005, 2006; Thomas et al., 1980). Periodic refreshment of the dilbit source in the exposure system used here replenished these LMW hydrocarbons in exposure tanks, possibly resulting in repeated activation of neural pathways in the HPI axis.

Cortisol elevation can directly increase energy expenditure by escalating aerobic and anaerobic metabolism (De Boeck et al., 2001), suppressing growth rates, and reduce feeding and food conversion efficiency (Gregory and Wood, 1999; Madison et al., 2015b), all of which may be responsible for reduced growth in this study. Consistent with cortisol's key role in mediating the peripheral mobilization of energetic substrates during stress, dilbit-induced stress may have resulted in the catabolism of body carbohydrate, protein, and lipid reserves at whole body level. Cortisol elevation typically leads to an increase in the utilization of hepatic and muscle glycogen storage, as well as an inhibition of muscle glycogen restoration following exercise (Milligan, 2003; Mommsen et al., 1999), which may explain the lowered baseline glycogen level in dilbit-exposed fish. Cortisol can induce protein catabolism and decrease protein synthesis in white muscle and liver tissue (reviewed in Mommsen et al., 1999), leading to the reduced whole body soluble protein content seen in this study. However, elevated cortisol generally increases peripheral and hepatic lipolysis in chronically stressed fish (Mommsen et al., 1999), through increases in lipase activity (Baltzegar et al., 2014), increased glycerol utilization (Vijayan et al., 1991), and reductions in hepatic lipogenic potential (López-Patino et al., 2014). The higher lipid and triglyceride content in fish exposed to dilbit appear to conflict the general effects of high circulating cortisol levels.

Exhaustive exercise typically results in increased circulating cortisol levels, accompanied by a decrease in muscle glycogen stores, and short-term hyperglycemic responses (increases in plasma glucose and lactate concentration). Fish in control and low exposure groups exhibited these classical biochemical changes following the exercise test. However, fish exposed to the two higher dilbit concentrations had elevated baseline cortisol pre-exercise, levels which did not increase further in response to exercise. Previous studies have demonstrated that the stress from acute exposure to crude oil and exhaustive swimming were additive (Kennedy and Farrell, 2006; Thomas and Rice, 1987), but chronic exposures caused a decreased or muted cortisol response following exercise (Kennedy and Farrell, 2005, 2006). Similarly, Hontela et al. (1992) found that fish sampled from sites polluted by high level of PAHs and other organic contaminants showed an abolished cortisol response to acute stress when compared with fish from reference sites. Since the main role of cortisol-induced stress response is to supply immediate energy source for fuel-intensive behaviours and physiological processes, a deemphasized cortisol response can be considered maladaptive. Several hypotheses have been proposed to explain the lack of cortisol response with repeated, pulse exposures to dilbit: 1) hyperactivity of cortisol-producing cells associated with long-term xenobiotic exposure lead to HPI axis exhaustion (Hontela et al., 1997), and 2) chemicals acting directly as endocrine disruptors, targeting pituitary or adrenocortical tissues (Dorval et al., 2003) and affecting multiple sites in the HPI axis (Kennedy and Farrell, 2005). It also has been shown that exposure to PAH such as naphthalene cause severe necrosis in the interrenal tissues of mummichogs (Fundulus heteroclitus), linking tissue damage to reduced cortisol production during stress ((DiMichele and Taylor, 1978).

5.5. Conclusion

The present study simulated a prolonged dilbit exposure in freshwater utilizing a dosing system that generating a WSF containing PACs at environmentally relevant concentrations and examined the effects on sockeye ELS. Chronic exposure significantly reduced survival and growth, impaired both aerobic and anaerobic swimming performance, altered body biochemical composition (including pre- and post-

exercise body energetics), as well as cardiac gene expression, providing evidence that this complex mixture likely has multiple targets resulting in a complex suite of toxicological outcomes. Dilbit release into the natural habitat of Pacific salmon, under similar exposure scenarios are likely to produce adverse effects that will affect the viability and sustainability of local salmon populations.

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Table 5. 1. Wet weight and fork length of fish exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Time of exposure was 147 d for embryo to swim-up fry, and 90 d for swim-up stage to 7-month fry. Data are means ± S.E. for n = 20 fish. Bars that do not share a common letter are statistically different ($p<0.05$).

Life stage	Treatment	Body weight (mg)	Fork length (mm)	N
Alevin	**Control**	182.9 ± 2.0^{a}	22.6 ± 0.2^{a}	70
	Low	184.1 ± 2.1^{a}	22.2 ± 0.1^{a}	70
	Medium	178.9 ± 1.9^{ab}	22.4 ± 0.1^{a}	70
	High	173.3 ± 2.4^{b}	22.3 ± 0.2^{a}	70
Swim-up fry	**Control**	161.9 ± 0.9^{a}	28.8 ± 0.1^{a}	227
	Low	163.7 ± 0.7^{a}	28.4 ± 0.1^{b}	179
	Medium	140.2 ± 0.9^{b}	27.0 ± 0.1^{c}	177
	High	137.4 ± 1.6^{b}	26.9 ± 0.1^{c}	92
7M fry	**Control**	673.8 ± 6.9^{a}	39.7 ± 0.2^{a}	368
	Low	651.3 ± 6.9^{a}	39.3 ± 0.2^{a}	373
	Medium	561.5 ± 6.8^{b}	39.4 ± 0.2^{a}	367
	High	583.8 ± 7.1^{b}	39.6 ± 0.2^{a}	363

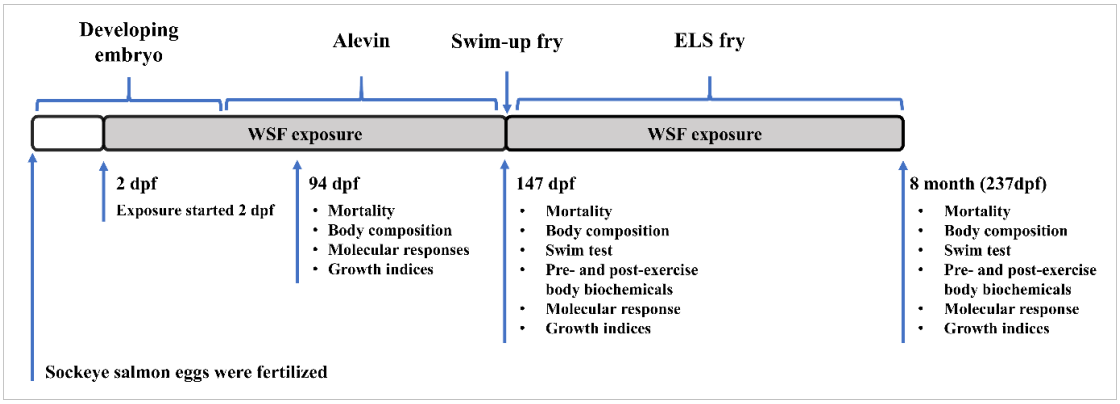

Figure 5. 1. Schematic representation of the experimental design.

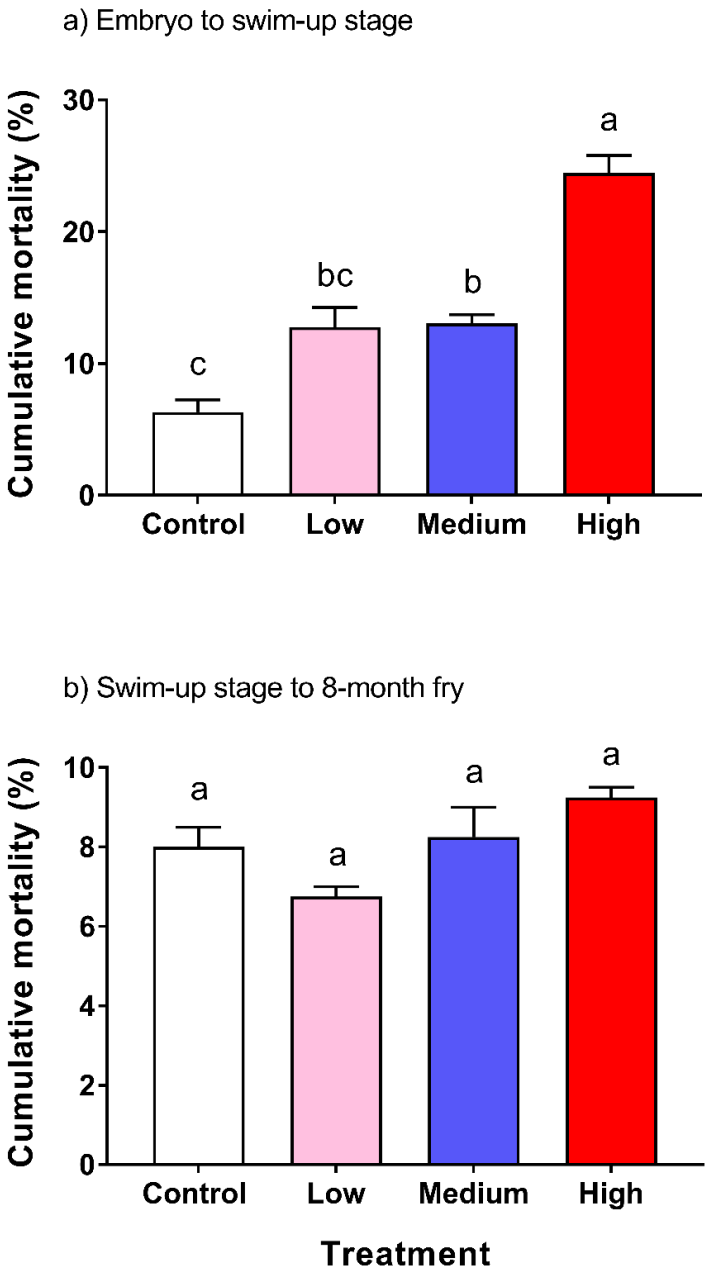

Figure 5. 2. Cumulative mortality of fertilized sockeye embryos exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Time of exposure was 147 d for embryo to swim-up fry, and 90 d for swim-up stage to 8-month fry. One-factor ANOVA and Tukey's multiple comparison test were used to test the effects of different WSF concentrations. Bars that do not share a common letter are statistically different ($p<0.05$).

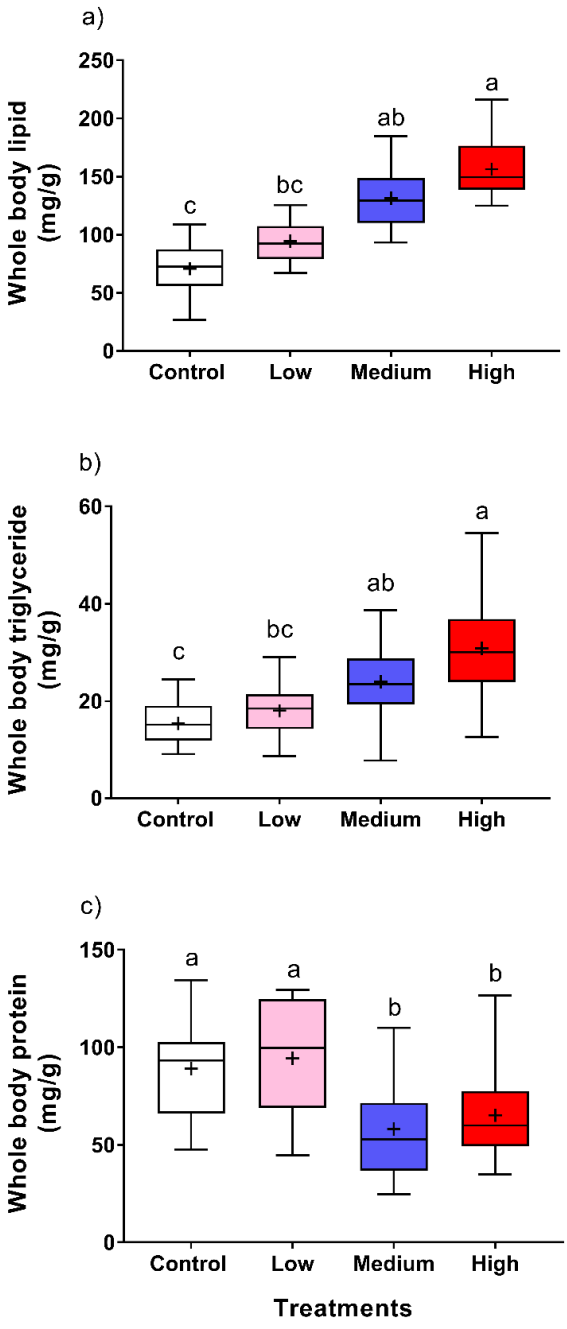

Figure 5. 3. Boxplots of whole body total (a) lipid, (b) triglyceride, (c) protein content in sockeye alevins exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 20 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

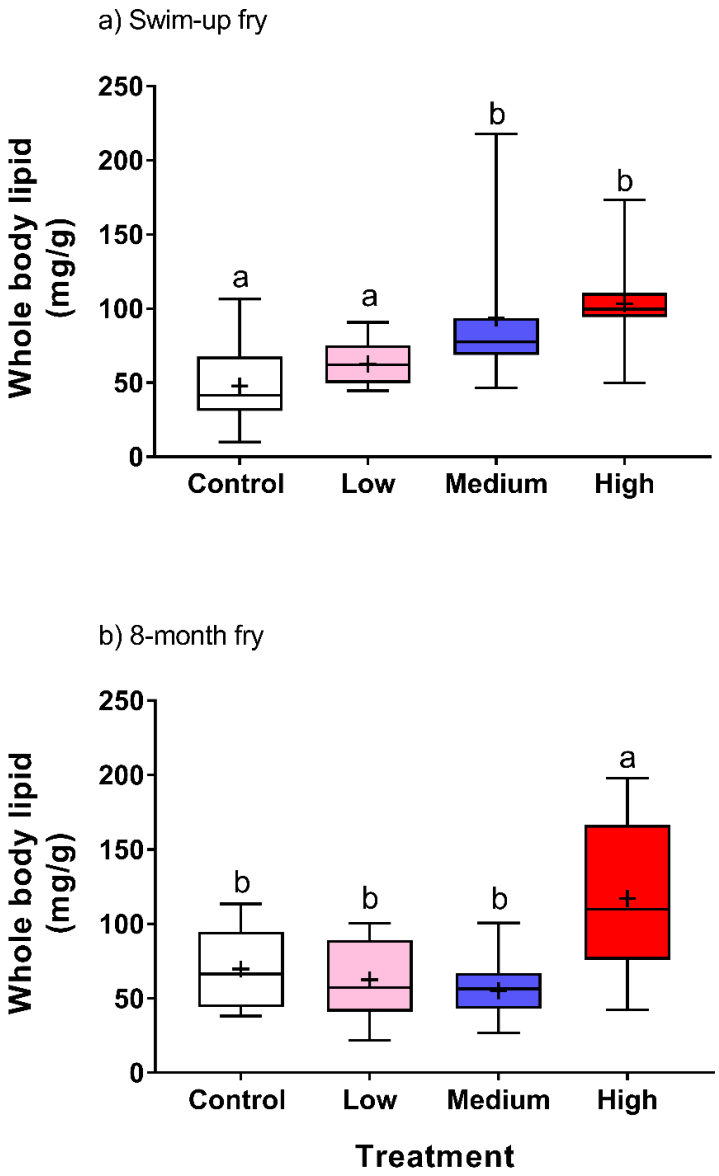

Figure 5. 4. Boxplots of total lipid content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, **+** indicates mean for n = 16 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

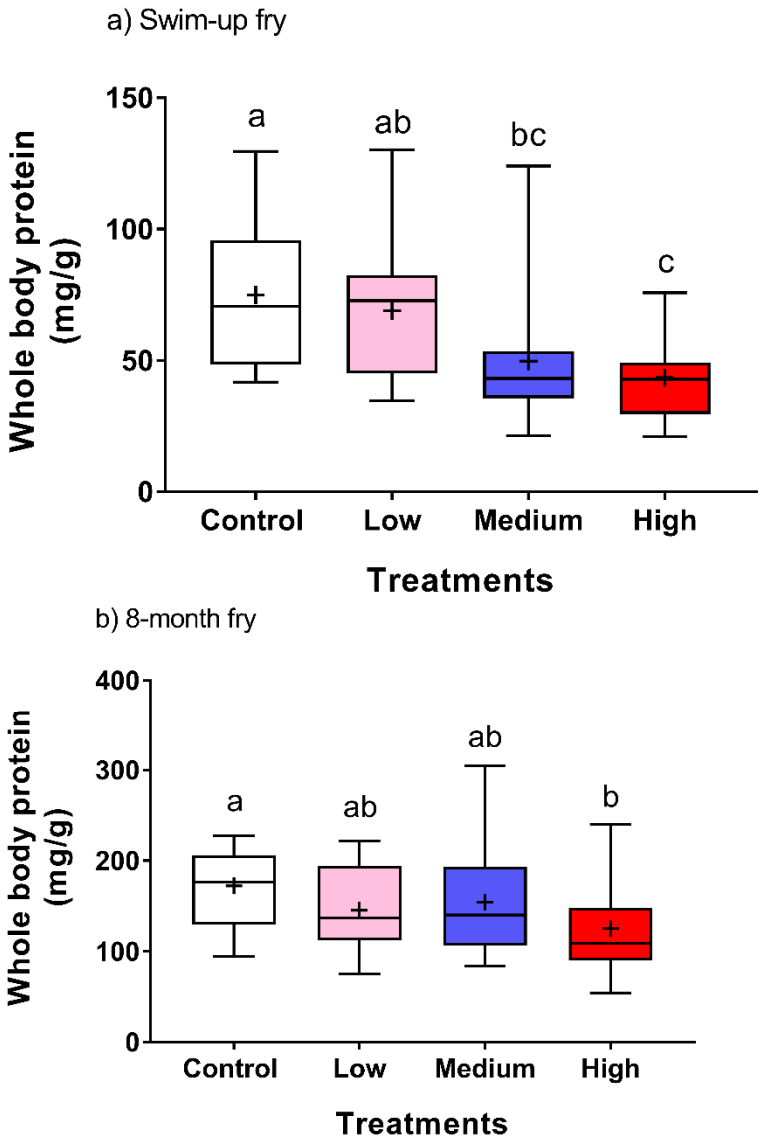

Figure 5. 5. Boxplots of total protein content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 16 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

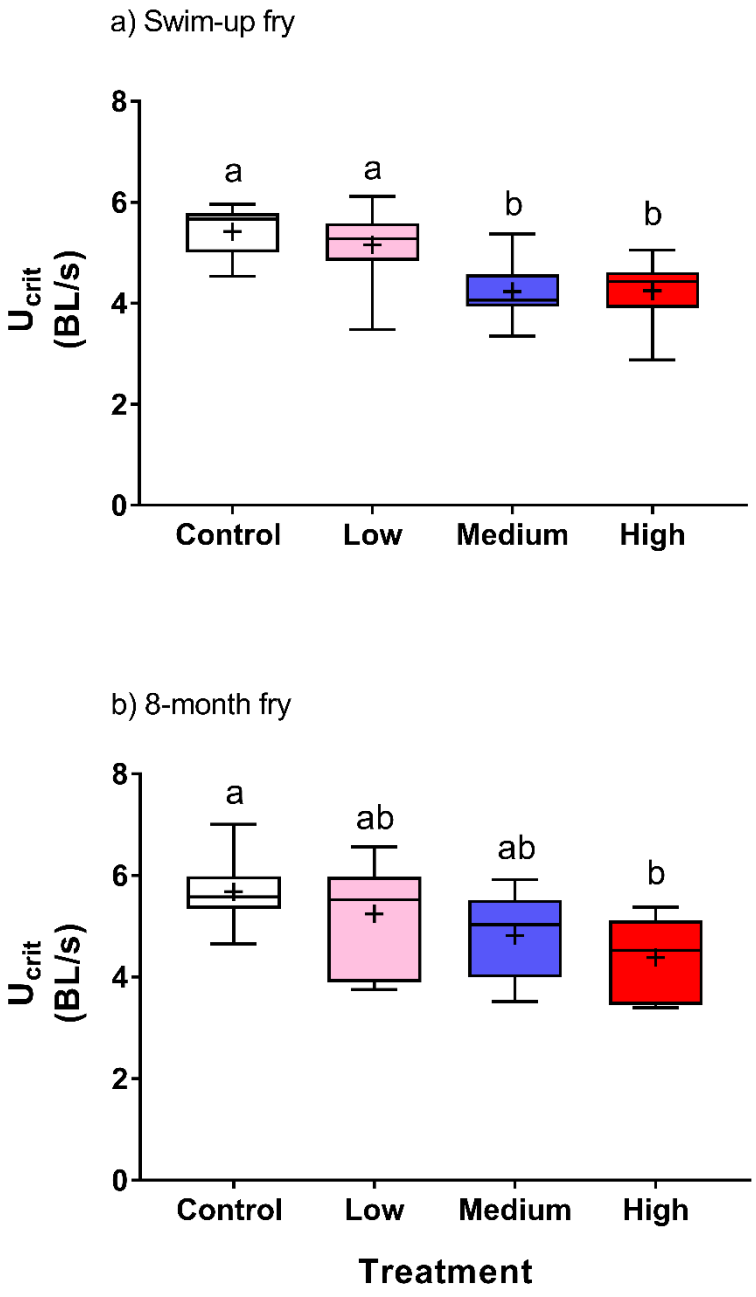

Figure 5. 6. Boxplots for U_{crit} of sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, **+** indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

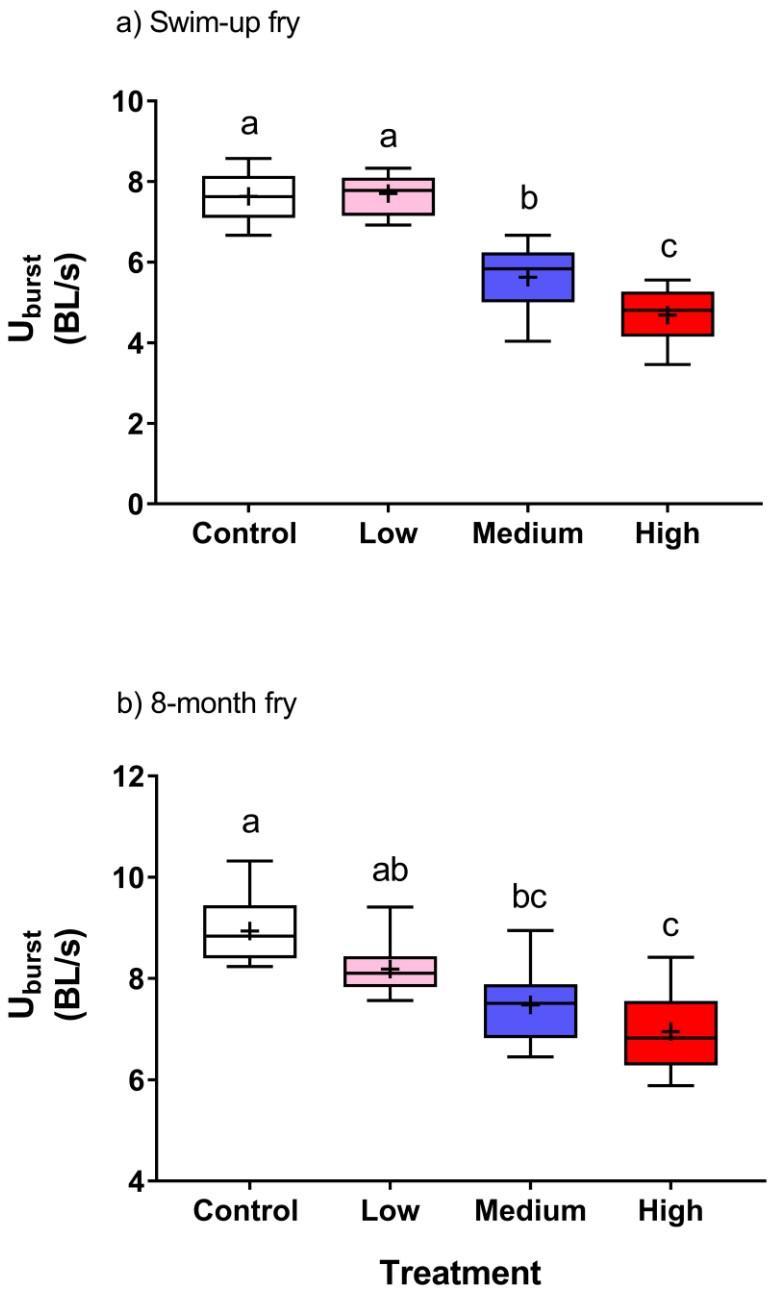

Figure 5. 7. Boxplots for U_{burst} of sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

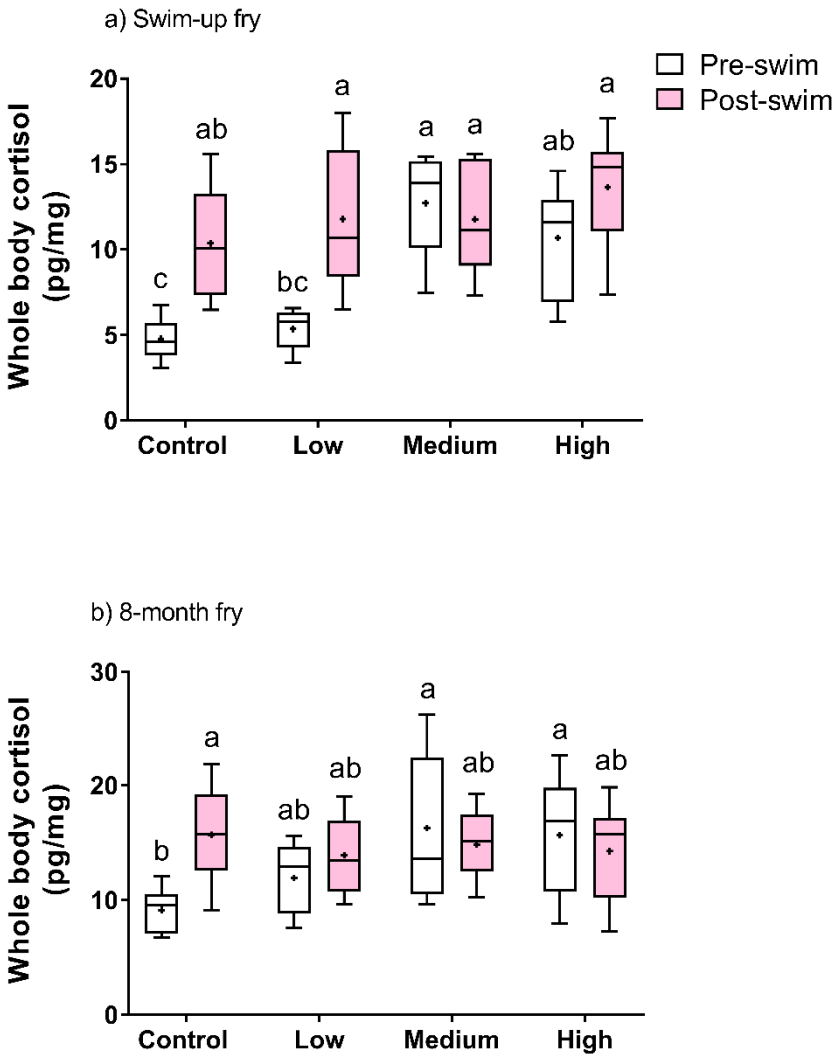

Figure 5. 8. Boxplots of pre-exercise and post-exercise whole body cortisol content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

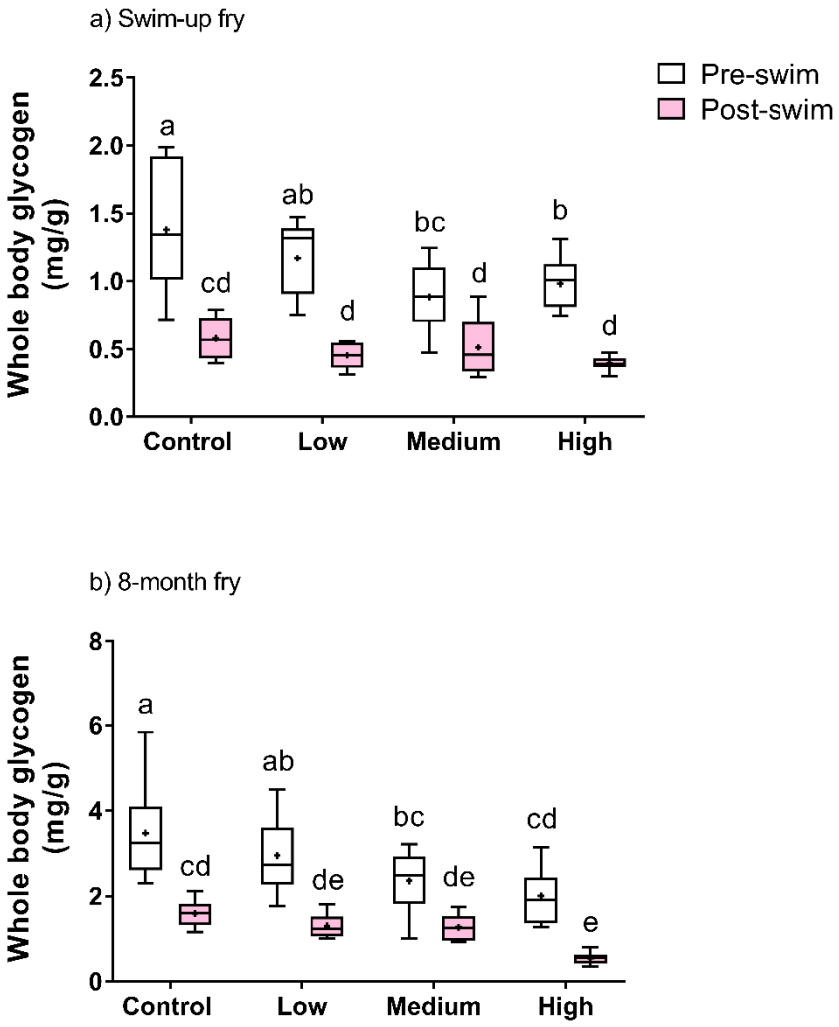

Figure 5. 9. Boxplots of pre-exercise and post-exercise whole body glycogen content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

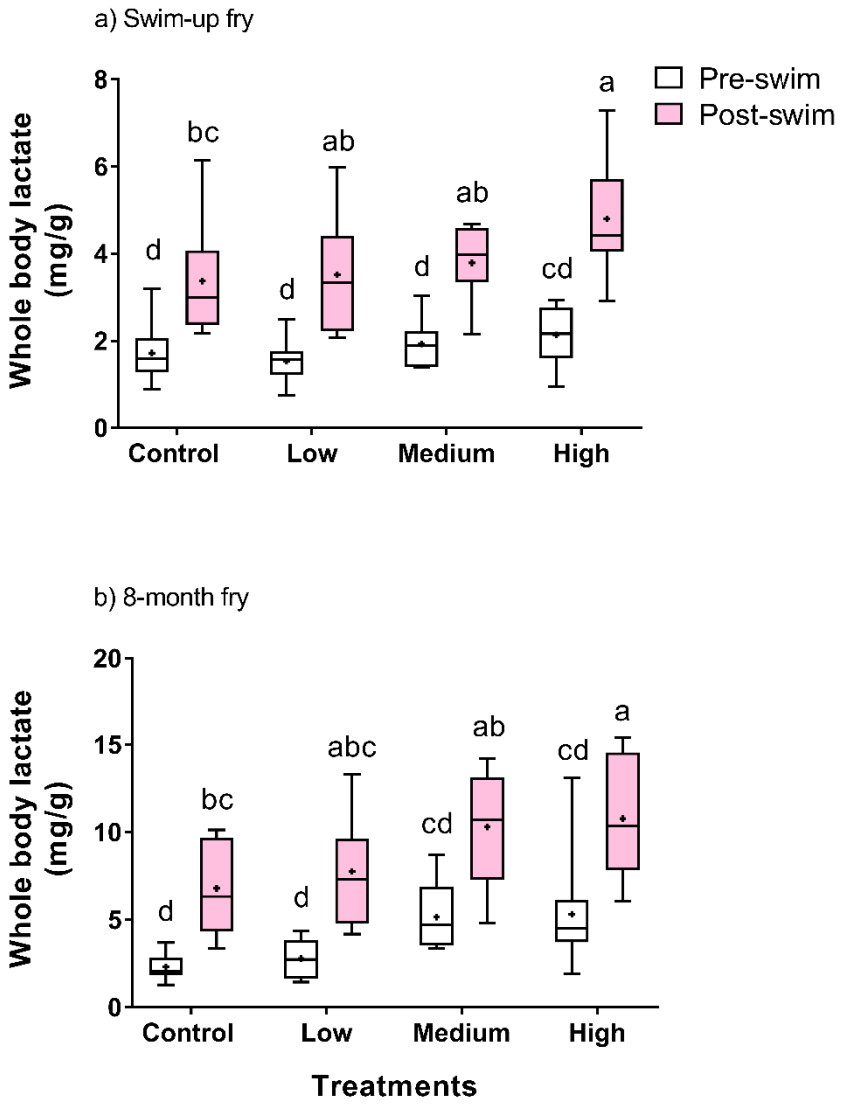

Figure 5. 10. Boxplots of pre-exercise and post-exercise whole body lactate content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

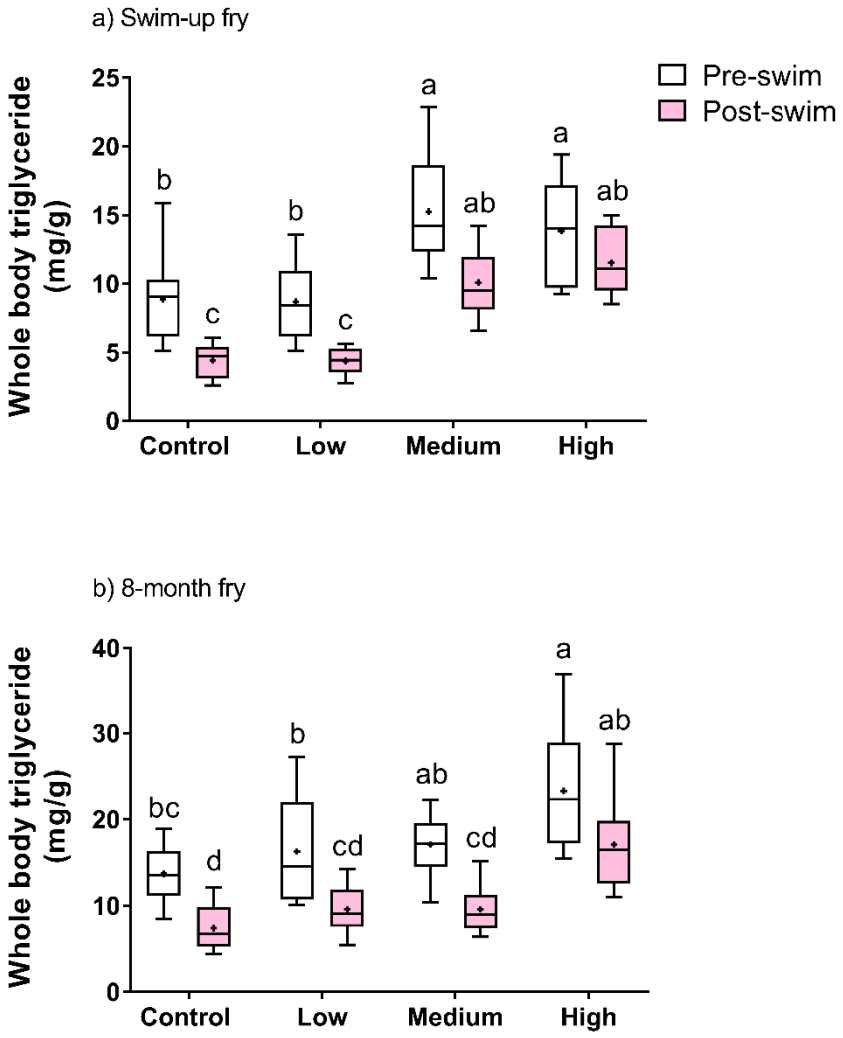

Figure 5. 11. Boxplots of pre-exercise and post-exercise whole body triglyceride content in sockeye (a) swim-up frys and (b) 8-month frys exposed to WSF of CLB dilbit. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Within each plot, ✚ indicates mean for n = 10 fish, boxes that do not share a common letter are statistically different ($p<0.05$).

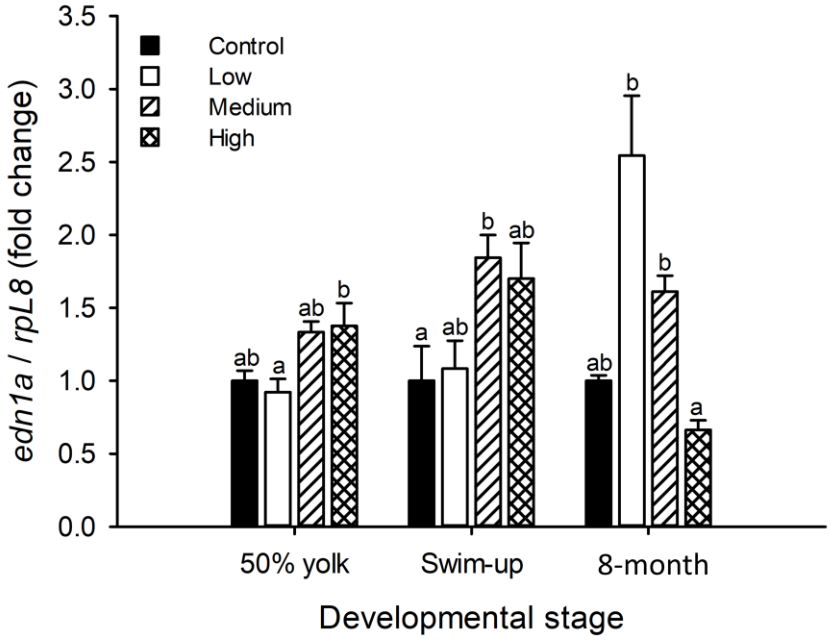

Figure 5. 12. Endothelin 1a (*edn1a*) expression in developing sockeye alevin head regions (50% yolk) or isolated whole hearts (swim-up, 8-month) exposed continuously to various concentrations of WSF, normalized to the housekeeping gene *rpl8*. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Expression is shown as fold-change from control (set to 1) at each developmental stage, with letters indicating significant changes (one-way ANOVA and Bonferroni post-hoc test; n=8; 50% yolk P=0.007, Swim-up P=0.016, 5 month p<0.001).

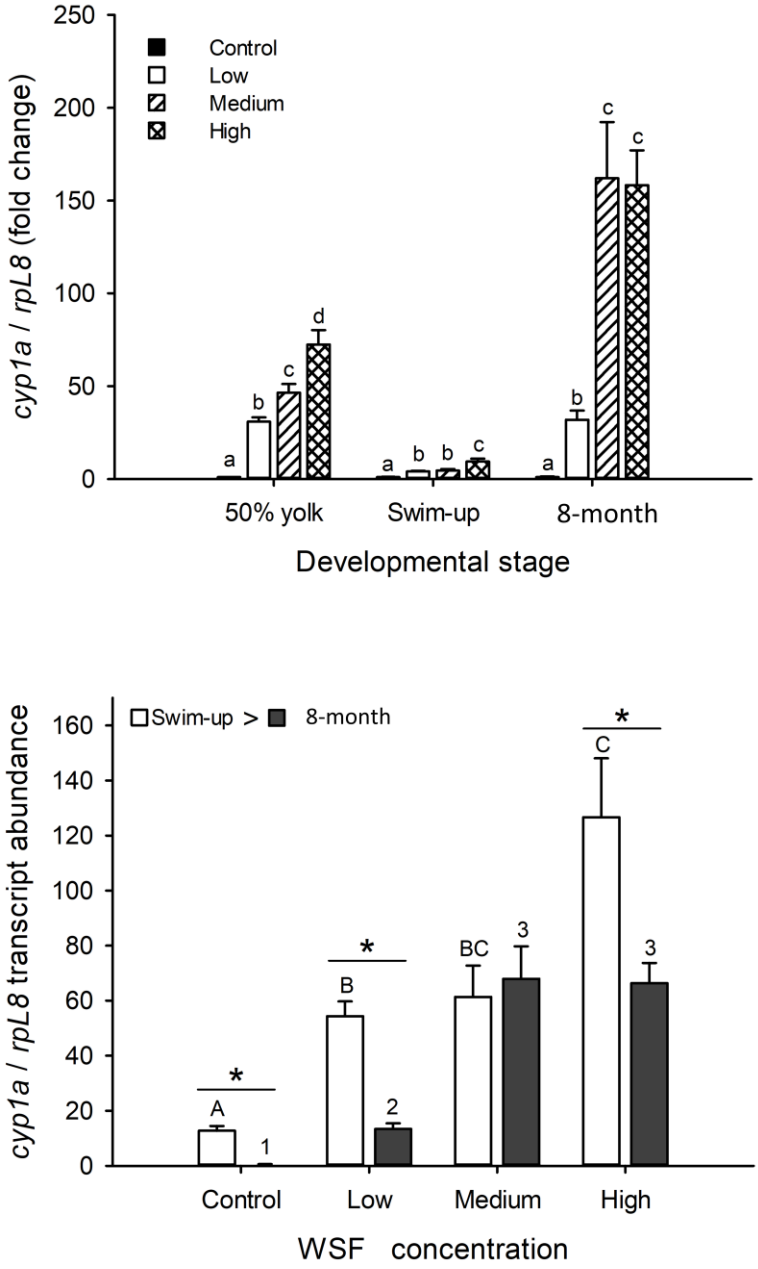

Figure 5. 13. Cytochrome p450 1a (*cyp1a*) expression in developing sockeye alevin head regions (50% yolk) and isolated whole hearts (swim-up, month) exposed to various concentrations of WSF. The initial TPAC concentrations for each WSF were 0.2 (Control), 13.7 (Low), 34.7 (Medium) and 124.5 (High) µg/L. Expression was normalized to the housekeeping gene, *rpL8*. The top panel presents data as fold-change from controls (set to 1) for each developmental stage, and bars that do not share a common letter are significantly different (one-way ANOVA and Bonferroni post-hoc test; n=8;

$p<0.001$). This shows a clear dose-dependent increase in cyp1a at each developmental stage, with the greatest induction evident in the oldest fish. The bottom panel presents raw values of cyp1a to permit comparison between developmental stages (the 50% yolk data is excluded here since it is a mixed tissue sample).

Chapter 6. The effects of diluted bitumen on the swimming performance, aerobic scope, and post-exercise metabolic recovery of juvenile sockeye salmon (*Oncorhynchus nerka*)

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Abstract

Diluted bitumens (dilbits) are heavier types of crude oils produced by mixing the bitumen extracted from Canadian oil sands with lighter petroleum diluents (e.g., oil-gas condensate). The transportation of dilbit *via* transmission pipeline or railway has increased significantly as the demands for oils sands products continue to grow, increasing the risk of exposure to aquatic biota following a spill event. In the present study, juvenile sockeye salmon (*Oncorhynchus nerka*) were exposed either acutely (96 h) or subchronically (28 d) to the water-soluble fraction (WSF) of Cold Lake Blend dilbit containing initial total polycyclic aromatic compound (TPAC) concentrations of 0, 13.7, 34.7, and 124.5 µg/L. The biological effects of dilbit exposure on swimming speed, intermediary metabolism, and biochemical post-exercise recovery were both time- and concentration-dependent. A significant induction (>3-fold) of hepatic ethoxyresorufin-O-deethylase (EROD) activity was seen in fish exposed to [PAC] ≥ 34.7 µg/L at 96 h, while the threshold for induction was lower (≥13.7 µg/L) for a 28 d exposure. Mean critical swimming speed (U_{crit}) was reduced by 28.4% in acute exposures to 124.5 µg/L, with reductions of 14.2% and 35.4% in fish subchronically exposed to the two higher concentration groups. Their reductions in U_{crit} were likely due to their significantly diminished aerobic scope (by 24.3-46.6%) in the 34.7 and 124.5 µg/L exposure groups. Both acute and subchronic exposure to WSF of dilbit induced marked elevations in baseline plasma [cortisol], [lactate], [Na^+], and [Cl^-], and a significant reduction of [muscle glycogen], indicating an activation of the physiological stress response and a disturbance of ion homeostasis. Subchronic exposure to the two higher WSF concentrations did not impair the ability to mount a secondary stress response following burst exercise; however, the time required for these biochemical and ionic parameters to return to baseline values was prolonged. The high dependence of salmonids on swimming performance, the capacity to recovery from exhaustive exercise, and ability to mount a stress response are key aspects of ecological fitness, and impairments to these critical physiological functions following dilbit exposure may result in significant population effects.

Keywords: Oil sands; diluted bitumen (dilbit); crude oil; toxicity; fish; sockeye salmon; swimming performance; stress response; exercise recovery; biochemistry

6.1. Introduction

The extraction of oil sands in Canada reached 2.9 million barrels per day (MB/d) in 2018, contributing to over 63% of the national crude oil production and averaging 4.6 MB/d (CAPP, 2019a). The primary petroleum product from the oil sands is bitumen, a mixture naturally high in viscosity and density (Crosby et al., 2013). Bitumen is diluted with lower-density hydrocarbon mixtures (e.g., natural gas condensate and naphtha) to produce diluted bitumen (dilbit) (Dew et al., 2015) for transport *via* pipeline. Pipeline networks traverse large distances across North America and a small but increasing volume is shipped by railway (CER, 2018a). As the global demand for crude continues to grow, the production of Canadian oil sands is anticipated to rise by 46%, reaching 4.25 MB/d by 2035 and potentially exceed the capacity of current pipeline networks (CAPP, 2019b). In British Columbia (BC), the existing and expanding pipeline traffic near important freshwater and estuarine habitats, as well as migratory routes for multiple species of Pacific salmon (*Oncorhynochus spp.*), are raising environmental concerns of oil spills in ecologically sensitive areas (CER 2018b; Levy, 2009; RCF, 2018).

The fate and behavior of dilbit in aquatic environments can vary significantly depending on the blend and the characteristics of the spill site (Alsaadi et al., 2018a). Information from the Kalamazoo River spill suggests that dilbit may bind to particulate matter and become entrained in sediments (Lee et al., 2015; NASEM, 2016) leading to potential acute as well as chronic exposure scenarios (Dew et al., 2015; Dupuis and Ucan-Marin, 2015) and likely different degrees and categories of toxic effects. The impacts of exposure to conventional crude oils on both freshwater and marine teleosts, include documented effects that include developmental and functional defects in early life stages (ELS), the suppression of growth and development, reduced swimming performance and post-exercise recovery, compromised immune systems, endocrine disruption, alterations in gene expression, bioenergetic effects, and impaired reproduction (reviewed in Kennedy, 2015). These oil-induced effects are primarily attributed to several groups of petrogenic compounds (e.g., BTEX, NAs, and PACs) that

are also the prominent components of dilbit (ECCC et al., 2013). The chemical constituents in different blends of oils may vary significantly in terms of their composition, therefore the extrapolation of toxicological data from other commonly transported crudes to predict outcomes following dilbit exposure may prove difficult (NASEM, 2016).

Although dilbit has been transported in North America for decades, existing data on its toxicity to fish remains limited (Dupuis and Ucan-Marin, 2015). Previous studies show that sublethal exposure to the water-accommodated fraction (WAF) of dilbit (10 to 100 µg/L total PAC [TPAC]) increased the prevalence of post-hatching deformities and altered the expression of genes involved in biotransformation and cellular stress in fathead minnow, *Pimephales promelas* (Alsaadi et al., 2018b), Japanese medaka, *Oryzias latipes* (Madison et al., 2015, 2017), yellow perch, *Perca flavescens* (McDonnell et al., 2019). Similarly, Philibert et al. (2016) reported developmental toxicity in WAF-exposed embryonic zebrafish (*Danio rerio*); hatched larvae exhibited altered patterns in shelter-seeking and continuous swimming behavior. In salmonids, exposure to the water-soluble fraction (WSF) of dilbit (at 35 µg/L TPAC) decreased the hatching success, impaired development, induced changes in bioenergetic composition, and the expression of biomarker gene in ELS sockeye (*Oncorhynchus nerka*) Alderman et al. (2018). Fish exposed to WSF containing 100 µg/L total PAC experienced higher mortality rates during a 2-month depuration period in clean water following exposure, and latent effects were observed in the brain morphology of surviving fish after 8 months (Alderman et al., 2018). While most laboratory studies center on sensitive ELS of fish, investigations regarding the sublethal toxicity of dilbit to juvenile fish are rare. 1+ year old sockeye exposed to dilbit WSF (66.7 µg/L TPAC) for 4 weeks exhibited reduced swimming performance accompanied by cardiac remodeling and upregulation of biomarker genes, as well as alterations in the serum proteome (Alderman et al., 2017a, b). Avey et al. (2020) exposed Atlantic salmon smolts (*Salmo salar*) to WSF containing 67.9 µg/L TPAC and found alterations in energy metabolism in cardiac and skeletal muscle, as well as changes in tissue gene expression and enzyme activity. Most of these immediate biological effects persisted only through a 7 to 14 d depuration period (Avey et al., 2020). Sockeye parr acutely (24 h and 96 h) and subchronically (21 d) exposed to WSF containing similar TPAC concentrations showed significant

osmoregulatory disturbances, activation of the internal stress response, and increased susceptibility against infectious pathogens (Lin et al., 2020).

There is a pressing research need to characterize the sublethal effects of dilbit to Pacific salmon due to their unique migratory life history. The knowledge gap regarding dilbit's immediate and latent toxicity to salmonids remains significant. The objective of this study was to examine the effects of an acute and subchronic exposure to dilbit on the locomotor capability and metabolic recovery capacity following exhaustive exercise of juvenile sockeye salmon. The measurement of swimming performance (e.g., critical swimming speed [U_{crit}]) is a widely adopted physiological endpoint for assessing the sublethal effect of various environmental contaminants on teleost due to its extensive involvement in a variety of important life events including food acquisition, predator-prey interactions, reproduction, schooling and migration between habitats (Hammer, 1995; Little and Finger, 1990). The ability to efficiently recover from intense exercise is also considered an ecologically relevant endpoint since avoidance from predators and advancing through rapid currents often involve several repeated swimming episodes (Kieffer, 2000). Previous studies have demonstrated that hydrocarbon exposure can significantly impair the post-exercise metabolic and ionic recovery capacity of juvenile fish, as well as alterations to pre-exercise parameters related to hypothalamic-pituitary-interrenal (HPI) axis-mediated stress responses (Kennedy and Farrell, 2005, 2006; Lin et al., 2020).

Post-exposure respirometry analyses for routine metabolic rate (RMR), maximum metabolic rate (MMR), and aerobic scope (AS) were also conducted here to evaluate oil-induced impacts on the immediate metabolic rates and aerobic capacity due to their close links to high-intensity swimming activities in teleosts. Particularly, reduced MMR and/or AS and have been linked to decreased aerobic swim performance in oil-exposed juvenile and adult teleosts (Johansen and Esbaugh, 2017; Nelson et al., 2017; Stieglitz et al., 2016), and may potentially contribute to other observed reductions in performance indices such as decreased prey capturing ability (Rowsey et al., 2019), increased susceptibility to predation (Johansen et al., 2017), and lowered marine survival in pink salmon (*Oncorhynchus gorbuscha)* (Heintz et al., 2000). The integration of these crucial fitness traits may help better characterize the potential disturbances of dilbit to multiple

physiological functions in juvenile salmonids, providing the necessary toxicological data for ecological risk assessments related to dilbit storage and transportation, as well as the management plans for remediating potential spills.

6.2. Materials and Methods

6.2.1. Fish

Sockeye salmon gametes were obtained from adults cultured at the Upper Pitt River Hatchery (Fraser Valley, BC) and fertilized following standard protocols (OMNR, 2009). Fertilized embryos were incubated in the dark in flow-through Heath trays (MariSource, Fife, WA) supplied with dechlorinated water (flow 5.5 L/min, mean temperature 10.5 °C, dissolved oxygen (DO) > 90%, hardness 6.0 mg/L $CaCO_3$, pH 6.8). When the external yolk sac of embryos was absorbed, phenotypically normal fry were transferred to grow-out tanks (250 L) supplied with flowing water at ambient temperature (12.3 ± 0.7 °C). Fish were fed twice a day *ad libitum* with commercial salmonid feed (Skretting, Vancouver, BC); photoperiod was 12h light:12h dark. At 6 months, juveniles (mean ± SE, 6.8 ± 0.5 g) were randomly collected and distributed into flow-through experimental tanks (250 L) for a minimum 2-week acclimation period; fish were fed once daily at a maintenance ration of 1.5% bw/d (Meador et al., 2005). The care and use of experimental fish were approved by the Animal Care Committee of SFU in accordance with the guidelines of Canadian Council on Animal Care (CCAC).

6.2.2. Diluted Bitumen Exposure

Juvenile sockeye were exposed to 4 concentrations of the water-soluble fraction (WSF) of Cold Lake blend (CLB) dilbit for 96 h or 28 d in a flow-through exposure system described elsewhere (Alderman et al., 2017b; Lin et al., 2020). Briefly, WSFs of dilbit (supplied by COOGER, Fisheries and Oceans Canada) were generated by continuously passing fresh dechlorinated water through PVC columns (16 cm diameter x 80 cm length) containing Siproax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) which were pre-soaked in unweathered dilbit for 24 h. The nominal dilbit load in each WSF-generator varied through changes in the number of coated beads. Three WSFs

were generated with initial aqueous TPAC concentrations in two replicate exposure systems. Each column delivered water to 4 250 L fiberglass tanks (n = 25 fish per tank); control fish were exposed to water passing through uncontaminated ceramic beads. Oiled beads were maintained in generator columns without light exposure. Fish were fed once daily with a maintenance ration during exposure; food was withheld 24 h prior to swim test or respirometry measurements (Mager et al., 2014).

Water samples were taken from duplicate generator columns at 0, 7, 14 d following the initiation of exposure. The concentrations of individual PACs were determined using gas chromatography–mass spectrometry analysis (SGS Axys Analytical Services Ltd., Sidney, BC); detailed methods and protocols have been described (Alderman et al., 2017b).

6.2.3. Swim Performance

A 1.5 L Brett-type swim tunnel system (Loligo® Systems, Tjele, Denmark) was used to measure the swimming performance of fish (Mager et al., 2014) following a 96 h and 28 d WSF exposure. Individual fish from experimental tanks were gently placed into the tunnel filled with aerated dechlorinated freshwater (maintained at 12 °C, DO > 95% for the test and measured in real-time using separate probes connected to a Witrox 1 minisensor oxygen meter [Loligo® Systems]). Fish were allowed to acclimate in the chamber for 30 min at a water velocity of 5 cm/s [approximately 0.5 body length per second (BL/s)]. A ramp-U_{crit} test was performed according to published protocols (Jain et al., 1997; Farrell, 2008). The test was initiated by accelerating water velocity at increments of 5 cm/s in 1 min intervals, reaching 50% of the estimated U_{crit}. This estimate was determined from preliminary tests conducted using unexposed individuals, and those U_{crit} values were shown to range within 40-58% of the measured U_{crit}. After the ramping phase, velocity increments were fixed at 10 cm/s in 20 min intervals until fish were fatigued. Fish were considered fatigued when they consistently lay the caudal fin on the rear of tunnel for over 5 seconds. The system was isolated from the surrounding lab environment by black curtains to minimize any disturbance; the activity of fish was monitored by a HD video camera mounted above the chamber. Fish (n = 8 per treatment) were swum in random order; exhausted fish were immediately removed from

the tunnel and euthanized by an overdose of buffered MS-222. Wet weight (g), fork length (cm), and cross-sectional dimensions of fish were recorded after swimming. U_{crit} (in BL/s) was calculated according to Brett (1964). Water velocity was not corrected as the solid blocking effect was determined to not be significant (< 10%) for all tested individuals (Bell and Terhune, 1970).

6.2.4. Respirometry

Oxygen consumption rate (MO_2; $mgO_2/kg/h$) analyses were conducted following a 96 h and 28 d exposure using an automated intermittent-flow respirometry system (Loligo® Systems) that contained 4 horizontal glass chambers (~ 90 mL, ID = 28 mm). Chambers were submerged in an aerated water bath (8 L) in which water temperature was maintained at 12.0 °C using a custom-built water bath circulator. The oxygen tension (%) within each chamber was measured each sec using a fiber-optic probe connected to a Witrox 4 minisensor oxygen meter, and real-time oxygen levels were recorded using AutoResp™ 2 software (Loligo® Systems). Individual fish (n = 8 for each treatment or control group) were randomly removed from experimental tanks, and held in a chamber for a 2.5 h acclimation period at a minimal water flow rate. Results from preliminary studies with unfed juvenile sockeye indicated that a minimum of 2 h habituation period was required to for recovery from handling stress and to obtain a stable routine metabolic rate (RMR). Each RMR measurement consisted of two 10-min loops which were automatically set to complete in a 240 s flushing, 60 s mixing, and 300 s measuring cycle. An average oxygen consumption rate value from the two measuring loops were adopted as the final RMR. Upon the completion of RMR measurements, fish were then transferred into a 40 L glass aquarium and subsequently chased until exhaustion which was determined at the point when fish failed to avoid the chasing (Fang et al., 2019). Exhausted fish were immediately returned to the respirometry chamber, and the maximum metabolic rate (MMR) determined with a 6-min loop consisting of a 60 s mixing and 300 s measuring cycles.

6.2.5. Exercise Recovery

The exercise recovery ability of sockeye was measured following exposure when forced to perform burst swimming for 5 min according to Milligan and Wood (1986). Whole blood samples, liver and white muscle tissues were sampled (n = 8 per treatment group) pre-exercise and then at 0, 2, 6, and 12 h after exhaustive exercise and an overdose with buffered MS222. Blood was sampled from the caudal vasculature using heparinized microcapillary tubes and briefly centrifuged at 13,000 *x g* for 1 min (Kennedy and Farrell, 2006). Hematocrit (H_{crit}) was measured using a digital caliper, and the separated plasma was transferred into 1.5 mL microcentrifuge tubes and snap-frozen in liquid N_2. Collected whole blood (5 µL) was diluted in 1.25 mL Drabkin's reagent (D5941; Sigma-Aldrich, St. Louis, MO). Liver and white muscle were stored in microcentrifuge tubes and snap-frozen in liquid N_2. All samples were stored at -80 °C until further analysis.

6.2.6. Biochemical Analysis

Concentration of plasma L-lactate (Catalog# 120001400P; Eton Bioscience, San Diego, CA) were determined spectrophotometrically using commercial colorimetric assay kits according to respective protocols provided by manufacturers. Plasma cortisol concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Catalog# 402710; Neogen Corp., Lexington, KY). Concentration of plasma Cl^- was quantified following Osachoff et al. (2014). Plasma [Na^+] was measured using a flame photometer (Model# Varian AA240FS; Palo Alto, CA; Love, 2016). White muscle glycogen content was measured using a colorimetric kit (Catalog# K646-100; BioVision, Milpitas, CA). The background glucose concentration in the muscle tissues were quantified using a colorimetric assay kit (Catalog# 120003400P; Eton Bioscience) [Hemoglobin] (Hb) was measured in the diluted whole blood sample with cyanmethemoglobin as the standards (Catalog# 0325006; Stanbio Laboratory, Boerne, TX) according to Drabkin and Austin (1932) and Lin et al. (2020). An Epoch™ 2 spectrophotometer (BioTek, Winooski, VT) and Corning® 96-well microplates (Corning Inc., Corning, NY) were used for all colorimetric assays.

Liver microsomes were prepared from pre-weighed frozen liver tissues according Gourley and Kennedy (2009). The isolated microsomal fraction was used to determine hepatic ethoxyresorufin-O-deethylase (EROD) activity using a fluorescence microplate reader following Hodson et al. (1991). Total protein concentration was quantified using a Bradford assay kit (Thermo Fisher Scientific, Mississauga, ON) with bovine serum albumin as the standard.

6.2.7. Statistical Analysis

The mean value for each quadruplicate exposure tank was calculated prior to statistical analysis. To account for variation in metabolic rates due to variations in size, values of RMR and MMR were normalized to wet mass (g); AS was then calculated as the difference between the RMR and MMR for each fish. A one-factor ANOVA followed by a Tukey-Kramer multiple comparison test was then used to compare means between the control group and WSF-treatment groups in their EROD activity, hematocrit, [Hb], U_{crit}, and respirometry endpoints ($\alpha = 0.05$). For the plasma and muscle biochemical measurements following exhaustive exercise, means were compared using a two-factor ANOVA and a Tukey-Kramer multiple comparison test ($\alpha = 0.05$). The two independent factors used here were the TPAC concentration (0, 13.7, 34.7, or 124.5 µg/L) and the exposure length (96 h or 28 d). Mean plasma [Na^+], [Cl^-], [cortisol], and [L-lactate] and white muscle glycogen content were compared for fish within the same treatment group across different sampling times using a two-factor ANOVA and a Tukey-Kramer multiple comparison test ($\alpha = 0.05$). The two independent factors used this test were the TPAC concentration (0, 13.7, 34.7, or 124.5 µg/L) and the timing of sampling in relation to exhaustive chasing (pre-exercise, 0, 2, 6, or 12 h after the exercise). This test also permitted a comparison between controls and WSF-treated groups for same biochemical endpoint at the same sampling time, but the data sets from acute exposure (96 h) and subchronic exposure (28 d) were not compared. All data analyses in the present study were performed using JMP 14.2 software (SAS Institute, Cary, NC).

6.3. Results

6.3.1. TPACs concentrations and composition

The system used in the present study delivered low water soluble PAC from dilbit to juvenile sockeye salmon both acutely (96 h) and subchronically (28 d). The aqueous TPAC concentrations delivered to the exposure tanks at time 0 were 0 (control), 13.7 (low), 34.7 (medium), and 124.5 µg/L (High). These values comparable to other previously reported TPAC concentrations in WSF of CLB dilbit (Alderman et al., 2017a, b, 2018; Avey et al., 2020), and crude oil (Kennedy and Farrell, 2005, 2006, 2008). Consistent with the known changes in the chemical composition of spilled dilbit through the weathering process (Alsaadi et al., 2018a; NASEM, 2016), the compounds detected in the generated WSFs are initially dominated by more volatile and lower-molecular-weight (LMW) hydrocarbons (e.g., BTEX, naphthalene), while higher-molecular-weight (HMW) became relatively more prominent as exposures progress; total TPAC concentrations decline with time (detailed chemical analysis is provided in Lin et al., 2020).

6.3.2. Hepatic EROD Activity

Fish exposed to the medium and high WSF of dilbit exhibited significantly increased hepatic EROD activity (Figure 6.1). At 96 h, the enzyme activity level had increased by 3.1-fold ($p < 0.01$) and 3.9-fold ($p < 0.01$) compared to the controls, respectively; exposure to the low WSF concentration did not alter EROD activity. As the time of exposure increased to 28 d, fish exposed to all three concentrations of WSF exhibited concentration-dependent increases in liver EROD activity, and the highest induction occurred in the high exposure group (4.5-fold, $p < 0.01$).

6.3.3. H_{crit} and [Hb]

No difference was seen in the Hcrit between control and dilbit-treated fish at either 96 h or 28 d of exposure. Fish in High group had significantly elevated [Hb]

comparing to the controls at 28 d ($p = 0.012$), while 96 h exposure to the same WSF concentration did not induce similar alterations.

6.3.4. Swimming Performance

A significant reduction in mean U_{crit} by 28.4% ($p < 0.01$) was seen in fish acutely exposed to the high WSF of dilbit (Figure 6.2a). In the 28-d exposure, a concentration-dependent decrease in U_{crit} was observed in fish exposed to the medium and high treatments (14.2% ($p < 0.01$) and 35.4% ($p < 0.01$, respectively) compared to controls.

6.3.5. Respirometry

Respirometry was conducted to determine RMR, MMR, and AS following dilbit treatment. In acute exposures, no significant effect on RMR was seen in fish in the low treatment group ($p < 0.05$), however fish in the medium and high treatment groups showed significant elevations in RMR by 51.7% ($p < 0.01$) and 60.7% ($p < 0.01$) compared to control fish, respectively (Figure 6.2b). MMR was not affected ($p < 0.05$; Figure 6.2c), however AS for fish in the medium and high treatment groups were significantly decreased by 24.3% ($p = 0.032$) and 35.5% ($p < 0.01$), respectively (Fig. 2d).

Subchronic exposure (28 d) resulted in a significant increase in RMR in all 3 exposure groups (Fig. 2b); the responses were concentration-dependent and the range in increase was between 38.8-77.3% ($p < 0.05$). The MMR medium and high treatment groups were reduced by 18.5% ($p = 0.034$) and 20.75% ($p = 0.013$), respectively compared to controls (Fig. 2c). Consistently, fish exposed to the two higher concentrations exhibited significantly lower AS values by 39.4% ($p < 0.01$) and 46.6% ($p < 0.01$), compared to controls, respectively (Fig. 2d).

6.3.6. Exercise Recovery

Exercise recovery (plasma cortisol, lactate, Na^+, and Cl^- concentrations, muscle glycogen content) was determined before and 12 h following burst swimming in fish from

the various treatment groups. For control fish in either the 96 h or 28 d exposure, cortisol concentrations increased significantly (≥ 3.3-fold; $p < 0.01$) at 0 h post-exercise (Table 6.1). These elevations persisted for 2 h post-exercise (≥ 4.2-fold; $p < 0.01$), and returned to baseline values by 6 h. Plasma [lactate], [Na^+], and [Cl^-] concentrations showed a similar pattern of response before and after exercise; these peaked at 0 h ($P < 0.05$) and returned to baseline levels by 6 h. Burst swimming also induced a significant depletion of glycogen content in white muscle ($p < 0.05$); its recovery in the control group was achieved by 12 h.

Acute exposure to the medium and high WSF concentrations resulted in significant elevations in baseline plasma [cortisol] and [lactate] before exercise ($p < 0.01$), as well as significant reductions in white muscle glycogen reserves compared to controls ($p < 0.05$; Table 6.1). Decreases of pre-exercise plasma [Na^+] and [Cl^-] were only observed in the high WSF group ($p < 0.05$). By 96 h, for fish in the medium and high WSF groups, plasma cortisol and lactate concentrations reached higher levels in response to exhaustive exercise at 0 h compared to controls ($p < 0.01$); exposure to dilbit did not alter the recovery for either parameter, which returned to pre-exercise levels 6 h following exercise. White muscle glycogen content in fish from these two higher exposure groups showed a similar depletion following burst swimming and returned to pre-exercise levels by 12 h as in controls. Plasma ions of fish in high WSF group exhibited a similar response pattern to exhaustive burst swimming and a similar timeframe to recovery when compared to fish in the control group. These ions were elevated immediately following burst exercise ($p < 0.05$), and then returned to pre-exercise levels by 6 h. Fish acutely exposed in the low WSF treatment group only exhibited significantly elevated baseline plasma [cortisol] ($p < 0.01$), with no other significant alterations to any other pre-exercise measurements, as well as their post-exercise biochemical responses and recovery capacity.

Fish subchronically exposed to the two higher concentrations of WSF exhibited significant increases in pre-exercise plasma [cortisol] and [lactate] ($p < 0.01$; Table 6.1), but marked reductions in baseline muscle glycogen content was seen in these groups compared to controls ($p < 0.05$; Table 6.1). By 28 d, all dilbit-exposed fish were able to immediately elevate plasma cortisol and lactate concentrations after the exhaustive

exercise at 0 h ($p < 0.01$), but the time required for baseline recovery was longer in the medium and high WSF group for both parameters. As mentioned above, post-exercise plasma [cortisol] and [lactate] for the control fish typically returned to baseline by 6 h. In comparison, these two parameters in fish in the medium WSF group failed to recover by 6 h. The inability to recover to baseline values was more pronounced fish from the 28 d high group, where a prolonged increase in circulating [cortisol] and [lactate] 12 h after the burst swimming was seen. In these fish, a further depletion in [muscle glycogen] was seen in response to exhaustive burst swimming ($p < 0.01$); however, the time required to recover to baseline values was similar to controls. Consistent with other metabolic variables, pre-exercise plasma [Na^+] were significantly decreased by exposure to the two higher dilbit concentrations ($p < 0.05$; Table 6.1); plasma [Cl^-] was only reduced in the high treatment group ($p < 0.05$). Exercise induced increases in post-exercise plasma ion concentrations comparable to controls, as well as recovery to baseline pre-exercise values.

6.4. Discussion

Given the uncertainties of the fate and behaviours of dilbit in freshwater habitats, acute or prolonged exposures could occur during a potential spill event. Currently, there is very limited information regarding the impacts of short-term and prolonged exposure to aqueous hydrocarbons derived from dilbit on the swimming performance, aerobic capacity, and exercise recovery capacity of freshwater and marine teleosts. The present study utilized a passive diffuser system to conduct both acute (96 h) and subchronic (28 d) exposures to WSF of dilbit at various environmentally relevant concentrations. A recent study using column exposure apparatus with identical design generated WSFs of Cold Lake Blend (CLB) dilbit containing initial TPAC concentrations at 13.7-124.5 µg/L (Lin et al., 2020), these values were comparable to other previously reported WSFs of CLB dilbit with concentrations at 3.5-100 µg/L TPAC (Alderman et al., 2017a,b, 2018; Avey et al., 2020), as well as WSFs of Alaska North Slope crude oil (ANSCO) at 7.4-127 µg/L total polycyclic aromatic hydrocarbons (TPAHs) (Kennedy and Farrell, 2005, 2006, 2008). Consistent with the known changes in the chemical composition of spilled dilbit over the weathering process (Alsaadi et al., 2018a; NASEM, 2016), the petrogenic

compounds detected in these WSFs were initially dominated by more volatile and lower-molecular-weight (LMW) hydrocarbons (e.g., BTEX, naphthalene), while higher-molecular-weight (HMW) became relatively more prominent as exposure progressed with the TPAC concentrations declined with time (detailed chemistry analysis was provided in Lin et al., 2020). The sublethal effects on Ucrit, aerobic scope, and metabolic recovery post-exercise were both concentration- and time-dependent with a clear threshold. Exposure to the low-WSF did not cause significant effect on juvenile sockeye by 96 h and 28 d, whereas fish in the medium- and high-WSF groups showed remarkable impairments to those physiological functions.

No mortality occurred in fish exposed to any concentration of dilbit WSF for up to 28 d, which is generally consistent with previous studies on dilbit and salmonids. To compare, acute (24 to 96 h) or subchronic (21 to 28 d) exposures to the WSF of dilbit containing 3.5 to 124.5 µg/L TPAC did not affect the survival of 1+ year juvenile sockeye salmon (Alderman et al., 2017a, b; Lin et al., 2020) or Atlantic salmon smolts (8 months; Avey et al., 2020). However, during embryonic development, sockeye salmon exhibit comparatively higher sensitivity to dilbit in this regard; a significantly elevated mortality was seen in ELS sockeye at exposure concentrations ≥ 35 µg/L PAC (Alderman et al., 2018). In contrast to embryonic exposures in salmonids, no acute lethality was reported in ELS fathead minnow, Japanese medaka, or yellow perch following exposure to WAFs of dilbit containing up to 100 µg/L TPAC (Alsaadi et al., 2018b; Madison et al., 2015; 2017; McDonnell et al., 2019). Barron et al. (2018) compared the toxicity of WAFs derived from unweathered CLB dilbit and Western Canadian Select (WCS) dilbit to larval fathead minnow and inland silverside (*Menidia beryllina*); acute lethality was observed at concentrations of 12.4-26.2 µg/L TPAH and 16.5-36.5 µg/L TPAH for these two blends, respectively. Based on existing data, the acute lethality of dilbit to teleosts is lifestage-dependent with ELS being the most sensitive life stage; lethal concentration threshold are both species- and exposure method-dependent (e.g., WAF *v.* WSF).

The bioavailability of hydrocarbons in exposure water was assessed indirectly by measuring the induction of hepatic EROD activity; this is a commonly adopted biomarker for exposure to petrogenic PAHs and planar halogenated hydrocarbons (Goksøyr and Förlin, 1992; van der Oost et al., 2003). Here, the induction of EROD activity in fish was

both concentration- and time-dependent, indicating that hydrocarbons in the water from the exposure system were bioavailable. The WSF containing ≥ 34.7 µg/L initial TPAC induced a significant increase in EROD (> 3-fold) by 96 h; the threshold for EROD induction was ≥ 13.7 µg/L when exposure duration was increased to 28 d. Notably, the maximum induction occurred in the highest treatment group by 96 h, but prolonging exposure duration to 28 d did not induce a further increase in EROD activity. For comparison, the threshold initial TPAC concentration that significantly induced liver EROD activity in 1+ year sockeye in a 7 d of exposure was 3.5 µg/L, elevations that were positively correlated with increasing TPAC concentrations (Alderman et al., 2017a). At 4 weeks of exposure EROD induction remained elevated, but no differences were seen across concentrations ranging from 3.5-66.7 µg/L TPAC (Alderman et al., 2017a). Lin et al. (2020) reported a similar response pattern in dilbit-exposed juvenile sockeye; in acute exposures (24 h and 96 h) EROD induction did not occur, however, subchronic exposure (21 d) to dilbit containing 13.7 to 124.5 µg/L TPAC resulted in similar induction levels.

Dilbit-induced impairment of swimming performance was observed in fish from the high concentration group (initial 124.5 µg/L TPAC) at 96 h of exposure. In the 28-d exposure, reductions in swimming speed occurred in a concentration-dependent manner, and the threshold for effects was lower at 34.7 µg/L initial TPAC. Both an acute and subchronic exposure in the high treatment group resulted in similar decrease in the U_{crit} value. In another dilbit study, older sockeye juveniles exposed to 66.7 µg/L initial TPAC resulted in no effects at 7 d but significant reductions in U_{crit} occurred at 4 weeks of exposure (Alderman et al., 2017). Similarly, repeated U_{crit} measures in Atlantic salmon were not affected after a 24-d exposure to dilbit WSF (67.9 µg/L TPAC) (Avey et al., 2020). Interestingly, both studies documented increases in U_{crit} values when fish were exposed to lower dilbit concentrations (3.5 to 9.65 µg/L); the improved U_{crit} of swimming speeds were suggested to be due to the observed remodeling of myocyte and collagen percentage compositions in the compact myocardium (Alderman et al., 2017a), or an increased reliance on anaerobic metabolic pathways in cardiac and red skeletal muscle (Avey et al., 2020). The present findings indicate that even short-term exposure to dilbit is sufficient to result in alterations in the physiological systems supporting swimming,

highlighting the sensitivity of young salmon and the importance of an immediate clean-up following a potential spill. An impaired U_{crit} could potentially lead to a decrease in fitness and survival as salmon parr predominantly utilize prolonged swimming for migration between optimal habitats, foraging, and escaping predators (Leavy and Bonner, 2009),

Decreased U_{crit} may be associated with a diminished aerobic scope (AS) resulting from an elevated routine metabolic rate (RMR) and/or a reduced maximum metabolic rate (MMR); these measurements demonstrated distinct time- and concentration-dependent alterations. AS is an integrated estimate of the overall metabolic budget that an animal can allocate for routine activities including somatic growth, sexual reproduction, food digestion, and locomotor movement (Killen et al., 2016; Norin and Clark, 2015), a decreased AS would therefore limit the performance of sustained activity, including U_{crit}. Combining swimming test and measurements of AS allow for a determination of whether the mechanism underlying the effect of contaminant exposure on Ucrit is caused by a loading stress (elevated RMR) or a limiting stress (reduced MMR) (Incardona et al., 2014; Stieglitz et al., 2016). Swimming impairment in the 96 h high-WSF group fish exhibited a significantly increased RMR and a decreased AS, with no change in MMR, suggesting that the diminished AS was only caused by a loading stress. In the 28 d exposure, the MMR of U_{crit}-impaired fish was reduced, concomitant with a significant elevation in RMR. This indicates that under subchronic exposure, dilbit exposure results in both a loading and limiting stress. Links between diminished AS and U_{crit} impairment have been made in marine pelagic fish exposed to Deepwater Horizon crude oil (DHCO) at low TPAC concentrations. U_{crit} and optimal swimming speed (U_{opt}) of young adult mahi-mahi (*Coryphaena hippurus*) decreased over 10% following an acute exposure (24 h) to 8.4 µg/L TPAH, in addition to a 20% reduction in MMR and an approximate 30% reduction in AS (Stieglitz et al., 2016). Similarly, a greater than 8% decrease in U_{crit} was reported in 24-h exposed red drum (*Sciaenops ocellatus*) at TPAH concentrations of 12.1 µg/L; swim-impaired fish exhibited a significant decrease in MMR and reduction in AS, impairments that persisted up to 6 weeks in uncontaminated water (Johansen and Esbaugh, 2017). It is worth noting that most of the DHCO studies show that reductions in AS were only accompanied by a

diminished MMR (limiting stress) (Johansen and Esbaugh, 2017; Stieglitz et al., 2016), effects which are not completely consistent with the present study.

This data suggests that dilbit causes limiting stress, reducing MMR and AS. This may occur *via* two mechanisms that limit both O_2 uptake and delivery. Oil-induced histopathological changes have been reported in fish gill epithelia (e.g., epithelial lifting, hyperplasia, hypertrophy, lamellar fusion [e.g., Kennedy and Farrell, 2005; Kochhann et al., 2014; Negreiros et al., 2011]), and can restrain oxygen uptake by reducing gas exchange surface area and/or increasing diffusion distance. Although a morphological assessment was not performed here, the significant reductions in plasma [Na^+] and [Cl^-] in unexercised exposed fish suggests a compromised gill function (Kennedy and Farrell, 2005). Exposure to hydrocarbons can also decrease cardiac output by compromising the cardiovascular system, including lowering stroke volume and heart contractility (Nelson et al., 2016, 2017), altering heart rate (reviewed in Pasparakis et al., 2019), decreasing cardiac power output (Cox et al., 2017), disrupting the excitation-contraction coupling in cardiomyocytes (Brette et al., 2014, 2017), altering myocytes and collagen content in the compact myocardium (Alderman et al., 2017a), and reducing ATP production efficiency in cardiac muscle fibers (Kirby et al., 2019).

Increased loading stress in dilbit-exposed fish may also be attributed to several mechanisms. Dilbit exposure may increase routine energy expenditures derived from induced and increased biotransformation reactions, metabolite transport, and damage repair processes (Kennedy, 2014; Whitehead, 2013). Oil exposure may also result in higher osmoregulatory costs due to altered gill morphology (Whitehead, 2013). The elevation in RMR may also be associated with a physiological stress response; elevated cortisol levels can increase both aerobic and anaerobic metabolic rates in fish (De Boeck et al., 2001). For example, acute exposures (2 to 72 h) to petroleum or PAH increase metabolic rates in juveniles of Antarctic bald notothen, *Pagothenia borchgrevinki* (Davison et al., 1992), Florida pompano, *Trachinotus carolinus* (dos Santos et al., 2006), chub mackerel, *Scomber japonicus* (Klinger et al., 2015), and larval mahi-mahi (Pasparakiset al., 2016). Conversely, unaffected resting metabolic rates have been reported in several species of teleosts following short-term (24 to 48 h) or subchronic exposures to DHCO (Mager et al., 2014, 2018; Stieglitz et al., 2016; Nelson, 2017;

Johansen and Esbaugh, 2017) or petroleum products (Pan et al., 2018). Notably, the threshold concentration for RMR elevation here was 34.7 µg/L initial TPAC in an acute exposure. While there is clear inter- and intra-species variability in this response, the TPC concentration ranges reported in those studies were much lower (8.4 to 23.2 µg/L) than in the present study; the loading stress resulting from these exposure concentrations may simply not be sufficient to initiate a similar response in RMR under short-term exposure scenarios.

Studies directly assessing oxygen consumption rates of larval and juvenile mahi-mahi and adult red drum acutely exposed to DHCO (-4.2 µg/L TPAH) demonstrated that impaired Ucrit could be uncoupled from constrained MMR and AS (Mager et al., 2014, 2018; Johansen and Esbaugh, 2017), suggesting potential mechanisms other than AS injury could conceivably play a role in reducing swimming performance. In the present study, acutely exposed fish exhibited a significant decrease in AS with no effect on Ucrit, indicating that oil-induced effects on oxygen consumption occur prior to swimming impairment. Alternative mechanisms (to altered aerobic capacity) could underly Ucrit alterations following oil exposure. These include reduced energy production efficiency resulting from PAH-induced toxic stress to swimming musculature (Johansen and Esbaugh, 2017), suppressed contraction efficiency in skeletal muscle (Mager et al., 2014) due to similar excitation-contraction coupling inhibition as seen in cardiomyocytes in oil-exposed tuna (Brette et al., 2014, 2017), altered swimming behaviours (Johansen et al., 2017; Kawaguchi et al., 2012; Philibert et al., 2016) which are potentially related to the developmental abnormality and transcriptomic responses in the nervous system of oil-exposed ELS fish (Kawaguchi et al., 2012; Xu et al., 2016, 2017), and/or disrupted HPI axis stress responses and ionic homeostasis (Kennedy and Farrell, 2006).

Interpreting the effects of dilbit on post-exercise recovery is complex because of its effects on pre-exposure levels of metabolic and ionic recovery indicators. In the present study, an acute or subchronic exposure of fish to dilbit significantly increased plasma cortisol and lactate concentrations and reduced white muscle glycogen in non-exercised fish. In teleosts, activation of the HPI axis during the physiological stress response, leads to cortisol secretion from interrenal tissue (Hontela et al., 1992) and hyperglycemia through enhanced glycogenolytic metabolism of glycogen reserves; these

energy sources fuel intensive behaviours and physiological processes including swimming (De Boeck et al., 2001; Mommsen et al., 1999). Consistent with the present study, studies show that oil exposure causes a classic stress response in fish (Alkindi et al., 1996; Kennedy and Farrell, 2005, 2006; Lin et al., 2020; Reddam et al., 2017; Thomas et al., 1980; Thomas and Rice, 1987). For example, Pacific herring (*Clupea pallasii*) exhibited a typical physiological stress response during exposure to Alaska North Slope crude oil (initial TPAC 40 to 120.2 µg/L); cortisol and lactate concentrations, and muscle glycogen content returned to baseline levels by 96 h (Kennedy and Farrell, 2005, 2006). This was attributed the short-term interrenal activation to volatile LMW compounds in oil (e.g., BTEX and naphthalene) because of their irritant properties (Kennedy and Farrell, 2005, 2006). Longer exposures did not elicit stimulatory responses (Kennedy and Farrell, 2006). Striped mullet (*Mugil cephalus*) also showed a similar corticosteroid adaptation to crude oil exposure (Thomas et al., 1980). In contrast, the present study indicated that the stress response was not mitigated with the decreasing concentration of LMW components in the exposure water with time or an attenuation or habituation to oil components; the remaining non-volatile, HMW PACs in WSF at 28 d resulted in a continual stress response. Similarly, Lin et al. (2020) reported that an acute exposure (24 h or 96 h) to dilbit (34.7 to 124.5 µg/L) resulted in a continuous activation of the physiological stress response, and that stress-related parameters did not recover with a subchronic exposure up to 21 d. While the underlying mechanism and physiological consequences for chronic HPI activation are not clear, the normal biosynthesis and secretion of cortisol did not seem to be affected by WSF exposure as was suggested by Kennedy and Farrell (2005, 2006).

The exercise protocol used here is considered a stress event (Pagnotta et al., 1994); high-intensity burst swimming increases plasma [cortisol] that usually peaks 1 to 2 h following exercise, initiating glycogenolysis and a classic hyperglycemic response (Gamperl et al., 1994; Kieffer, 2000; Wang et al., 1994). Gluconeogenic re-synthesis of glycogen often takes 4-6 h, and in some species may require up to 12 h; circulating [lactate] usually recovers to pre-exercise levels within 6 to 12 h (Milligan et al., 2000; Milligan, 1996; Pagnotta et al., 1994). Control fish exhibited this typical sequence paradigm. The time for post-exercise metabolic and ionic recovery was within the time

frames documented for other species of teleosts (e.g., Milligan et al., 2000; Milligan, 1996; Kennedy and Farrell, 2005, 2006). Sockeye acutely exposed to dilbit showed a large stress response by 96 h where the magnitude of post-exercise increases in plasma [cortisol] and [lactate] as well as ion concentrations were much higher in exposed fish compared to controls. The parameters returned to baseline levels slower compared to controls. The additive effects of dilbit exposure and exercise on the stress response and downstream effects have been previously reported in Pacific herring (*Clupea pallasi*) (Kennedy and Farrell, 2006) and coho salmon (*Oncorhynchus kisutch*) (Thomas and Rice, 1987) however no evidence of impairment to fish's metabolic and ionic recovery was noted under short-term exposure (Kennedy and Farrell, 2005, 2006), which is consistent with the present findings.

With the longer exposure, a lowered post-exercise cortisol response and a diminished capacity to recover from exercise were demonstrated. Specifically, control fish circulating cortisol concentrations increased (4-fold) in response to exhaustive swimming, however these cortisol elevations were less than 2-fold in dilbit-exposed fish despite their higher cortisol levels before exercise. The additive effect of WSF exposure and exercise seen in the acute exposure was not observed in the subchronic exposure. Exercise-induced alterations in plasma [lactate], [Na^+] and [Cl^-], as well as [muscle glycogen] exhibited a similar pattern to the acute exposure, however, fish subchronically exposed exhibited a slower recovery for some of these parameters. Similar results were seen in Gulf toadfish (*Opsanus beta*) intraperitoneally treated with naphthalene or phenanthrene; exposed fish had significantly increased resting circulating cortisol concentration after a 24 h exposure, and were unable to mount a proper secondary stress response when subjected to an additional crowding stress (Reddam et al., 2017). In comparison, Kennedy and Farrell (2005, 2006) showed that when exposed to crude oil for 4 to 8 weeks, Pacific herring showed no elevation in basal plasma cortisol concentration and other stress-related biochemical parameters ([glucose], [lactate], [muscle glycogen]). Interesting, chronically exposed (8 weeks) herring were not able to increase circulating cortisol level and initiate typical cortisol response to exhaustive exercise, but their plasma [lactate] and [muscle glycogen] returned to baseline more rapidly (Kennedy and Farrell, 2005, 2006). Multiple sites in the HPI axis may be

susceptible to various chemicals in oil, and several possible mechanisms may exist to explain the muting or desensitizing of the cortisol response and the decreased capacity to mount a secondary stress response. Prolonged PAH exposure may cause interrenal tissue hyperactivity, driving the cortisol-mediated neuroendocrine pathway to a state of exhaustion (Hontela et al., 1992). Evidence exists to support this hypothesis; fish sampled from sites contaminated by high levels of PAHs and other pollutants (e.g., polychlorinated biphenyls and mercury) showed a reduced ability to elevate circulating plasma cortisol following acute capturing stress, compared to fish collected from a reference site (Hontela et al., 1992; Girard et al., 1998). Both *in vivo* and *in vitro* studies have previously shown that treatment with PAH or β-naphthoflavone (BNF), an agonist of the aryl hydrocarbon receptor can significantly increase basal plasma cortisol concentrations in rainbow trout, however, treated fish had a reduced cortisol response when subjected to an acute stressor, as well as a decreased interrenal sensitivity to adrenocorticotropic hormone (ACTH) stimulation (Aluru and Vijayan, 2004, 2006; Gesto et al., 2008; Girard et al., 1998; Hontela, 1998; Wilson, et a., 1998). Long-term exposure to PAHs are also known to result in histopathological changes in interrenal tissues, including necrosis (DiMichele and Taylor, 1978) and atrophy (Hontela et al., 1992), both of which could result in a reduced ability to respond to secretagogues or to produce hormones (Kennedy and Farrell, 2005). Alternatively, the activation of AhR and induction of CYP1A in response to hydrocarbon metabolism have been shown to reduce the production and secretion of cortisol by inhibiting the expressions of steroidogenic acute regulatory (StAR) protein and 11β-hydroxylase (Aluru and Vijayan, 2006). However, in the present study, sockeye chronically exposed to dilbit WSF did not exhibit an evident reduction in cortisol production capacity, suggesting that cortisol biosynthesis was not affected directly. The functional integrity of the HPI-mediated stress response plays an essential role in the energy mobilization during sudden environmental changes; the reduced recovery ability and diminished capacity to initiate a necessary corticosteroid response is considered maladaptive, and may lead to decreased fitness.

6.5. Conclusion

Given the current understanding of dilbit fate and behavior in freshwater environments, both acute and prolonged exposures are possible in post spill scenarios, highlighting information gaps regarding the impacts these exposure conditions to dissolved components of dilbit in freshwater and marine teleost species. The present study clearly shows that exposure to WSF of dilbit affects both the swimming performance, intermediary metabolism of juvenile sockeye salmon, and their ability to recover from exhaustive bursting exercise at low PAC (ppb) concentrations. Alterations in biological endpoints exhibited unique time- and concentration-dependent relationships. In both acute and subchronic exposures, dilbit induced loading stress in fish, resulting in higher RMR, activated EROD activity, and a cortisol-mediated stress response, as well as disturbing ion homeostasis. Besides loading stress, subchronic WSF exposure also exclusively induced limiting stress to fish, causing a decrease in MMR, and a reduced capacity to recover from exhaustive swimming. Overall, these results indicate that exposure to dilbit can result in detrimental impacts on the fitness of juvenile sockeye salmon by greatly impairing important physiological functions.

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Table 6. 1. Pre- and post-exercise (at 0, 2, 6, and 12 h) muscle glycogen content, plasma cortisol, lactate, Na^+, and Cl^- concentrations in fish exposed to water-soluble fractions (WSF) of unweathered cold lake blend diluted bitumen (CLB dilbit) concertinaing initial aqueous total polycyclic aromatic compound (PAC) concentrations at 0 (Control), 13.7 (Low), 34.7 (Medium), and 124.5 (High) µg/L for 96 h or 28 d. Results are shown as tank means ± S.E., a * indicates a statistical difference from the pre-exercise level within the same treatment group ($p < 0.05$), a † indicates a statistical difference from the control group at each sampling time ($p < 0.05$).

96 h Exposure

(a)	Cortisol (ng/mL)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	17.3 ± 1.0	56.5 ± 2.5 *	73.2 ± 8.5 *	41.0 ± 2.8	19.1 ± 1.3
Low	47.3 ± 1.7 †	80.5 ± 5.7 *	97.7 ± 1.7 *	51.3 ± 3.9	57.5 ± 5.9 †
Medium	51.7 ± 4.4 †	87.4 ± 4.8 *†	95.6 ± 6.0 *	69.0 ± 4.7 †	59.1 ± 4.3 †
High	62.4 ± 6.5 †	90.8 ± 3.3 *†	103.5 ± 7.3 *†	86.4 ± 4.8 †	67.7 ± 5.6 †
(b)	Muscle glycogen (mg/g)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	16.5 ± 0.7	6.3 ± 0.5 *	5.0 ± 0.4 *	8.6 ± 0.2 *	15.0 ± 0.7
Low	14.5 ± 0.6	4.6 ± 0.6 *	4.0 ± 0.4 *	7.5 ± 0.7 *	13.8 ± 0.9
Medium	12.1 ± 1.1 †	3.4 ± 0.3 *	4.6 ± 0.5 *	7.0 ± 0.4 *	12.9 ± 0.4
High	8.8 ± 0.7 †	3.6 ± 0.7 *	4.0 ± 0.4 *	5.3 ± 0.4 *†	9.4 ± 0.9 †
(c)	Plasma lactate (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	2.7 ± 0.2	7.3 ± 1.0 *	12.7 ± 1.1 *	5.8 ± 0.3	4.0 ± 0.3
Low	5.4 ± 0.3	10.5 ± 0.8 *	15.3 ± 0.8 *	8.2 ± 0.2	5.8 ± 0.3
Medium	6.7 ± 0.3 †	12.9 ± 0.4 *†	18.0 ± 1.2 *†	9.2 ± 0.7	6.9 ± 0.7
High	6.8 ± 0.3 †	13.9 ± 1.3 *†	17.4 ± 1.4 *†	12.9 ± 0.5 *†	5.5 ± 0.4
(d)	Plasma Na^+ (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	153.4 ± 1.1	177.8 ± 1.3 *	172.7 ± 1.5 *	153.9 ± 5.1	151.1 ± 1.9
Low	148.9 ± 2.3	174.6 ± 2.5 *	170.6 ± 3.2 *	152.5 ± 2.6	153.5 ± 0.9
Medium	140.4 ± 4.3	173.2 ± 3.5 *	168.1 ± 4.1 *	150.8 ± 1.7	144.3 ± 5.6
High	135.6 ± 3.2 †	159.3 ± 4.2 *†	155.6 ± 2.4 *†	135.1 ± 4.5 †	128.8 ± 2.4 †
(e)	Plasma Cl^- (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	134.3 ± 1.8	161.2 ± 1.1 *	159.4 ± 1.2*	131.5 ± 2.4	133.3 ± 1.9
Low	129.2 ± 2.4	162.6 ± 3.8 *	158.0 ± 4.1 *	133.8 ± 1.7	130.4 ± 1.2
Medium	126.8 ± 0.7	158.3 ± 2.1 *	160.9 ± 4.7 *	128.7 ± 3.0	126.3 ± 2.5
High	119.8 ± 1.1 †	145.3 ± 4.2 *†	141.6 ± 2.4 *†	117.8 ± 1.7 †	118.9 ± 1.2 †

28 d Exposure					
(a)	Cortisol (ng/mL)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	16.6 ± 2.4	65.8 ± 5.4 *	87.8 ± 6.5 *	39.8 ± 3.1	14.8 ± 0.8
Low	21.4 ± 2.9	60.1 ± 4.2 *	93.0 ± 4.9 *	50.4 ± 2.7 *	43.5 ± 0.8 †
Medium	41.2 ± 1.8 †	75.4 ± 4.2 *	89.2 ± 4.1 *	92.0 ± 5.6 *†	64.9 ± 6.4 †
High	54.0 ± 7.7 †	85.6 ± 2.4 *	95.0 ± 6.1 *	99.3 ± 5.2 *†	88.5 ± 6.5 *†
(b)	Muscle glycogen (mg/g)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	15.8 ± 0.7	4.3 ± 0.3 *	7.2 ± 0.3 *	9.2 ± 0.7 *	14.3 ± 0.5
Low	14.8 ± 1.2	3.8 ± 0.2 *	5.8 ± 0.4 *	8.7 ± 0.7 *	13.9 ± 1.3
Medium	11.8 ± 1.3 †	3.2 ± 0.4 *	5.1 ± 0.4 *	7.0 ± 1.0 *	10.3 ± 0.6 †
High	10.1 ± 0.9 †	3.2 ± 0.3 *	4.3 ± 0.2 *	5.8 ± 0.7 *	9.1 ± 0.5 †
(c)	Plasma lactate (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	3.03 ± 0.3	8.4 ± 0.6 *	11.6 ± 0.6 *	3.8 ± 0.1	3.9 ± 0.2
Low	3.4 ± 0.4	9.6 ± 0.5 *	10.9 ± 0.8 *	4.6 ± 0.5	4.0 ± 0.8
Medium	6.5 ± 0.3 †	11.6 ± 0.4 *	14.7 ± 0.9 *	10.1 ± 0.6 *†	9.0 ± 0.3 †
High	7.1 ± 0.2 †	12.2 ± 1.2 *†	15.3 ± 0.7 *†	10.6 ± 1.1 *†	11.8 ± 0.8 *†
(d)	Plasma Na^+ (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	149.7 ± 0.6	172.1 ± 1.7 *	176.3 ± 1.9 *	148.8 ± 3.6	146.7 ± 1.1
Low	146.5 ± 1.8	171.9 ± 3.4 *	169.9 ± 1.8 *	146.3 ± 3.0	149.9 ± 1.6
Medium	131.9 ± 1.5 †	163.8 ± 3.3 *	159.9 ± 2.7 *†	134.2 ± 2.4 †	131.8 ± 0.6 †
High	134.1 ± 1.3 †	162.3 ± 2.5 *	155.8 ± 3.3 *†	128.0 ± 3.7 †	129.2 ± 4.4 †
(e)	Plasma Cl^- (mM)				
	Pre-exercise	0 h	2 h	6 h	12 h
Control	133.3 ± 0.7	161.7 ± 1.5 *	162.8 ± 3.0 *	135.8 ± 2.2	132.8 ± 2.1
Low	132.2 ± 2.6	164.6 ± 1.0 *	160.4 ± 3.4 *	133.6 ± 2.7	130.9 ± 2.5
Medium	129.1 ± 2.6	156.5 ± 2.5 *	156.0 ± 4.6 *	127.3 ± 2.0	124.4 ± 2.7
High	121.0 ± 1.7 †	144.2 ± 0.3 *†	147.7 ± 3.2 *†	119.4 ± 1.3 †	115.8 ± 1.9 †

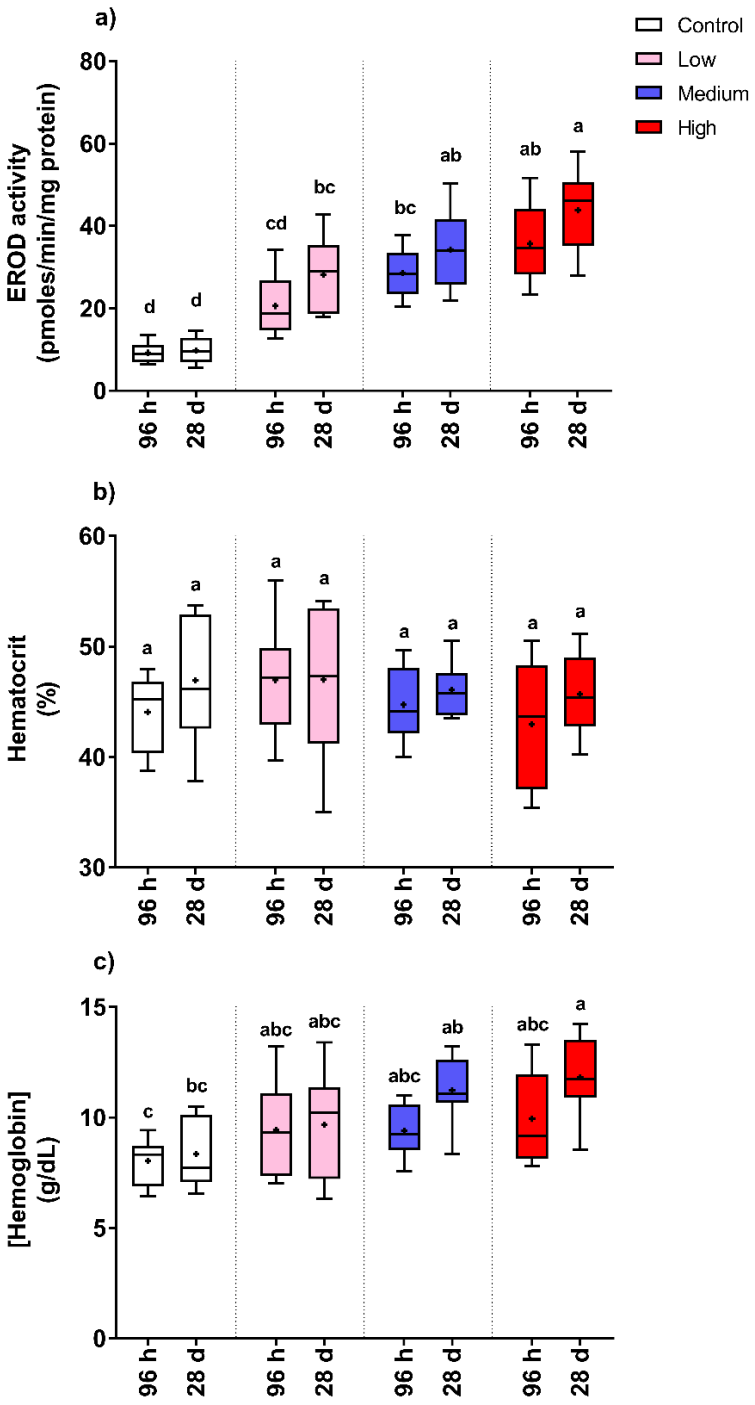

Figure 6. 1. (a) Hepatic ethoxyresorufin-O-deethylase (EROD) activity, (b) hematocrit, (c) [hemoglobin] of fish exposed to water-soluble fractions (WSF) of unweathered cold lake blend diluted bitumen (CLB dilbit) concertinaing initial aqueous total polycyclic

aromatic compound (PAC) concentrations at 0 (Control), 13.7 (Low), 34.7 (Medium), and 124.5 (High) µg/L for 96 h or 28 d. Results are shown as tank means ± S.E., treatments that do not share a common letter are statistically different ($p < 0.05$).

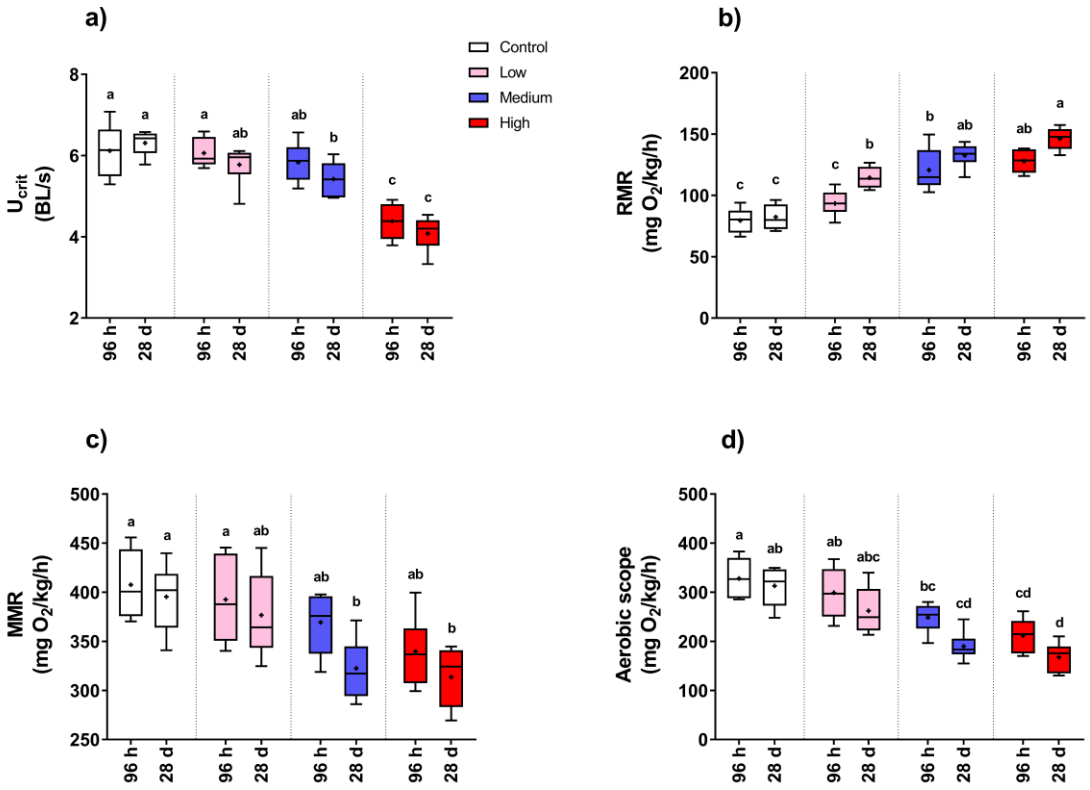

Figure 6. 2. (a) U_{crit}, (b) routine metabolic rate (RMR), (c) maximum metabolic rate (MMR), (d) aerobic scope (AS) of fish exposed to water-soluble fractions (WSF) of unweathered cold lake blend diluted bitumen (CLB dilbit) concertinaing initial aqueous total polycyclic aromatic compound (PAC) concentrations at 0 (Control), 13.7 (Low), 34.7 (Medium), and 124.5 (High) µg/L for 96 h or 28 d. Results are shown as tank means ± S.E., treatments that do not share a common letter are statistically different ($p < 0.05$).

Chapter 7. Final Review and Future Directions

While most studies have focused on the chronic toxicity of dilbit to ELS development and molecular responses (Alderman et al., 2018; Alsaadi et al., 2018a; Madison et al., 2015, 2017; McDonnell et al., 2019), this present research presents novel empirical data on a wide array of lethal and sublethal effects of dilbit to two Pacific salmonid species: sockeye (*Oncorhynchus nerka*) and pink salmon (*Oncorhynchus gorbuscha*) which are of great ecological, economic, and cultural importance in BC (Table 1). The toxic outcomes seen in juvenile pink salmon (fry stage) and multiple life stages of sockeye salmon (developing embryos to pre-smolt juvenile stage) following exposure to either WSF or WAF of dilbit include reduced survival and hatching success, ELS and latent developmental abnormalities, suppressed growth, decreases in both sustained (U_{crit}) and burst swimming (U_{burst}) performance, alterations to aerobic scope (AS) and oxygen consumption at different exercising statues [resting metabolic rate (RMR) and maximum metabolic rate (MMR)], lowered metabolic recovery capacity post-exhaustive exercise, altered energy stores, the upregulated expression of genes and enzyme activities associated with biotransformation reactions, induction of acute and chronic interrenal stress responses, disrupted iono-osmoregulatory ability, and a disrupted immunological defense against an infectious pathogen. These observed adverse impacts on above crucial life functions and physiological systems were seen at environmentally relevant concentrations of TPAC (ppb level, up to 124.5 µg/L). The findings from the present work are generally consistent with previously reported biological effects in a diverse species of marine and freshwater teleosts acutely or subchronically exposed to conventional crude oil containing similar levels of TPAC (reviewed in Alsaadi et al., 2018b; Kennedy, 2014; NASEM, 2016; Pasparakis et al., 2019). Despite the fact that CLB dilbit exhibited many similarities in the categories of sublethal effects as compared to crude oils and other petroleum products, the results here demonstrate that dilbit did not modulate these impacts with greater toxicity (Barron et al., 2018; Kennedy, 2014; NASEM, 2016).

Across all levels of biological organization in response described above, only several higher-order endpoints, such as chronic survival, growth, ELS and latent developmental abnormalities (Chapter 3, 4, 5) can be directly linked to the fitness of individuals and the long-term recruitment to populations. However, it is conceivable, but difficult to extrapolate the sublethal effects seen at lower-levels of biological organization to estimates of survivability or population-level impacts. For example, an oil-induced alteration to the interrenal hormonal stress response (Chapter 2, 5, 6) may translate to changes in fish's behavior patterns when challenging events such as avoidance of predation, capture of food, and social competition occur (e.g., Khursigara et al., 2018, 2019). U_{crit}, U_{burst}, and exercise recovery capacity (Chapter 4, 5, 6) are closely related to the migratory life history of Pacific salmon; reductions in these important performance criteria may directly affect the success seaward-migrating juveniles and returning spawn-ready adults. Notably, juvenile salmon populations residing in the Salish Sea, Lower Fraser River, and its adjacent estuaries are frequently challenged by various environmental stressors, such as fluctuating salinity, temperature, oxygen levels, and the presence of opportunistic pathogens. In particular, oil-induced iono-osmoregulatory (Chapter 2, 4) and immunological (Chapter 2) compromisation may potentially interfere with a fish's ability to cope with suboptimal conditions posed by natural stressors, and thus affect their survivability in the wild. This hypothesis is supported by the data in Chapter 4, in which higher salinities synergistically interact with WAF exposure, resulting in an enhanced reduction in survival and growth of pink fry. It is even more challenging to apply endpoints at the molecular and cellular levels to accurate or realistic assessment of their ecological consequences at the whole-animal and population levels. However, molecular studies and in-depth investigations of biochemical alterations are definitely required for exploring the toxic mechanisms of dilbit / crude oil in teleosts. Future studies should attempt to integrate laboratory testing with field studies, mesocosm studies, modeling, in an effort to link molecular initiating events to altered behavioral responses (e.g., swimming performance), and ultimately to impacted survivorship under natural conditions. Efforts such as those would provide the necessary toxicological information required for the development of risk assessment plans for managing salmon populations and restoring habitat in the event of potential pipeline failures or tanker spills.

There are several further research directions that could be highlighted according to the current knowledge regarding dilbit's toxicity and environmental fate and behavior. Firstly, given its unique hydrocarbon composition, dilbit tends to exhibit greater adhesivity, the potential for rapid weathering, submergence, and association with suspended particulates and sediments compared to other crude oils. Additional research on the toxicity of weathered dilbit or dilbit-sediment mixtures are needed to help generate more informed predictions regarding the impact of benthically-entrenched dilbit that may persist in aquatic environments. As indicated by the Kalamazoo River dilbit spill, local biota could experience prolonged exposures to petrogenic hydrocarbons if residual oils are not removed immediately. Further studies using subchronic or chronic exposure regimes would be desirable to better characterize the biological effects during long-term oil contamination. Secondly, the available toxicity data for dilbit to fish species are still extremely limited, particularly those are universally adopted in ecological risk assessment (e.g., LC20, LC50). It is therefore necessary to establish the basic dilbit toxicity data to a greater diversity of both standard test species and native residents in the waters of the Pacific Northwest. The present results show that many dilbit-induced adverse outcomes are not only concentration-dependent, but also highly life stage-dependent (Chapter 3, 5). Salmon are species with complex life histories; fish of different ages may exhibit remarkable variations in sensitivity to oil exposure. Research characterizing dilbit toxicity using salmon at multiple life stages would further assist in identifying the most susceptible age group(s) in a population and provide long-term conservation implications. Thirdly, the combined effects of oil contamination and naturally-encountered stressors (e.g., temperature, salinity, hypoxia, pathogens, and competition) to teleosts still remain unknown. Some have hypothesized that the toxic stress induced by oil could compromise fish's tolerance to the physiological challenges from those environmental stressors in the real world, contributing to impacts on fitness, populations, and communities, that may not be predicted from the direct toxicity of hydrocarbons alone (Whitehead, 2013). Further studies must consider the inherent complexities in interactions between oil contamination and the effects of natural stressors on organisms.

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Table 7. 1. A brief summary and potential implications of the adversely affected endpoints observed in the present work.

Adversely Affected Endpoints	Affected Life Stage(s)	Potential Implication
• Mortality • Growth and development	• Developing ELS salmon (embryo to fry) • Juvenile salmon (fry to yearling parr)	• Crucial impacts on the survival and fitness of fish at individual and population level under natural conditions • Straightforward information for risk assessment and habitat restoration following oil spill accident
• Swimming performance, cardiac capacity, recovery from exhaustive exercise	• Juvenile salmon (fry to yearling parr)	• May directly affect fish's fitness under natural environment • Relevance to impaired ability to capture food, avoid predators, migrate between habitats, and schooling
• Osmoregulation, immune function, endocrine stress response	• Juvenile salmon (fry to yearling parr)	• May indirectly affect fish's fitness in nature • Evidence of reduced osmoregulatory capacity at varying salinity levels • Evidence of suppressed immunological defense against infectious opportunistic pathogen • Evidence of altered metabolic stress response and behavior when encountered by environmental stressors
• Gene expression, enzyme activity (e.g., EROD)	• Juvenile salmon (fry to yearling parr)	• Information needed for elucidating Molecular and cellular mechanisms related to effects observed at higher levels • Potential biomarkers for contaminant exposure and habitat quality assessment

Appendix.

Supplemental Information

Table A.1. Custom designed primer pairs for quantitative real-time PCR analysis in this study.

Associate Biological functi	Gene of Interest	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Reference
Housekeeping gene	*ef1a*	CTGTTGCCTTTGTGCCCATC	TTCCATCCCTTGAACCAGCC	This study
Xenobiotic metabolism Phase I	*hsp90*	GCTGAACAAGACCAAGCCCA	AGCCAGGTGTTCCTCCCA	This study
Xenobiotic metabolism Phase I	*cyp1a*	GATGTCAGTGGCAGCTTTGA	TCCTGGTCATCATGGCTGTA	This study
Xenobiotic metabolism Phase II	*gst-π*	CTACTTTGGAGTCCGAGGGC	CGTCCAGTCAGCAAAGTCCA	This study
Xenobiotic metabolism Phase II	*sod1*	TTACTGGGAGCCCTGGTACA	ACAGAGCCTTCTATGGGGGT	This study
Oxidative metabolism	*cs*	GGCCAAGTACTGGGAGTTCA	CTCATGGTCACTGTGGATGG	This study
Oxidative metabolism	*cox*	TACGTGGGGGACATGGTGTT	CCCAGGAGCCCTTCTCCTTC	This study
Stress temperature	*hsp70*	GATGTGGTGTTGGGGCAGTA	GGACAGTGGGGTCATCAAGG	This study
Stress hypoxia	*hif1α*	GACTCCGCCCCAGATTTCC	GGTGCTGGTGATGAGGTGAG	This study
Immune system	*il1β*	TAAAGGGTGGCGAGGGGGT	ACCTTGCTCCCTACCTTCCA	This study
Lipid metabolism	*pparα*	CTGGAGCTGGATGACAGTGA	GGCAAGTTTTTGCAGCAGAT	This study

Table A.2. The concentration and proportion breakdown of individual polycyclic aromatic compound (PAC) detected in the 100% stock WAF of CLWB dilbit at 7, 14, and 28 ‰.

Individual PAH	CLWB-100% at 7 ‰		CLWB-100% at 14 ‰		CLWB-100% at 28 ‰	
	ng/L	% TPAHs	ng/L	% TPAHs	ng/L	% TPAHs
Naphthalene	3530	2.74	3320	3.41	2070	7.09
Acenaphthylene	0.00	0.00	0.00	0.00	1.04	0.00
Acenaphthene	456	0.35	416	0.43	228	0.78
C2 Phenanthrenes/Anthracenes	4060	3.15	3240	3.33	855	2.93
Fluorene	621	0.48	514	0.53	275	0.94
Phenanthrene	1590	1.23	1220	1.25	503	1.72
Anthracene	70	0.05	51.2	0.05	21.5	0.07
C1 Phenanthrenes/Anthracenes	5060	3.92	3740	3.84	957	3.28
Fluoranthene	52.3	0.04	40.1	0.04	12.8	0.04
Pyrene	85.5	0.07	69.4	0.07	20.8	0.07
Benz[a]anthracene	75.1	0.06	57.9	0.06	10.3	0.04
Chrysene	280	0.22	191	0.20	35.6	0.12
Benzo[b]fluoranthene	58.9	0.05	39.1	0.04	6.74	0.02
Benzo[j,k]fluoranthenes	25.8	0.02	18.4	0.02	3.1	0.01
Benzo[e]pyrene	98.7	0.08	70.7	0.07	10.4	0.04
Benzo[a]pyrene	52.2	0.04	33.6	0.03	5.74	0.02
Perylene	180	0.14	131	0.13	20.9	0.07
Dibenz[a,h]anthracene	11.9	0.01	8.67	0.01	1.2	0.00
Indeno[1,2,3-cd]pyrene	28.6	0.02	19.5	0.02	3.51	0.01
C4-Fluoranthenes/Pyrenes	558	0.43	543	0.56	106	0.36
C1-Benzo[a]anthracenes/Chrysenes	1000	0.78	704	0.72	120	0.41
C2-Benzo[a]anthracenes/Chrysenes	1390	1.08	966	0.99	157	0.54
C3-Benzo[a]anthracenes/Chrysenes	506	0.39	319	0.33	56.3	0.19
C4-Benzo[a]anthracenes/Chrysenes	211	0.16	148	0.15	24.2	0.08
C1-Benzofluoranthenes/Benzopyrenes	577	0.45	390	0.40	56.8	0.19
7-Methylbenzo[a]pyrene	44.1	0.03	29	0.03	4.05	0.01
C2-Benzofluoranthenes/Benzopyrenes	330	0.26	242	0.25	35.1	0.12
TPAHs	**128998.2**	**100.00**	**97334.27**	**100.00**	**29206.5**	**100.00**

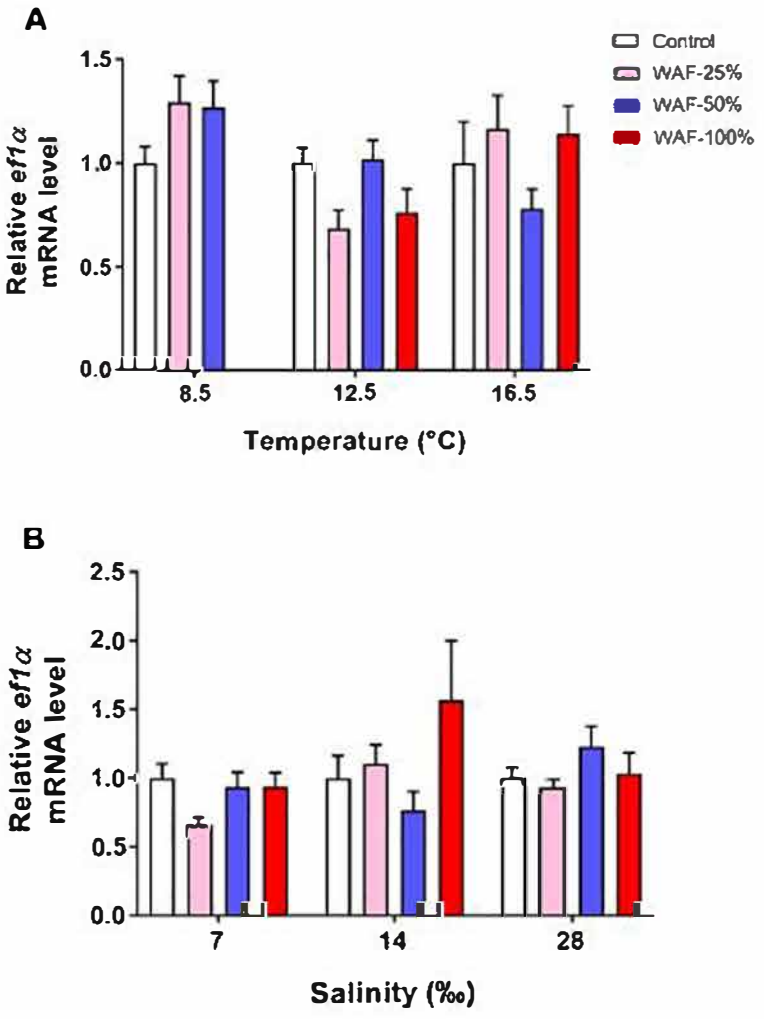

Figure A.1. Housekeeping gene *ef1α* stability of juvenile pink salmon exposed to WAF of CLB dilbit at different temperature and salinity conditions. As in 8.5 °C, one group was missing due to the lack of data, a one-way ANOVA and Dunnett's multiple comparisons test were used to test, in each salinity or temperature condition group, the effects of CLWB-WAF and temperature or salinity ($p < 0.05$). Data are mean ± SE for n = 5-8 fish. Within a salinity or temperature group, an asterisk indicates a difference with their respective control.

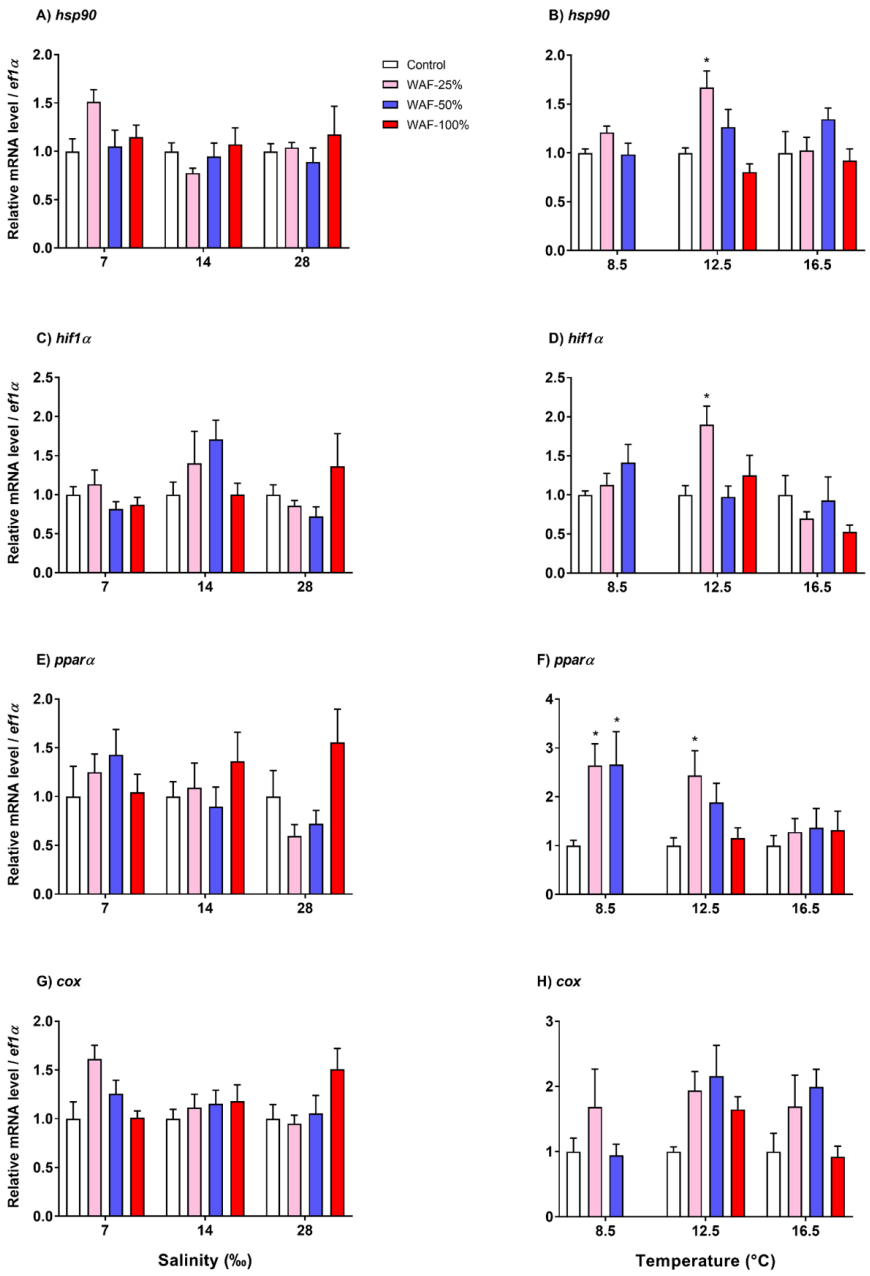

Figure A.2. The effect of exposure to WAF of CLB dilbit on the regulation of A) *hsp90*, B) *hif1*, C) *pparα*, D) *cox* mRNA level on juvenile pink salmon fish exposed to varying of salinities and temperatures. As in 8.5 °C, one group was missing due to the lack of individual (n = 2), unpaired t-test was used and run between each condition with their respective control, in each salinity or temperature condition group, the effects of CLWB-WAF and temperature or salinity ($p < 0.05$). Data are mean ± SE for n = 5-8 fish. Within a salinity or temperature group, an asterisk indicates a difference with their respective control.

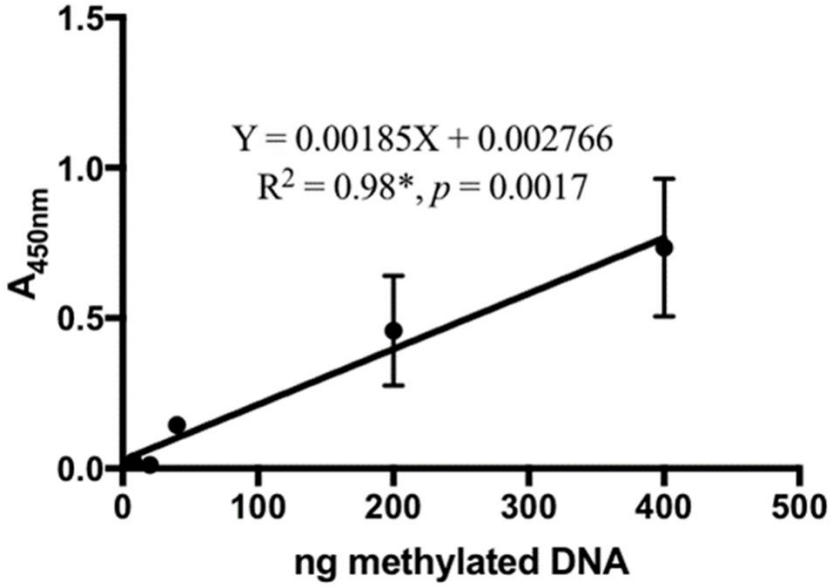

Figure A.3. Linear regression of ng 5-methylcytosine DNA versus absorption at 450 nm (data are mean ± SE for n = 3 technical replicates). The coefficient R^2, the slope equation and level of significance of the correlation are reported on graph.

Complete methodology for the Global CpG methylation assay

The methylation protocol was modified from Pierron et al. (2014). In order to facilitate maximum binding of DNA, 96-well microplates (Corning UV Transparent Flat Bottom) were incubated overnight at 4 °C with 200 µL per well of a 0.1% aqueous solution of protamine sulfate (Sigma-Aldrich, Oakville, ON). The plates were then emptied by simple inversion and washed five times with 200 µL of ultrapure Milli-Q water. These coated plates were then dried at 37 °C for 10 min and stored at 4 °C in dark before use. To establish a standard curve, DNA from several eels was treated with CpG methyltransferase M.SssI (New England Biolabs, Ipswich, MA) according to manufacturer instructions. Briefly, 1 µL of DNA solution at 1 µg/µL was incubated at 37 °C for 90 min with 2 µL of SAM at 32 mM, 2 µL of 10X NEBuffer, 1 µL of SssI methylase at 4 U/µL, and 14 µL of nuclease free water. DNA standard was diluted with TE to obtain a standard solution at 10 ng/µL. Brielfly, 200 µL of standard solutions containing 8, 20, 40, 100, 200, and 400 ng of methylated-DNA as well as DNA samples containing 400 ng of DNA were heated at 94 °C for 2 min and immediately cooled in ice. Then, 50 µL of heat-treated DNA was added in the desired number of wells and incubated 1 h at 37 °C. After DNA attachment, wells were washed five times with 200 µL of PBS (NaCl 0.14 M, Na_2HPO_4 0.01M, pH 7.3). To diminish nonspecific antibody binding, each well was then filled with 200 µL of PBS-BSA solution (2% BSA), and the plate was incubated 1 h at 37 °C. The plate was then washed five times with PBS. Primary antibodies, i.e., anti-5-methylcytosine monoclonal antibody, were diluted with PTB-BSA solution (PBS solution with BSA 2% and Tween-20 0.02%) at a final concentration of 0.5 ng/µL. Wells were then filled with 50 µL of primary antibody solution, and the plate was incubated 1 h at 37 °C. After incubation, the plate was washed three-times with PTB solution (i.e., PBS solution with Tween-20 at 0.02%) followed by two-times with PBS. Secondary antibodies, i.e. Goat anti-Mouse IgG1 Antibody HRP Conjugated, were diluted with PTB-BSA solution at a final concentration of 0.2 ng/µL. Wells were then filled with 50 µL of secondary antibody solution, and the plate was incubated 30 min at 37 °C. After incubation, the plate was washed three-times with PTB solution followed by two times with PBS. Then, 150 µL of TMB solution (Pierce) was added to each well, and the plate was incubated between 10 to 20 min at room temperature according to the fish species.

The reaction was stopped by the addition of 50 µL of H_2SO_4 at 2N. The absorbance was then read at 450 nm in a plate reader (Infinite M1000 Pro, Tecan, Morrisville, NC).