The Influence of Chemical Dispersion and Temperature on the Physiological Responses to Physically Dispersed Crude Oil Exposure in Larvae of a Commercial Cold-Water Marine Invertebrate, the American Lobster (*Homarus americanus*)

by

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ABSTRACT

Pelagic larvae of marine animals are vulnerable to oil spills because they are a sensitive early life stage that inhabit near-surface waters. Yet, there is a poor understanding of the physiological effects of petroleum exposure and spill response measures in cold-water marine larvae, especially of invertebrates and during variation in natural factors such as temperature. I found that relevant concentrations of physically and chemically dispersed crude oil had no effect on the metabolic rate and heart rate of larval American lobster (*Homarus americanus*). Subsequently, I found that exposure to relevant concentrations of physically dispersed crude oil at three environmentally relevant temperatures (9,12,15°C) caused no effect at 12°C compared with an increased metabolic rate at 9°C and high mortality and low heart rate at 15°C. Overall, metabolic and cardiac function of lobster larvae are resilient to oil and dispersant exposure, but there is temperature-dependency in responses to physically dispersed oil.

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List of Symbols, Nomenclature or Abbreviations

m^3: Cubic meter
SIMA: Spill impact mitigation assessment
API: American Petroleum Institute
MAH: Monocyclic aromatic hydrocarbon
PAH: Polycyclic aromatic hydrocarbon
AhR: Aryl hydrocarbon receptor
CYP1A: Cytochrome P450 1A
SMR: Standard metabolic rate
MMR: Maximum metabolic rate
RMR: Routine metabolic rate
µmol: Micromole
O_2: Oxygen
h: Hour
mg: Milligram
$\dot{M}O_2$: Oxygen consumption rate, a proxy of metabolic rate, measured in µmol O_2 h^{-1} mg^{-1}
min: Minute
f_H: Heart rate, measured in beats min^{-1}
SV: Stroke volume
WAF: Water-accommodated fraction
CEWAF: Chemically enhanced water-accommodated fraction
m: meter
°C: Degrees Celsius
g: Gram
L: Liter
µm: Micrometer
µL: Microliter
dw: Dry weight
DO: Dissolved oxygen
cm: Centimeter
rpm: Revolutions per minute
v/v: Volume per volume
s: Second
RFU: Relative fluorescence units
∑PAH: Sum of polycyclic aromatic hydrocarbons
GLM: Generalized linear model
CoV: Coefficient of variation

Chapter 1: General Introduction

The Risk of Oil Spills in Canadian Waters

Petroleum exploration and extraction occurs in oceans worldwide, and the risk of an oil spill poses a threat to marine organisms by causing damage through direct fouling and oil toxicity. Canada is the fifth largest oil producer and fourth largest crude oil exporter in the world (Natural Resources Canada, 2020). Atlantic Canada produces in excess of 233,000 barrels of oil per day and experiences approximately 17,000 oil tanker transits each year (CAPP, 2019a; ClearSeas, 2020). An oil spill off the coast of Nova Scotia in 1970 marked the first major oil spill in Canadian waters, which initiated research on the fate and effects of oil in the ocean and the development of spill response measures (Lee *et al*., 2020). Despite the presence of environmental protection regulations and measures to prevent oil spills, the ever-developing Canadian offshore oil industry still poses a risk to Canada's marine environment.

The estimated risk of an oil spill in a Canadian region is calculated based on the estimated probability of an oil spill and the environmental sensitivity of the region. The probability of an oil spill is estimated from shipping traffic, cargo volume, and spill frequency data. Because oil spills in Canadian waters occur infrequently, spill frequency estimates are based on both federal and international incident data, which could overstate the likelihood of a spill in Canada. The environmental sensitivity of the region is determined based on the physical environment (e.g., water depth, tide, physical oceanography), biological environment (e.g., aquatic species living in waters of study), and human environment (e.g., commercial fishing, aquaculture, tourism) (WSP Canada

Inc., 2014). The combination of the probability and environmental sensitivity calculations produce the Environmental Risk Index (ERI). The ERI is calculated along four classes of spill volumes (ranging from 10-10,000 m^3) and five classes of risk level (very-low to very-high). Although the risk of a large oil spill (i.e., >10,000 m^3) in Canada overall is considered to be low, ERI values vary across the country (WSP Canada Inc., 2014). Three regions within the Atlantic Canadian sector have ERI values that place them at the highest estimated risk of a large-scale crude oil spill in Canada, given the higher probability of a spill and high environmental sensitivity in the regions (WSP Canada Inc., 2014). Furthermore, all sectors in Canada are at a high risk of small and medium oil spills, especially in the 100-999 m^3 range.

When oil spills into a marine environment, it undergoes physical, chemical, and biological changes that ultimately determine its fate; these processes are known as oil weathering. Factors that affect oil weathering include weather and sea conditions (e.g., wind speed, wave action, currents, and atmospheric and water temperature) and degree of sunlight (i.e., photo-oxidation of oil) (Ramseur, 2010). Some natural processes, such as natural dispersion and dissolution/evaporation, help to remove oil from the marine environment, while others lead to its persistence (e.g., formation of water-in-oil emulsions). It was estimated that 37% of spilled oil from the Deepwater Horizon blow-out in the Gulf of Mexico in 2010 was remediated by natural processes, while 41% was remediated by human efforts (Ramseur, 2010).

Responding to an Oil Spill in Canadian Waters

The Canadian oil spill response is built on three pillars of defence: prevention, preparedness, and response (CAPP, 2019b). Spill prevention and preparedness measures include monitoring, maintaining, and repairing equipment, employee training, implementing new research and technology, identifying potential risks and preparing detailed oil spill response and contingency protocols (CAPP, 2019b). If prevention measures fail and an oil spill occurs, the first step in Canada's clean-up response is to contain the spill by booming (i.e., sorbent, floating barriers used to contain the oil spill). A Spill Impact Mitigation Assessment (SIMA) is then used to select feasible and effective response options that minimize impacts on the environment and community (Delaney, 2021). Approved response options in Canada include mechanical and natural recovery. Mechanical recovery is the most frequently used oil spill response method in Canada; along with containment booms, it includes the deployment of response vessels that use skimmers, sorbent pads, and vacuums to remove oil from the water. During rough weather conditions (e.g., strong currents and high waves), mechanical recovery strategies are difficult and unsafe, thus the oil is left to evaporate, emulsify, dissolve, and naturally disperse, followed by bioremediation (ClearSeas, 2020). Furthermore, the use of spill-treating agents (e.g., chemical dispersants) can be approved for use by the Canada Energy Regulator depending on the unique characteristics of the spill (Government of Canada, 2016; CAPP, 2019c; ClearSeas, 2020).

Dispersants are made up of surfactants and solvents and are sprayed onto the surface of the oil slick to lower the surface tension between the water and oil (Bejarano, 2018; Lessard and Demarco, 2002). This promotes the formation of low-concentration

droplets, which accelerates natural dilution of oil in the water column (Lessard and DeMarco, 2000). While chemical dispersion reduces the impact of oil spills on shorelines, it does not reduce the amount of oil entering the marine environment. Currently, there is only one approved chemical dispersant in Canada: Corexit® EC9500A (Government of Canada, 2016). This product is particularly effective at dispersing slicks of heavy oil on seawater and was approved by Environment and Climate Change Canada based on the results of laboratory tests and field studies, documented experiences from real-life spill response activities, and its overall designation of best-in-class performance from oil spill researchers in North America (Government of Canada, 2016; Corexit, 2022). Other chemical dispersants are under study in Canada with the intention of expanding the list of available response options; approval is largely based on results of toxicity and effectiveness tests, relative to the currently approved Corexit® product (Government of Canada, 2016). Slickgone EW is among the list of dispersants being tested and will be a focus of my MSc thesis. This product is effective at emulsifying a broad-spectrum of oils (e.g., crude oils, intermediate/heavy fuel oils, water-in-oil emulsions) and is currently approved for use in the United Kingdom and Australia. To date, the effect of dispersant addition on marine organisms has largely focused on Corexit EC9500A. Bejarano et al. (2018) estimated the 5% hazard concentration (i.e., HC5; concentration that protects 95% of species included in a species-distribution curve) from seven aquatic species acutely exposed (6-96 hours) to Slickgone EW (dispersant-only exposure); an HC5 of 15 mg/L was calculated, compared to Corexit EC9500A's estimate of 5.8 mg/L. This could suggest that Slickgone EW is less acutely toxic to aquatic

organisms compared to the currently approved Corexit EC9500A, which justifies further testing of Slickgone EW for approval in Canada.

Production and Composition of Crude Oil

Crude oil is a fossil fuel composed primarily of hydrocarbons and is found in large quantities in underground reservoirs beneath land or the ocean floor. It is refined into useable petroleum products such as gasoline, diesel fuel, and heating fuel (EIA, 2021). Canada produced 5.9% of the total global supply of crude oil in 2019, with the majority being exported to the United States (Canada Energy Regulator, 2021). Crude oil is typically categorized as conventional or unconventional, which simply refers to the technique used to produce and extract the oil. Conventional crude oil is found in pools within a porous, permeable rock formation, yet below impermeable, impervious rock barriers. It is liquid at atmospheric temperature and pressure, so traditional drilling methods (i.e., vertical well bores and a pipeline) are used to extract oil from the pools (CAPP, 2019c). In contrast, unconventional crude oil does not flow on its own; it is found within low-permeability rock formations (e.g., shale, sandstone) and cannot be recovered using conventional drilling methods. Rather, novel methods are used to extract the oil (e.g., oil sands, hydraulic fracturing) that are more energy intensive than conventional oil extraction techniques (Government of British Columbia, 2021). The production of unconventional crude oil is becoming increasingly relied on in Canada due to the depletion of conventional crude oil reserves, particularly in Western Canada. However, the offshore oil industry in Newfoundland and Labrador is still a large producer and exporter of conventional crude oil, producing an average of 260,000 barrels per day in

2021 (Canada Energy Regulator, 2021). As such, a conventional heavy crude oil product will be the focus of my MSc thesis.

Depending on its specific gravity, crude oils can be classified as light, medium, or heavy. The American Petroleum Institute (API) gravity number is the industry's standard to measure how light or heavy a petroleum product is in comparison to water and is inversely proportional to the product's specific gravity (CAPP, 2019c). Because heavy oils have a higher proportion of long hydrocarbon chains, it increases the specific gravity of the oil; thus, the heavier the oil, the lower the API gravity number. Consequently, portions of heavy crude oils tend to sink, which increases the difficulty of the spill response and leads to the persistence of oil in the environment (ClearSeas, 2020).

Though crude oil is a complex mixture of organic compounds, its composition is dominated by hydrocarbons of varying molecular weights. Of these hydrocarbons, monocyclic aromatic hydrocarbons (MAHs) and polycyclic aromatic hydrocarbons (PAHs) are major contributors to the toxicity of crude oil to organisms (Albers, 2003; Saadoun, 2015). MAHs (e.g., benzene, toluene, ethylbenzene, xylenes) are composed of single aromatic rings, and are relatively water-soluble; however, they have low-molecular weights, which makes them volatile and highly biodegradable (McGrath and di Toro, 2009). In contrast, PAHs in crude oil (e.g., naphthalene, phenanthrene, fluoranthene) have configurations ranging from two- to six-rings, have low volatility and are slow to degrade from aquatic environments; volatility decreases as the number of rings increases, and thus heavy PAHs (four or more rings) are highly resistant to biodegradation and may have a long-term impact on the environment (McGrath and di Toro, 2009). As a result, concentrations of crude oil in toxicity studies are typically characterized by the sum of

PAHs in the sample and the PAH content increases with the specific gravity of the oil (Albers, 2003).

Mechanisms of Acute and Chronic PAH Toxicity

Toxicity refers to sublethal or lethal effects of exposure to a toxicant and can be acute or chronic. Chronic PAH toxicity refers to the sublethal or lethal effects of prolonged (e.g., weeks to years) and/or repeated exposure to PAHs (United States Environmental Protection Agency, 1994). In early aquatic life stages (e.g., embryos and larvae), PAH chronic toxicity may be explained by the alkyl phenanthrene model, which proposes that alkyl phenanthrenes drive toxicity. The specific pathway is unknown, though oxidative stress and effects on cardiovascular morphogenesis have been hypothesized as underlying mechanisms (Barron *et al*., 2004; Turcotte *et al*., 2010). In general, chronic effects of PAH exposure are less understood compared to acute effects (Pasparakis *et al*., 2019). Chronic sublethal effects of PAH exposure in marine fishes and invertebrates include decreased larval condition, reduced growth, changes in diet, trophic level shifts, and decreased reproduction (Suchanek, 1993; Robidoux *et al*., 2018; Pulster *et al*., 2020).

Acute PAH toxicity typically refers to immediate, and often reversible, adverse effects of a single, short-term exposure (typically 24-96 hours) to PAHs (United States Environmental Protection Agency, 1994). The acute sublethal and lethal toxicity of PAHs in marine animals is generally attributed to nonpolar narcosis, which is a non-specific reversible anaesthetic effect that occurs when hydrophobic chemicals partition into the cell membrane and disrupt membrane function (di Toro *et al*., 1999; Saadoun, 2015). As

a nonpolar narcotic chemical (i.e., type I narcotic chemical), PAHs typically accumulate in the hydrophobic core of cell membranes (Wezel and Opperhuizen, 1995). Once a narcotic chemical is taken up by an organism, it is distributed to different organs and tissues until it reaches its target site (i.e., where the chemical exerts its toxicity). The concentration of a chemical at a target site is referred to as the target burden, and toxicity is hypothesized to be additive (i.e., the sum of individual PAH effects). Sublethal and lethal effects of exposure occur when the target burden is sufficient to cause a change at the target site, which in turn affects the functioning of the organism. This change is hypothesized to be an increase in membrane lipid fluidity, which occurs as a result of narcotic chemicals lowering the transition temperature between the gel phase and the liquid-crystalline phase of phospholipids. The result can be perturbation of the membrane's functional components, such as membrane-bound enzymes, which are vital for cellular life (Wezel and Opperhuizen, 1995). The target site of type I narcotic chemicals is hypothesized to be a lipid fraction that is non-species specific (i.e., the target lipid has the same lipid-octanol linear free energy relationship in all organisms) (di Toro *et al*., 1999; McGrath and di Toro, 2009). However, the concentration of chemical required to bring about a change at this target site is species-specific due to varying membrane compositions and varying levels of sensitivity (di Toro *et al*., 2000).

PAHs may also exert acute toxic effects through the aryl hydrocarbon receptor (AhR) pathway (Incardona *et al.*, 2004). During exposure, PAHs bind to the aryl hydrocarbon receptor (AhR), which regulates the expression of cytochrome P450 1A (CYP1A). CYP1A is an enzyme involved in metabolizing xenobiotics such as PAHs, yet much of the acute toxicity of PAHs has been found to arise from the oxidative stress and

cellular damage resulting from CYP1A catalytic activity (Incardona et al. 2005). The AhR is present in all vertebrates, although is not found in all invertebrates (e.g., American lobster), and thus it is not a universal mechanism for PAH toxicity.

Acute sublethal effects of PAH exposure in marine fishes and invertebrates include reduced shell growth and fertilization success, decreased swim performance, reduced hatching success, impaired predator avoidance, and decreased feeding rates (Suchanek, 1993; Mager *et al*., 2014; Boulais *et al*., 2018; Hansen *et al*., 2019; Laurel *et al*., 2019; Cresci *et al*., 2020). Impairments to the cardiorespiratory pathways, including cardiac function and metabolic rate, have also been identified as markers of acute sublethal PAH toxicity (Pasparakis *et al.*, 2019).

Metabolic Rate

Metabolic rate is the amount of energy an animal expends per unit of time. It is bound by minimum and maximum limits, defined as standard metabolic rate (SMR) and maximum metabolic rate (MMR), respectively (Norin and Speers-Roesch, 2020). SMR is the basic cost of living of an animal (i.e., in a post-absorptive, non-reproducing, and quiescent state), while MMR is the upper limit of aerobic metabolic capacity (Norin and Clark, 2015; Chabot *et al*., 2016). The difference between MMR and SMR is an animal's aerobic scope, which is the aerobic metabolic capacity to support important life processes such as digestion, exercise, and activity (Norin and Speers-Roesch, 2020). Though a variety of levels of metabolic rate exist within these bounds, routine metabolic rate (RMR) is most commonly measured in marine fish larvae. RMR includes the cost of growth and maintenance functions (e.g., spontaneous movement), but excludes costs of

food processing (i.e., specific dynamic action), reproduction, and active movement. Because metabolic rate fundamentally determines an organism's energy demands and functional capacities, it is a major focus for research on the impacts of environmental change on animals.

Aerobic metabolism is the dominant type of metabolism under normal, well-oxygenated conditions, and thus the measurement of oxygen consumption rate (i.e., $\dot{M}O_2$) can be used as a proxy for metabolic rate. $\dot{M}O_2$ is estimated by placing an organism in a sealed chamber (respirometer) and measuring the rate of oxygen decline within the chamber as the organism respires. $\dot{M}O_2$ measurements can be performed on tiny organisms such as eggs, embryos, and larvae, though it is important to use equipment that ensures a high signal-to-noise ratio to capture the respiratory signal from such small individuals (Peck and Moyano, 2016). Throughout my thesis experiments, I used a microplate respirometry system to measure $\dot{M}O_2$ of larval lobster (~1 mg). For this type of respirometry, individual animals are placed within small gas-tight wells of a glass microplate fitted with oxygen sensor spots that contain a luminescent dye. The microplate is then placed on a 24-channel optical fluorescence oxygen reading device; when this reader fluoresces, oxygen interferes with the luminescent properties of the sensors' dye through a process known as quenching. The reduction in fluorescence intensity is compared to a reference reading, which allows for real-time measurements of oxygen partial pressure in the wells over time (Wang and Wolfbeis, 2014). To prevent oxygen leakage during the measurement period, the microplate is sealed with a film, silicone gasket, and compression block; however, this also prevents the ability to monitor animal activity in the wells. Consequently, it is impractical to measure SMR using this system

because larval activity must be measured to ensure its absence in measurements. SMR estimates are also challenging to obtain in early life stages because it is difficult to exclude the cost of growth from such rapidly and constantly growing organisms (Peck and Moyano, 2016). For this reason, I measured routine $\dot{M}O_2$ in my experiments.

Cardiac Function

The circulatory system plays an important role in sustaining metabolic rate by pumping circulatory fluid (i.e., blood or hemolymph) to deliver oxygen and fuels to tissues and remove waste. The heart is the driver of blood circulation, and its function and performance are tightly linked to metabolic rate. Cardiac output is the amount of circulatory fluid pumped by the heart per unit time and ultimately determines circulatory oxygen delivery to the tissues (King and Lowery, 2022). Cardiac output is modulated to meet the energetic demands of an animal and is thus linked to $\dot{M}O_2$ and metabolic rate. Cardiac output is a product of stroke volume (SV), which is the volume of circulatory fluid ejected by each heartbeat, and heart rate (f_H) (King and Lowery, 2022). In many species, changes in cardiac output are largely driven by changes in f_H, rather than SV (Gore and Burggren, 2012). As such, I measured f_H as a proxy for cardiac performance in my thesis experiments.

Effects of Crude Oil Exposure on Cardiac Function and Metabolic Rate

Following the Deepwater Horizon blowout that released four million barrels of crude oil into the Gulf of Mexico in 2010, there was a rapid expansion in research investigating the effects of oil exposure on marine animals, in particular adult and early life stages of pelagic marine fishes. The heart emerged as a main site of toxic and

sublethal effects of oil exposure. Common cardiac effects of crude oil exposure observed included reduced heart rate (i.e., bradycardia), irregular heartbeat (i.e., arrhythmia), and fluid accumulation in the pericardial area (i.e., pericardial edema) (Incardona *et al*., 2014). Three-ringed PAHs have been directly linked to cardiotoxicity; although the underlying mechanism is not fully elucidated, it is thought to be independent of narcosis and AhR modes of toxicity, and rather a result of ion channel blocking activity. Specifically, PAHs disrupt excitation-contraction (E-C) coupling by blocking potassium repolarizing currents and reducing intracellular Ca^{2+} levels, thus affecting cardiac rhythm (Incardona and Scholz, 2018). Crude oil effects on cardiac function are often accompanied by impairments in metabolic rate, or vice versa, leading to a reduction in overall cardiorespiratory performance (Incardona and Scholz, 2018). Changes in $\dot{M}O_2$ in oil-exposed individuals could reflect an impairment of physiological function (e.g., cardiotoxicity) or a cost of PAH detoxification that causes energy to be consumed that otherwise could be allocated to fitness-related performance, such as growth, development, foraging and swim performance/predator avoidance (Klinger *et al*., 2015; Pasparakis *et al*., 2019; Cresci *et al*., 2020).

The American Lobster (*Homarus americanus*)

Oil extraction and risk of oil spills occurs in cold oceans worldwide, and although studies on warm-water species dominate the literature, there is an emerging interest in understanding impacts of oil exposure on cold-water marine species. Given the abundance of cold-water species in Canadian waters, there is a need to develop a more comprehensive understanding of potential effects of an oil spill on local, native species

(Echols *et al*., 2015). For example, American lobster (*Homarus americanus*) are an ecologically and commercially important species on the East Coast of North America, where the value of commercial landings exceeded $1.2 billion in 2020 (Fisheries and Oceans Canada, 2020; NOAA Fisheries, 2020). For my thesis, I investigated the physiological effects of oil exposure on lobster larvae, whose planktonic stage makes them vulnerable to a spill.

Approximately one year after mating, female lobsters enclose their fertilized eggs in egg envelopes and carry them under their pleopods throughout embryonic development. Females carry their eggs for 9-12 months before the eggs hatch and moult into the first of three strictly planktonic larval stages (i.e., stages I, II, and III). My thesis will focus on stage I larvae, largely for ease of supply and a higher rate of survival in laboratory settings. Though the morphology, behaviour, and distribution of larval lobsters is generally well understood, little is known about the ontogenetic development of the cardiorespiratory system of larval crustaceans, and in particular American lobsters.

Adult American lobsters have a single-chambered neurogenic heart composed of striated cardiac muscle fibers that receives innervation from a cardiac ganglion. Despite strict neurogenic control of heartbeat in adult decapods, there is evidence of initial myogenic control in embryonic and larval stages of some species, and thus it is not known whether larval lobster hearts are under neuronal control, spontaneously active, or a combination of the two (Spicer, 2001; McMahon *et al*., 2002; Fitzgibbon *et al*., 2015). Adult American lobster hearts circulate hemolymph (i.e., circulating fluid) in an open circulatory system and hemocyanin is the oxygen-carrying protein. During contraction, the heart pumps oxygenated hemolymph through seven arteries to the body, then to the

gills, and back to the heart (Martin and Hose, 1995). Crustacean larvae can also rely on diffusion of gases via the exoskeleton due to their small size (Oellermann *et al*., 2022). American lobster is not a standard toxicity test species and its responses to crude oil exposure are less well understood compared to other crustaceans and invertebrates, such as mysid shrimp (*Americamysis bahia*) (de Jourdan *et al*., 2022). Crude oil exposure has caused reduced growth, delayed molting, and decreased food consumption in larval lobsters, and the lethal toxicity of PAH exposure has been increasingly studied (Wells and Sprague, 1976; Capuzzo *et al*., 1984; Philibert *et al*., 2021; de Jourdan *et al*., 2022). Little work has been done on the effects of crude oil exposure on physiological traits that underlie survival and performance, such as $\dot{M}O_2$ and heart rate, of larval American lobster. The absence of an aryl hydrocarbon receptor and lack of CYP1A-like biotransformation capacity in lobsters suggests the presence of a different pathway of PAH metabolization compared to vertebrates (James and Boyle, 1998; Koenig *et al*., 2012; de Jourdan *et al*., 2021). Due to the lack of a strong detoxification system, it is expected that crustaceans, and other invertebrates, will be more sensitive to toxicants compared to other species with well-established mechanisms of detoxification (e.g., vertebrates) (Bejarano *et al*., 2014). Indeed, acute lethal toxicity studies have identified American lobster among the most sensitive species to petroleum hydrocarbon exposure, relative to fishes and other crustaceans (di Toro *et al*., 2000; Philibert *et al*., 2021; de Jourdan *et al*., 2022).

The Influence of Temperature on the Physiological Effects of Crude Oil Exposure

As ectotherms, American lobster are particular sensitive to changes in environmental temperature because their body temperature closely matches that of the environment. Consequently, changes in temperature can have profound impacts on their physiological performance (Norin and Speers-Roesch, 2020). Because of thermodynamic effects on biochemical reaction rates, biological rates (e.g., metabolic rate, heart rate) are expected to increase with increasing temperatures until a peak temperature is reached; beyond this peak temperature, performance will be impaired (Norin and Speers-Roesch, 2020). Elevated temperatures may increase the susceptibility of aquatic organisms to oil exposure (Pasparakis *et al*., 2016; Perrichon *et al*., 2018). For example, an increase in metabolic rate could lead to an increase in ventilation rate to increase oxygen uptake at the gills, which are also a primary route for xenobiotic entry in aquatic organisms (Hayton and Barron, 1990; Woods *et al*., 2020). Thus, it is possible that a temperature-dependent increase in metabolic rate could lead to increased PAH uptake across the gills.

Changes in temperature may also affect cell membrane fluidity; at higher temperatures, the membrane becomes more fluid, and consequently more permeable to the passive diffusion of molecules through the lipid bilayer (Blewett *et al*., 2021). Therefore, it is possible that an increase in membrane permeability could also increase the uptake of PAHs via passive diffusion, given the non-polar nature of these compounds. Furthermore, PAH solubility in water increases with increasing temperature, thus increasing the bioavailability to aquatic organisms (Nagpal, 1993). Because PAHs are expected to disrupt cell membrane function (i.e., increase membrane lipid fluidity), the combined effect of increased temperature and increased PAH uptake could theoretically

result in a loss of cell membrane function (Wezel and Opperhuizen, 1995). Membrane function is required for life, in particular for establishing vital electrochemical gradients across the cell's membrane and driving electrical activity in excitable cells such as heart muscle cells. Thus, membrane disruption can be detrimental to an organism, potentially resulting in death. Given the pervasive effect of temperature on whole-animal performance, and the wide variation in environmental temperatures seen in the ocean, it is important to develop a better understanding of the effect of temperature on the sublethal toxicity of crude oil to marine early life stages.

Thesis Objectives

The mechanisms and markers of physiological sublethal toxicity that may culminate in lethal responses or deleterious impacts on performance in larval lobsters are not well studied. Such knowledge is important for a comprehensive understanding of the potential impact of oil spills on this commercially important native species of Atlantic Canada. The overarching objective of my thesis was to evaluate the sublethal physiological impacts of crude oil exposure in larval lobsters, and whether additional impacts were incurred by the addition of a chemical dispersant or a change in temperature. Specifically, I assessed metabolic rate and cardiac function because these are temperature-sensitive endpoints and thought to mediate, and/or reflect, responses to oil exposure in vertebrate larvae. The outcomes of my research will contribute knowledge to our understanding of potential adverse effects of crude oil exposure in Canadian marine environments.

Chapter 2: The Effect of Chemical Dispersion and Temperature on the Metabolic and Cardiac Responses to Physically Dispersed Crude Oil Exposure in Larval American Lobster (*Homarus americanus*)

INTRODUCTION

The risk of an oil spill associated with petroleum exploration and extraction poses a potential threat to marine organisms worldwide. Early life stages of marine animals have been identified as particularly vulnerable; embryos and larvae of many species reside and develop in the epipelagic zone, which is impacted by oil spills (Forth *et al*., 2021). Following an oil spill, a small fraction of oil is mechanically mixed below the water's surface by wave and current action, producing a water-accommodated fraction (WAF) of oil (i.e., physically dispersed oil; McIntosh *et al*., 2010). During spill clean-up, chemical dispersants can be applied at the surface or subsea to atomize oil (Lee *et al.*, 2013). The formation of these low-concentration droplets facilitates dilution and biodegradation in the water column and produces a chemically enhanced WAF (CEWAF) of oil (i.e., chemically dispersed oil; Lessard and DeMarco, 2000). The potential for combined toxicity of crude oil and dispersant remains unclear; some studies found that CEWAF exposure increased sublethal and lethal toxicity to marine early life stages compared to WAFs (Ramachandran *et al*., 2004; Anderson *et al*., 2014; Li *et al*., 2018, 2021), while others found equal or less effect (Fuller *et al*., 2009; Hemmer *et al*., 2011; Gardiner *et al*., 2013; Esbaugh *et al*., 2016; Garcia *et al*., 2020).

While crude oil is a complex mixture of hydrocarbon and nonhydrocarbon compounds, polycyclic aromatic hydrocarbons (PAHs) are recognized as particularly toxic constituents. Crude oil-derived PAH exposure has been shown to cause impaired swimming ability and metabolic function, morphological defects, cardiotoxicity, reduced growth and increased mortality in early life stages of marine fishes and invertebrates (Suchanek, 1993; Carls *et al*., 1999; Heintz *et al*., 1999; Incardona *et al*., 2004; Takeshita *et al*., 2021). In particular, cardiac effects of crude oil exposure are well-studied in early life stages of warm-water marine vertebrate species, and include bradycardia, arrhythmias, pericardial edemas, reduced stroke volume, and reduced cardiac contractility, all of which could cause knock-on effects on performance (Mager *et al*., 2014; Incardona *et al*., 2014; Edmunds *et al*., 2015; Esbaugh *et al*., 2016; Khursigara *et al*., 2017; Perrichon *et al*., 2018). The effect of crude oil exposure on cardiac function also appears to occur in early life stages of cold-water marine fishes, with similar cardiotoxic symptoms observed (Kocan *et al*., 1996; Carls *et al*., 1999; Nahrgang *et al*., 2016; Laurel *et al*., 2019; Bender *et al*., 2021).

Cardiac function is linked to oxygen uptake and delivery to tissues and is thus involved in sustaining the energy demands of an animal. Disruptions to cardiac function therefore have the potential to perturb metabolic rate, and, conversely, changes in tissue energy demand and supply processes can feed back to affect cardiac function. Because most energy turnover under normal conditions is aerobic, measurement of whole-animal oxygen consumption rate ($\dot{M}O_2$) approximates metabolic rate and thus the energy requirements, or cost of living, of an organism. As such, $\dot{M}O_2$ is widely recognized as a major indicator of the effects of environmental stress on animals, which can influence

energy availability and/or the energetic demands of an individual (Norin and Speers-Roesch, 2020). Given the rapid growth and development rates of marine larvae, which requires high $\dot{M}O_2$ to sustain high energy demands, investigating how $\dot{M}O_2$ is impacted by environmental stressors is especially important to understand environmental constraints on larval growth and survival (Peck and Moyano, 2016).

The most common level of metabolism measured in marine larvae is routine metabolic rate (RMR) (Peck and Moyano, 2016). RMR is the rate of oxygen consumption measured in individuals in a post-absorptive state within semi-confining chambers that allow for spontaneous swimming (i.e., routine movement), but not active movement (Houde and Schekter, 1983; Peck and Moyano, 2016). Little is known about the effect of oil exposure on $\dot{M}O_2$ of marine early life stages, despite its potential importance as a marker of sublethal toxicity. Available studies on a cold-water and a warm-water fish species show varying responses, with acute exposure to WAF causing increased $\dot{M}O_2$ of embryonic and larval mahi-mahi (*Coryphaena hippurus*; Pasparakis *et al*., 2016, 2017) but decreased $\dot{M}O_2$ of Atlantic cod larvae (*Gadus morhua*; Serigstad, 1987). It has been hypothesized that oil-induced increases in $\dot{M}O_2$ are a result of an increased metabolic demand necessary to metabolize oil components and undergo detoxification processes, which could decrease energy allocation to other important life processes such as growth and development (Cohen *et al*., 2001; Klinger *et al.*, 2015; Pasparakis *et al*., 2016). A decrease in $\dot{M}O_2$, on the other hand, could indicate a lack of energetic investment in detoxification and/or an impairment of oxygen supply or demand mechanisms (Davoodi and Claireaux, 2007; Khursigara *et al*., 2021). Alternatively, a

depression of $\dot{M}O_2$ could be indicative of an adaptive response to decrease overall energy requirements to help cope with acute stress.

Few studies have measured $\dot{M}O_2$ and heart function in larval marine invertebrates, let alone in response to crude oil exposure. To date, only two studies have investigated the effect of crude oil exposure on the oxygen consumption rate of a larval marine invertebrate (the American lobster, *Homarus americanus*), with decreased $\dot{M}O_2$ observed in response to both physical and chemical oil dispersions (Capuzzo and Lancaster, 1981, 1982). Furthermore, to our knowledge, it is not known how crude oil exposure affects the cardiac function of larvae of a cold-water crustacean. Yet, many marine invertebrates have planktonic larvae susceptible to oil spills and spill response measures. For example, American lobster are an important species that hatch from female-carried eggs between May and late August and swim as stage I larvae into the upper water column (i.e., depths of 15-30 m during daylight and 0-10 m at night), where they will undergo two other stages of planktonic development before transitioning to the postlarval stage IV (Capuzzo and Lancaster, 1979; Harding *et al*., 1987). Unlike vertebrates such as fishes, American lobsters lack the aryl hydrocarbon receptor (AhR) that binds PAHs and regulates the expression of CYP1A in vertebrate species, and do not appear to use CYP1 enzymes for PAH biotransformation (James and Boyle, 1998). Lobster larvae may also have neurogenic cardiac control, as opposed to myogenic control in vertebrates. Thus, metabolic and cardiac responses to oil exposure seen in vertebrates may not be relevant nor good predictors of the response in lobsters.

Temperatures encountered by early life stages of marine animals vary greatly across time and space (e.g., latitude); for example, across their range, lobster larvae may

experience sea surface temperatures from below 10°C to above 20°C (Richaud *et al*., 2016). Because temperature has profound impacts on rates of metabolism and other cellular processes, potentially including detoxification pathways, there is a need to understand the temperature dependence of the response to crude oil exposure in early life stages including lobster larvae. Indeed, increasing temperature has been found to increase the vulnerability of early life stages of Arctic and warm-water fishes to sublethal effects of crude oil exposure (Pasparakis *et al*., 2017; Perrichon *et al*., 2017; Perrichon *et al*., 2018; Bender *et al*., 2021). However, the combined effect of temperature and oil exposure on cold-water larval invertebrates is not known.

In the present study, I used respirometry and video recording to assess the effect of acute exposure to environmentally relevant levels of physically and chemically dispersed crude oil on the routine metabolic rate and heart rate of stage I American lobster larvae. I then investigated whether temperature modifies the effect of physically dispersed crude oil on stage I lobster larvae, using three environmentally relevant temperatures. I hypothesize that there is a physiological (metabolic and cardiac) cost of exposure to increasing PAH concentrations of physically dispersed crude oil, and that this cost is exacerbated by dispersant addition and warming.

METHODS

Experimental Animals

Local fishers captured adult commercial-size (0.5-2.0 kg) berried female American lobsters (*Homarus americanus*, family Nephropidae) from the Bay of Fundy Fishing Area 36 under a Fisheries and Oceans Canada license in 2020 (Experiment 1 and 2) and 2021 (Experiment 3). Females were held in communal tanks at Huntsman Marine Science Centre (St. Andrews, New Brunswick, Canada) in ambient flow-through seawater (8-14°C) on a 16:8-hr light:dark cycle, where they were staged weekly based on visual inspection of embryo development. Berried females were transferred to an individual tank with heated seawater (18 ± 2°C) when larval release was imminent (i.e., late stage 3 embryos). Released larvae were collected daily and maintained in 60-L incubators containing aerated ambient flow-through seawater. Larvae were fed artemia daily. For Experiments 1 and 2, exposures were performed on larvae from the same female within an experiment, however a different female was used for each experiment. For Experiment 3, larvae from the same female were used within a temperature trial, however a different female was used for each of the three temperature trials. Dry weights of experimental lobster larvae were not significantly different across control and treatment groups in each experiment (Appendix A, Table A1).

Experiment 1: The Effect of Physically Dispersed Crude Oil on the Metabolic Rate and Heart Rate of Lobster Larvae

Exposure media preparation

A benchtop mixing method was used to prepare exposure media and was a modification of the baffled flask method for dispersant effectiveness used by Environment Canada and the United States Environmental Protection Agency (Venosa *et al*., 2002). A water-accommodated fraction (WAF) of crude oil was prepared to represent physical dispersion of oil in the environment (i.e., WAF is physically dispersed crude oil). The WAF was prepared by adding 1.6 g of oil to the central surface of the water (1.6 L of 1 µm UV filtered seawater, in a 2 L baffled flask) at a loading of 1 g of oil (conventional heavy crude oil) per L of seawater. The flask was sealed with DuraSeal and secured on an orbital shaker (MaxQ SHKE2000 digital shaker, ThermoFisher Scientific, Massachusetts, USA), where it was shaken at 150 rpm for 20 hours. Following a settling time of four hours, the first 100 mL of stock was discarded to remove any water trapped in the spout that was not representative of the WAF. The required volume of stock was subsequently poured out from the spout of the baffled flask to prepare exposure media with nominal concentrations of 72%, 37%, 19%, and 10% WAF by gradient dilution. A seawater (0.22 µm) control was also prepared. All media were prepared in a temperature-controlled room maintained at 12 ± 1°C.

Exposure protocol and post-exposure larval assessment

Exposures were performed on stage I larvae <24 h post-release from a single female lobster (n=10 larvae per treatment) in a temperature-controlled room maintained

at approximately 12 ± 1°C. Lighting for the entire test duration was a photoperiod of 16:8-h (light:dark). Larvae were randomly allocated into the exposure vessels (25 mL scintillation vials; one larva per vial to prevent cannibalism), which were filled with 20 mL of exposure media (i.e., allowing 20% headspace for exchange of surface O_2). The exposure duration was 24 ± 2 h and larvae were fasted during exposure.

At the end of the exposure duration, each larva was blindly assessed and assigned a score of 0, 1, or 2 based on activity level (see Table 1). Only individuals scored 0 or 1 were used to measure the physiological endpoints of interest; individuals that scored a 2 were excluded from measurements because sublethal endpoints cannot be measured on dead organisms. Larvae were then transferred to a microplate respirometry system immediately post-exposure to measure oxygen consumption rate ($\dot{M}O_2$) in a temperature-controlled room maintained at 12.4 ± 0.2 °C (see $\dot{M}O_2$ protocol below). Following $\dot{M}O_2$ measurements, larvae were recovered for a minimum of one-hour (see Validation of Heart Rate Assessment section for justification) prior to recording heart rate (f_H) videos (see heart rate protocol below). After obtaining heart rate measurements, each larva was rinsed with distilled water, blotted dry with a light-duty tissue wiper (VWR, Pennsylvania, USA), and placed in a disposable aluminum weigh boat. Larvae were placed in a drying oven (ThermoFisher Scientific Isotemp Oven, Massachusetts, USA) at 100°C for 24 h and individual dry weights (dw) were obtained using a 5-point microbalance (Sartorius AG, Göttingen, Germany).

Experiment 2: The Effect of Chemically Dispersed Crude Oil on the Metabolic Rate and Heart Rate of Lobster Larvae

A WAF of conventional heavy crude oil was prepared as described in Experiment 1. Additionally, a chemically enhanced WAF (CEWAF) was prepared to represent a chemical dispersion of oil. The CEWAF was prepared by adding dispersant (Slickgone EW; Dasic International, Hampshire, UK) to the centre of the WAF surface oil slick at a dispersant-to-oil ratio of 1:20. Following orbital shaking and settling (refer to Experiment 1), exposure media were prepared with nominal concentrations of 37%, 19%, and 10% WAF and 10%, 5.6%, and 3.2% CEWAF by gradient dilution. A seawater (filtered to 0.22 µm) control and dispersant-only control (nominally 15 mg/L) were also prepared.

Following the methods and trial validity criteria described for Experiment 1, 24-hour exposures were performed on stage I larvae <24 h post-release from a single female lobster at 12 ± 1°C (n=10 per treatment), followed by a post-exposure larval assessment. $\dot{M}O_2$ was measured immediately post-exposure in a temperature-controlled room maintained at 12 ± 0.3°C, followed by a minimum one-hour recovery period and f_H measurements (see protocols below). Dry weights of individual larvae were then obtained as previously described.

Experiment 3: The Combined Effect of Physically Dispersed Crude Oil Exposure and Temperature on the Metabolic Rate and Heart Rate of Lobster Larvae

WAFs were prepared using the CROSERF method described by Singer et al. (2000), which differed from the baffled flask method used for WAF preparation in Experiments 1 and 2. Briefly, 16 g of oil (conventional heavy crude oil) was added to the

central surface of the water (1.6 L of 0.22 µm filtered seawater in a 2 L aspirator bottle) at a loading of 10 g of oil per L of seawater. The bottle was sealed with Duraseal and placed on a magnetic stirrer (ThermoFisher Scientific, Massachusetts, USA). The mixing speed was determined by achieving a 20% vortex depth, and it was mixed for 20 hours. Following a settling time of 4 hours, the required volume of stock was poured from the bottle to prepare exposure media. A seawater control (filtered to 0.22 µm) was also prepared. Three exposure trials were performed on different groups of stage I larvae <24 h post-release in a temperature-controlled room maintained at 9, 12, or 15 ± 1°C, using the same methods and trial validity criteria described in Experiment 1. For the experiment performed at 12°C, exposure media were prepared with nominal concentrations of 100%, 32%, 10%, 3.2%, 1%, and 0.32% WAF by gradient dilution (n=10 exposed per treatment). For the experiments performed at 9 and 15°C, exposure media were prepared with nominal concentrations of 100%, 32%, 10%, and 3.2% WAF by gradient dilution (n=15 exposed per treatment). After the 24 h exposure, larvae were assessed using the scoring scheme described in Experiment 1 and transferred into 25-mL scintillation vials filled with 0.22 µm filtered seawater. Unfortunately, eight individuals (n=2 per treatment) were disposed of accidentally after the 15°C exposure prior to $\dot{M}O_2$ and f_H measurements (resulting in n=13 per treatment at 15°C).

$\dot{M}O_2$ was measured immediately post-exposure in a temperature-controlled room maintained at 9.3 ± 0.09, 12.1 ± 0.3, or 15.4 ± 0.2°C, followed by a minimum one-hour recovery period and f_H measurements (see protocols below). Dry weights of individual larvae were then obtained (see Experiment 1).

Trial Validity Criteria

Water quality (temperature, dissolved oxygen (DO), pH, and salinity) was measured at the start and end of the exposure in pooled samples. The exposure trial was considered invalid if: the combined mortality in the controls was greater than 20%, dissolved oxygen was less than 60% saturation, or temperature variation was greater than 1.5°C between units on the same day. No exposure trials were excluded from analysis based on these criteria.

Measurement of Oxygen Consumption Rate ($\dot{M}O_2$)

Routine metabolic rate was estimated by measuring oxygen consumption rate ($\dot{M}O_2$, μmol O_2 h^{-1}) using closed respirometry. $\dot{M}O_2$ was measured immediately post-exposure using a 24-well glass microplate (Loligo Systems, Viborg, Denmark) situated on an optical fluorescence oxygen reading device (SDR SensorDish Reader, Regensburg, Germany). Each well was fitted with an oxygen sensor spot and sensors were calibrated at the experimental temperature prior to trials with a zero solution and air-saturated water sample (0 and 100% air saturation calibration, respectively), according to the manufacturer's instructions. The $\dot{M}O_2$ measurement protocol was modified between Experiments 1 & 2 (performed in 2020) and Experiment 3 (performed in 2021), thus methods differ slightly and are described below.

Experiments 1 and 2

The loading and running set-up of the microplate system was as follows: glass microplates with well volumes of 500 μL (8 x 10 mm inner diameter x depth) were used for $\dot{M}O_2$ measurements. This well volume resulted in an 83:1 ratio of respirometer

volume (mL) to wet weight of larva (g), which is similar to the recommended ratio for larval measurements (100:1-300:1) (Peck and Moyano, 2016).

Measurement of larval $\dot{M}O_2$ was randomized by treatment and ten individuals were measured per plate (measuring individual blind to treatment group). Four random wells were designated blanks to measure background respiration. Stage I larvae had rapid $\dot{M}O_2$, so, to prolong the measurement period, 0.22 µm filtered seawater was oxygenated and diluted to 120 ± 5% air saturation for use in respirometry. 500 µL of seawater was pipetted into the blank wells, and 200 µL was pipetted into the treatment wells (to be topped up to 500 µL after larval addition). Air bubbles were removed from the wells using a micropipette. A modified plastic transfer pipette (~0.5 cm cut from tip and burned to smooth jagged edges) was used to transfer the larva from the exposure scintillation vial onto a light-duty tissue wiper to remove any residual oil on the individual. A spatula was then used to transfer the larva into its allocated well, and the well was topped up with 300 µL of seawater. Few studies on larval $\dot{M}O_2$ have employed an acclimation period in the respirometer prior to measurement (Peck and Moyano, 2016). Previously, preliminary $\dot{M}O_2$ trials with larval European lobster (*Hommarus gammarus*) indicated that there was no difference in measurements between larvae that received a 20-minute acclimation period, and individuals that were not acclimated (Small *et al*., 2015). Thus, we did not incorporate an acclimation period prior to measurement. Once all wells were loaded, the plate was immediately sealed with sealing film (DuraSeal, VWR, Pennsylvania, USA), a silicone pad, and a compression block following the manufacturer's instructions. The microplate was placed on an orbital shaker set to 100 rpm to prevent oxygen stratification in the wells during the measurement period. The decline in dissolved oxygen content (%

air saturation) in the wells was recorded at 15 s intervals in the MicroResp™ v.1 Automated Microplate Respirometry Software (Loligo Systems, Viborg, Denmark). The plates ran for 30 minutes, or until oxygen readings in the wells reached 70% air saturation. Then, forceps were used to gently transfer the larvae from the wells to 25 mL scintillation vials with 20 mL of 1 µm filtered seawater (one larva per vial) prior to heart rate measurements. Between trials, the glass microplate was thoroughly rinsed with distilled water and chlorinated tap water. After trial completion, the microplate was sterilized with ethanol (10% v/v), rinsed with distilled water, and dried at room temperature for 24 h.

Experiment 3

Microplate respirometry was performed as described for Experiments 1 and 2, with the following modifications. A glass microplate with well volumes of 1700 µL (12 x 15 mm inner diameter x depth) was used for larval $\dot{M}O_2$ measurements and fifteen individuals were measured per plate. The larger well volumes provided a greater respirometer volume to larval mass ratio (280:1), allowing for longer measurement durations. Consequently, there was no longer the need to use 120% air saturated seawater as in Experiments 1 and 2; rather, filtered seawater aerated to 100% air saturation was used. 1700 µL of 0.22 µm filtered seawater was pipetted into the blank wells, and 1000 µL was pipetted into the treatment wells. A modified plastic transfer pipette was used to transfer the larva from the scintillation vial directly into the appropriate microplate well, and the well was topped up with 700 µL of 0.22 µm filtered seawater. Once all wells were loaded, the plate was sealed and placed on an orbital shaker set to 115 rpm to

prevent oxygen stratification in the wells. The plates ran for 60 minutes, or until oxygen readings in the wells reached 70% air saturation. A modified transfer pipette was used to transfer the larvae from the wells to 25 mL scintillation vials with 20 mL of 1 µm filtered seawater (one larva per vial). The use of a plastic transfer pipette for the addition and removal of larvae to and from the wells was more efficient than the use of forceps in Experiments 1 and 2 and posed less risk to damaging the larva during transfer.

Measurement of Heart Rate (f_H)

Lobster larvae heart rates (f_H) were visualized using a stereomicroscope system (Olympus SZX7 microscope, Tokyo, Japan) and microscope software platform (Lecia Application Suite V.4.8, Ontario, Canada). Camera settings were optimized (i.e., image brightness, gamma, and saturation) using a non-experimental lobster larva prior to recording videos. To visualize heart rate, the larva was transferred from the scintillation vial to the bottom of a petri dish using a modified plastic transfer pipette. An unmodified plastic transfer pipette was used to remove the residual water surrounding the larva, which positioned the individual on its side. The beating heart was located in the microscope software and a 30 s video was recorded at a 20x magnification (Leica MC190 HD, Ontario, Canada). Heart rate recordings had to be performed at room temperature (19 ± 2°C), therefore only one larva was removed from the temperature-controlled room and recorded at a time to minimize length of exposure to the warmer temperature (i.e., maximum amount of time exposed to room temperature was one minute).

Validation of Heart Rate Assessment

To validate the reliability of the 30 s recording time for heart rate assessment, videos of stage I larvae (n=11) were recorded for 5 minutes. Heart rate was calculated (see Data Analysis) from the first 30 seconds and last 30 seconds of the video and a paired t-test was used to compare the counts. There was no significant difference between heartbeat counts in the first 30 seconds and last 30 seconds of 5-minute videos recorded on control larvae in preliminary trials (Appendix B, Figure B1), supporting my use of a 30 s recording time.

To validate whether one-hour of recovery post-respirometry was adequate before measurement of heart rate, 24 h fasted larvae at 15 ± 1°C were placed in the respirometry microplate for 40 mins, followed by a one-hour or two-hour recovery in individual scintillation vials with 0.22 µm seawater. Heart videos were then recorded on each of the recovered larvae groups (n=12 per group) and a subset of control larvae (i.e., not held beforehand in microplate; n=12), and heart rates were calculated and compared using a one-way ANOVA to determine if heart rate was similar between the three treatments. An outlier was present in the two-hour recovered group and was removed (Grubbs' test). The f_H of one- and two-hour recovered larvae was not significantly different than control larvae, nor from each other (Appendix B, Figure B2). Thus, we proceeded with a minimum one-hour post-respirometry recovery time.

Heart rates of individual recovered stage I larvae <24 h post-release were also assessed over three days to examine intrinsic intraindividual variability and the repeatability of heart rate measurement within individuals. 24 h fasted larvae at 12 ± 1°C (n=12) were placed in the microplate for 40 minutes, recovered for one-hour, and heart

videos were recorded. The subset of larvae was maintained unfed at 12 ±1°C for a subsequent two days in 0.22 µm seawater, with heart videos being obtained at the same time (±1 hr) on each day. Heart rates were then calculated and compared using a one-way repeated measures ANOVA with Tukey's pairwise comparisons. There was no difference between f_H measurements on day 1 and 2, but day 3 was different from both day 1 and 2 (Appendix B, Figure B3). This supports that there is consistency in f_H measurements within an individual over the first two days after oil exposure and $\dot{M}O_2$ measurements and increases the reliability of my measurements on day 1 (i.e., after a one-hour post-respirometry recovery period). Because larvae were fasted throughout this trial, the significant decrease in f_H on the third day was likely not reflective of inherent variability or a lack of repeatability, but rather a response to being without food for three days, which is a challenge for rapidly developing/metabolizing larvae.

Chemical Characterization of Exposure Solutions

Samples (~700 mL) were collected from the highest WAF and/or CEWAF test concentrations at the beginning of Experiment 1, 2, and 3 exposure trials, and sent to an external laboratory (Research & Productivity Council, Fredericton, NB) for analytical characterization of polycyclic aromatic hydrocarbons (PAHs) and alkyl PAHs by solvent extraction and gas chromatography-mass spectrometry (GC-MS; using the method previously described in the United States Environmental Protection Agency 3510C/8270C document) (Edgell and Wessellman, 1989). Three-dimensional emission fluorometry was also performed on all exposure solutions pre- and post-24 h exposure

using a Horiba Aqualog (Horiba, Ltd., Kyoto, Japan) to validate analytical determination of PAH concentrations.

Data Analysis

Quantification of PAHs

For each experiment, the concentrations (µg L^{-1}) of individual PAHs analytically characterized in the highest concentration test solution were summed to determine the sum of PAHs ($\sum PAH_x$, where x represents the total number of PAHs; µg L^{-1}). Dilution factors were used to estimate $\sum PAH_x$ of other treatment concentrations in each experiment as described by Forth et al. (2017a).

Oxygen consumption rate ($\dot{M}O_2$) analysis

Oxygen partial pressure (% air saturation) in each individual well during the $\dot{M}O_2$ measurement period was plotted against time. The first five minutes of oxygen measurements were excluded from analysis, along with sporadic oxygen values outside the range of 100-70% air saturation, which are likely due to mechanical interference of the larvae with the oxygen sensor. The respirometry package in RStudio (Version 1.2.5033, RStudio Inc., Boston, MA, USA; http://www.rstudio.com) was used to calculate the $\dot{M}O_2$ of each larva (µmol O_2 h^{-1}) from the slope of oxygen decline in the well over a ten-minute (Experiment 1 and 2) or twenty-minute (Experiment 3) interval within the measurement period (Schwemmer *et al*., 2020). R^2 values were calculated for each slope to confirm the reliability of the trace (see below). The $\dot{M}O_2$ calculation accounted for well volume, O_2 solubility at the seawater temperature and salinity, atmospheric pressure, and background respiration. To account for background

respiration, the slopes of oxygen change in the blank wells were calculated for each trial from the same time interval used for each individual larval $\dot{M}O_2$ calculation. The mean of these slopes was subtracted from the slope for the larva well prior to calculation of larval $\dot{M}O_2$. $\dot{M}O_2$ were normalized to mass by dividing by the individual's dry weight, resulting in the reported values for $\dot{M}O_2$ (μmol O_2 h^{-1} mg dw^{-1}). $\dot{M}O_2$ values calculated from the selected slope intervals were also compared to $\dot{M}O_2$ values calculated from the slope of the full measurement period (excluding the first five minutes of oxygen measurements and values outside the range of 100-70% air saturation); if there was >10% difference between these $\dot{M}O_2$ values, R^2 values were compared and the $\dot{M}O_2$ value associated with the highest R^2 was used in analysis.

Larvae where the slopes of oxygen decline had R^2 <0.8 were flagged and the slope was visually assessed (Chabot et al., 2020). If the slope showed an inconsistent decline in oxygen upon visual assessment (i.e., an unreliable reading), the individual was removed from analysis. Based on these removal criteria, across all treatments, 3 individuals (6%) were removed from Experiment 1, 7 individuals (9%) were removed from Experiment 2, and 9 individuals (4%) were removed from Experiment 3 (n=5 from 9°C trial, n=3 from 12°C trial and n=1 from 15°C trial). No more than two larvae were removed from a single control/treatment group, resulting in a minimum of n=8 in all groups for analyses.

Heart rate (f_H) *analysis*

Heart rates (f_H) were counted visually (randomized and observer blind) from the recorded video using VLC media player (Version 3.0.16, VideoLan Organization, Paris,

France, https://www.video lan.org). Video speed was reduced to 40%, and heartbeat counts from each 30 s video recording were multiplied by 2 to calculate f_H in beats min $^{-1}$. If the heart was difficult to visualize in the video and a heartbeat count was unable to be obtained, the video was removed from analysis. Based on this removal criterion, across all treatments, 2 videos (4%) were removed from Experiment 1 analysis, 5 videos (6%) were removed from Experiment 2, and none were removed from Experiment 3. Larvae were also removed from f_H analyses if they died during the one-hour minimum recovery period following $\dot{M}O_2$ measurements and prior to heart rate recordings. During the recovery period, two larvae (4%) died in Experiment 1, one larva (1%) in Experiment 2, and seven larvae (3%) in Experiment 3 (n=1 in 9°C trial, n=4 in 12°C trial, and n=2 in 15°C trial).

Statistics

All statistical analyses were performed in R (version 1.2.5033), and the level of significance was set at $p<0.05$. Values presented in the text are means ± standard deviation, unless otherwise stated.

Oxygen consumption rate ($\dot{M}O_2$) statistics

Because there were inherent differences in the PAH concentrations between WAF and CEWAF preparations in Experiment 2, and also across temperature trials in Experiment 3, concentration could not be used as a common categorical variable. Thus, one-way models were fit to the data for each experiment. Based on visual check of model assumptions, the homogeneity of variance and normality assumptions of an analysis of variance test were violated. As a result, one-way generalized linear models (GLMs) were

fit to the data specifying a Gamma distribution (i.e., $\dot{M}O_2$ is a positive, continuous variable). Multiple one-way GLMs were used to assess WAF concentration effects on $\dot{M}O_2$ (Experiment 1), WAF or CEWAF concentration effects on $\dot{M}O_2$ (Experiment 2), and WAF concentration effects at each temperature on $\dot{M}O_2$ (Experiment 3). A one-way GLM was also used to compare the $\dot{M}O_2$ of seawater control larvae across all three experiments to assess the repeatability of my methods. Type II Wald-chi square tests were performed on all models using the "car" package to identify if there was a significant difference in larval $\dot{M}O_2$ between concentrations. Bonferroni post hoc multiple comparison tests were carried out using the "emmeans" package when significant effects of concentration were found to identify where significant differences occurred.

Heart rate (f_H) statistics

For the same reasons mentioned above, separate one-way GLMs (family=Negative binomial) were fit to the data to evaluate WAF concentration effects on larval f_H (Experiment 1), WAF and CEWAF concentration effects on f_H (Experiment 2), WAF concentration effects at each temperature on f_H (Experiment 3) and compare the f_H of seawater control larvae across all three experiments. A negative binomial distribution was chosen because heart rate is a positive, discrete variable, and the data were over-dispersed. Post hoc multiple comparison tests were carried out as described above (see $\dot{M}O_2$ statistics).

RESULTS

Water Quality and PAH Analysis

The coefficient of variation (CoV) between replicates did not exceed 4% for any water quality variable in any experiment, and all trials were considered valid (Appendix C, Table C1).

Thirty-one PAHs were characterized in Experiments 1 and 2 and the sums of PAHs (µg L^{-1}) in solutions are reported in Table 2. In Experiment 2, measured PAH concentrations were higher in the CEWAF sample compared to those in the WAF sample; in the overlapping nominal concentration for WAF and CEWAF (10%), $\sum PAH_{31}$ was approximately ten times higher in the CEWAF preparation. Thirty-two PAHs were characterized in Experiment 3 and although $\sum PAH_{32}$ of the WAF preparations differed slightly across the three temperature experiments, the fold-increase between concentrations was the same (i.e., same nominal concentrations were used to prepare WAFs) (Table 2).

Experiment 1: Effects of Physically Dispersed Crude Oil Exposure on the Oxygen Consumption Rate ($\dot{M}O_2$) and Heart Rate (f_H) of Lobster Larvae at 12°C

During assessment scoring of lobster larvae (n=53) exposed to four concentrations of WAF and a seawater control, 74% of individuals scored a 0, and 24% scored a 1 (n=2, 5, and 6 at $\sum PAH_{31}$=5.38, 19.90, and 38.72 µg L^{-1}, respectively). One larva died (i.e., score of 2) from the highest tested concentration ($\sum PAH_{31}$=38.72 µg L^{-1}) (Appendix D, Figure D1). There was no significant difference in $\dot{M}O_2$ values between individuals with

an assessment score of 1 compared to control individuals, all of which were assigned a score of 0 (Welch's t-test, p=0.41).

Larval lobster $\dot{M}O_2$ and f_H were not significantly affected by acute exposure to physically dispersed conventional heavy crude oil (WAF). There was no significant difference in $\dot{M}O_2$ or f_H between the tested WAF concentrations/control (Wald chi-square tests, p=0.18 and p=0.51, respectively; Figure 1A and 1B). $\dot{M}O_2$ values and variation were similar across the tested WAF concentrations and ranged from approximately 0.080 – 0.12 µmol O_2 h^{-1} mg dw^{-1}.

Experiment 2: Effects of Chemical Dispersion of Physically Dispersed Crude Oil on $\dot{M}O_2$ and f_H of Lobster Larvae at 12°C

During assessment scoring of lobster larvae (n=80) exposed to three WAF concentrations and a seawater control, and three CEWAF concentrations and a dispersant control, 99% of individuals scored a 0, and one larva scored a 2 (at $\sum PAH_{31}$= 56.02 µg L^{-1}; CEWAF) (Appendix D, Figure D2).

As in Experiment 1, there was no significant difference in $\dot{M}O_2$ estimates between WAF concentrations/control (Wald chi-square test, p=0.44, Figure 2A). Larval lobster $\dot{M}O_2$ was also not significantly different between the tested concentrations of chemically dispersed conventional heavy crude oil (CEWAF made with Slickgone EW; Wald chi-square test, p=0.71, Figure 2B). $\dot{M}O_2$ of seawater control and dispersant-only control larvae did not differ significantly (Welch's t-test, p=0.31).

Larval lobster f_H did not significantly differ between the tested WAF concentrations/seawater control (Wald chi-square test, p=0.47, Figure 2C), nor between

the tested CEWAF concentrations/dispersant-only control (Wald chi-square test, p=0.45, Figure 2D).

Experiment 3: The Temperature Dependency of the Effect of Physically Dispersed Crude Oil Exposure on $\dot{M}O_2$ and f_H of Lobster Larvae

At 9°C, of the 75 individuals measured in the post-exposure activity assessment, 99% of individuals received a score of 0, and one larva received a score of 1 (at $\sum PAH_{32}$=1.27 µg L^{-1}) (Appendix D, Figure D3A). Following exposure at 12°C (n=70), 94% of larvae were given a score of 0 and 6% were given a score of 1 (n=1, 1, and 2 at $\sum PAH_{32}$=0.85, 8.54, and 26.70 µg L^{-1}, respectively) (Appendix D, Figure D3B). At 15°C, of the 75 individuals exposed, 91% received a score of 0, and six larvae died (i.e., score of 2) (Appendix D, Figure D3C). These six larvae were all exposed to the highest tested concentration ($\sum PAH_{32}$=33.82 µg L^{-1}), resulting in 40% mortality in this exposure group at 15°C, compared to no mortalities at the other temperatures.

Larval $\dot{M}O_2$ was significantly different between the WAF concentrations of conventional heavy crude oil at 9°C (Figure 3A). Larvae exposed to the highest WAF concentration ($\sum PAH_{32}$=39.66 µg L^{-1}) had an $\dot{M}O_2$ of 0.12 ± 0.099 µmol O_2 h^{-1} mg dw^{-1}, which was significantly higher than the $\dot{M}O_2$ of control larvae (0.059 ± 0.013 µmol O_2 h^{-1} mg dw^{-1}; Bonferroni post hoc, $p<0.01$) and the other exposure concentrations. More variation was observed in $\dot{M}O_2$ measurements of larvae exposed to the highest WAF concentration compared to the other concentrations and control; specifically, four individuals drove the observed increase, and I confirmed these were not statistical outliers (Bonferroni Outlier, $p>0.05$). At 12°C, there was a significant difference in larval

$\dot{M}O_2$ between the WAF concentrations/control (Wald chi-square test, $p<0.01$). However, there was no significant difference in $\dot{M}O_2$ estimates between the control and any exposure concentration (Bonferroni post hoc, $p>0.05$), nor was there a linear, concentration-dependent response between exposure concentrations (Figure 3B) The $\dot{M}O_2$ estimates of larvae exposed to PAH concentrations of 0.085, 0.85, and 8.54 µg L^{-1} were significantly higher than the $\dot{M}O_2$ of larvae exposed to the highest tested concentration ($\sum PAH_{32}$=26.70 µg L^{-1}), yet there were no significant differences between the estimates of larvae exposed to 0.27 and 2.67 µg L^{-1} compared to the highest concentration (Figure 3B). At 15°C, there was an outlier among larvae exposed to the highest WAF concentration ($\sum PAH_{32}$=33.82) that increased the average $\dot{M}O_2$ by 63% (Bonferroni outlier test, $p<0.001$). The outlier was removed, though its inclusion did not affect the overall result that $\dot{M}O_2$ was not significantly different between the tested WAF concentrations/control at 15°C (Bonferroni post hoc, $p=0.17$; Figure 3C).

The $\dot{M}O_2$ of seawater control larvae was similar across Experiments 1, 2, and 3 (12°C trial) (one-way GLM, $p=0.47$; CoV between estimates = 6.7%).

The heart rate of exposed larvae did not significantly differ between the tested WAF concentrations/control at 9°C (Wald chi-square test, $p=0.85$; Figure 3D). Control larvae had an average heart rate of 350 ± 27 beats min^{-1}, and mean heart rate of oil-exposed larvae were within 5% of controls. Larval heart rate also did not differ significantly between the PAH concentrations at 12°C (Wald chi-square test, $p=0.47$; Figure 3E). Control larvae had an average heart rate of 299 ± 23 beats min^{-1}, and mean heart rate of oil-exposed larvae were within 7% of the control. At 15°C, larval heart rate was significantly different between the tested WAF concentrations/control (Wald chi-

square test, $p<0.001$; Figure 3F). Seawater control larvae had a heart rate of 360 ± 14 beats min^{-1} and average heart rate decreased significantly (Bonferroni post hoc, $p<0.001$) by 13% following exposure to the highest WAF concentration ($\sum PAH_{32}$=33.82 µg L^{-1}). The heart rate of larvae exposed to this concentration was also significantly lower than the other exposure concentrations (Bonferroni post hoc, $p>0.05$), however there was no significant difference between the heart rate of seawater control larvae and these other tested concentrations (Figure 3F).

The f_H of seawater control larvae was similar across Experiments 1, 2, and 3 (12°C trial) (one-way GLM, p=0.62; CoV=3.6%).

DISCUSSION

Based on my measurements of routine metabolic rate (routine oxygen consumption rate, $\dot{M}O_2$) and cardiac function, I found that stage I lobster larvae are physiologically resilient to acute exposure to physically (WAF) or chemically dispersed (CEWAF) crude oil at 12°C. However, there were temperature-dependent effects of acute exposure to WAF at the highest tested concentrations, with an increased $\dot{M}O_2$ in larvae exposed at 9°C and decreased heart rate and increased mortality at 15°C. To my knowledge, my study is the first to measure the effect of crude oil exposure in combination with temperature on the oxygen consumption and heart rate of stage I American lobster larvae. The analytically determined PAH concentrations of physically dispersed crude oil across exposures in my study ranged from 0-39.66 µg L^{-1} ∑PAH. These were within range of ∑PAH observed in surface waters following the North Cape

oil spill off the coast of Rhode Island in 1996 ($\sum PAH_{31}$=13.7- 49.7µg L^{-1}) and the Deepwater Horizon oil spill in the Gulf of Mexico in 2010 ($\sum PAH_{50}$<0.01-237 µg L^{-1}), supporting the environmental relevance of my findings (Reddy and Quinn, 1999; Forth *et al*., 2021). Overall, lobster larvae appear relatively resilient to acute crude oil and/or dispersant exposure, but warming may increase their physiological sensitivity leading to increased mortality.

Estimating Respiration Rates and Heart Rates of Larval Lobsters

Obtaining reliable estimates of respiration rates (i.e., $\dot{M}O_2$) of marine larvae is challenging due to their small size and very high rates of mass-specific oxygen consumption (Peck and Moyano, 2016). The small size of larvae makes it difficult to design appropriately sized respirometers and to assess variability in activity levels (which influence $\dot{M}O_2$), especially in the closed microplate respirometry system used in the present study. Consequently, my estimates of $\dot{M}O_2$ could have unaccounted influences of activity that contributed to variation in routine metabolic rates among individuals. However, reliability of my methods is demonstrated by the similarity in $\dot{M}O_2$ values of seawater control larvae at 12°C across the three experiments, even though they were performed in different years and with larvae from different females. Furthermore, my measurements of $\dot{M}O_2$ in control (i.e., non-oil exposed) stage I lobster larvae are comparable with the few available measurements in the literature. Woods et al. (2020) found that stage I *H. americanus* larvae had a mean $\dot{M}O_2$ of 0.080 µmol O_2 h^{-1} mg dw^{-1} at 16°C, which corresponds closely to my 15°C mean estimate of 0.077 µmol O_2 h^{-1} mg dw^{-1}. The earlier measurements of Capuzzo and her colleagues showed $\dot{M}O_2$ in stage I

American lobster larvae of 1.5 and 1.1 O_2 h^{-1} mg dw^{-1}, respectively (= 0.067 and 0.049 µmol O_2 h^{-1} mg dw^{-1}, respectively) at 20 and 25°C (Capuzzo *et al*., 1976, 1984; Capuzzo and Lancaster, 1979, 1981). These $\dot{M}O_2$ are slightly lower than mine at a cooler 15°C, which could indicate increasing constraints on aerobic metabolism, given that the warm temperatures used by Capuzzo and colleagues approximated the upper lethal temperature for larval lobsters (i.e., 26°C) (Quinn, 2017). Another study found a much higher $\dot{M}O_2$ of ~225 nmol O_2 h^{-1} mg^{-1} (=0.225 µmol O_2 h^{-1} mg dw^{-1}) in *H. americanus* stage I larvae at 16°C (Waller *et al*., 2017). However, in their study, fifteen larvae were measured per respirometry container; because American lobster larvae are cannibalistic, it is possible that the higher $\dot{M}O_2$ value was a result of increased larval activity to avoid predation or short-term effects of specific dynamic action due to feeding. Overall, the $\dot{M}O_2$ of stage I larval lobster appears to be approximately 0.050-0.10 µmol O_2 h^{-1} mg dw^{-1} under routine conditions and dependent upon temperature.

There are no previously published measurements of heart rate for American lobster larvae. However, I observed similar f_H values in seawater control larvae across my three experiments at 12°C (i.e., ranging from 284-305 beats min^{-1}), which supports the reliability of my estimates. Furthermore, f_H measurements within an individual were consistent over two days (Appendix B, Figure B3), and standard methods were validated and used for recording videos (Incardona *et al*., 2009; Bender *et al*., 2021; Appendix B, Figure B1). Previous unpublished work at my institution found that stage I American lobster larvae had an average heart rate of 292 beats min^{-1} in seawater at 15°C, compared to my 15°C estimate of 360 beats min^{-1} (D. Philibert and A. Scovil, Huntsman Marine Science Centre, New Brunswick, Canada, unpublished data). Although not significant, I

observed an increase in f_H in recovered larvae (i.e., placed in the microplate prior to f_H measurements) relative to larvae not previously placed in the microplate (Appendix B, Figure B2), which, along with interindividual variability, could explain my higher estimate.

Cross-species comparisons of f_H measurements are challenging because decapod crustaceans are marked by different stages of larval development and they exhibit variation in heart rate and dry mass throughout these stages (McMahon *et al*., 2002; Fitzgibbon *et al*., 2015). However, f_H measured across larval stages of other decapod species ranged from 100-550 beats min^{-1} across temperatures of 11-27°C, which is comparable to my f_H measurements of 125-400 beats min^{-1} across temperatures of 9, 12, and 15°C (McMahon *et al*., 2002; Storch *et al*., 2009).

Physiological Resilience of Lobster Larvae to Physically Dispersed Crude Oil Exposure at 12°C

My findings in Experiment 1 demonstrate metabolic and cardiac resilience of stage I American lobster larvae in response to 24 h exposure to the tested concentrations of physically dispersed crude oil at 12°C. Neither larval oxygen consumption rate nor heart rate were affected by exposure to increasing concentrations of crude oil WAFs, nor were there differences in mortality (Figure 1 and Appendix D, Figure D1). At the highest tested concentration ($\sum PAH_{31}$=38.72 µg L^{-1}), 6 larvae (60%) were assigned an assessment score of 1, indicating that these larvae were moribund following oil exposure (Appendix D, Figure D1). This was not reflected in changes to routine $\dot{M}O_2$, as estimates did not differ between individuals with an assessment score of 1 compared to control

individuals (i.e., all of which were assigned a score of 0) (data not shown). Thus, there was no apparent physiological impact of the difference in scores; perhaps, behavioural responsiveness of the larvae to stimulation during scoring could have been affected rather than physiological function.

Although larvae were released from a different female in each experiment, we also observed similar metabolic and cardiac resilience following physically dispersed crude oil exposure at 12°C in Experiments 2 and 3, strengthening my conclusion of physiological resilience at 12°C. The sensitivity of lobster larvae to oil exposure appears to be consistent regardless of parentage. Specifically, de Jourdan et al. (2022) exposed stage I *H. americanus* larvae from 14 females to offshore crude oil and assessed variability in the acute toxicity response (i.e., degree of immobilization) between the different larval batches. The coefficient of variation of the 50% effective concentration (EC_{50}) values of larval batches was 37.8%, compared to a coefficient of variation of 216.7% in mysid shrimp, indicating low variability in the toxic response of lobster larvae to crude oil exposure (de Jourdan *et al*., 2022). As in my study, larvae used by de Jourdan and colleagues were collected from female lobsters of unknown relatedness that had been caught within the same geographical area (Bay of Fundy Fishing Area 36); thus, it is possible that the resilience of lobster larvae to crude oil exposure shown by both of our studies is specific to this population.

The response of cardiac function to oil exposure, or specifically the lack thereof, has not previously been measured in larval lobster. Respiration rates of American lobster larvae have previously been found to decrease following exposure to sublethal concentrations of physically dispersed crude oil (Capuzzo and Lancaster, 1981), but this

finding was made at a much warmer temperature of 20°C, raising the possibility of temperature dependent responses to crude oil exposure in lobster (see below).

Physiological Resilience of Lobster Larvae to Chemically Dispersed Crude Oil Exposure at 12°C

Based on their work with oyster larvae, Vignier et al. (2016) suggested an additive or synergistic effect of dispersant addition on oil toxicity in invertebrates. My findings in Experiment 2 do not support this notion with respect to the physiological endpoints measured in larval lobster. $\dot{M}O_2$ measurements of seawater control and dispersant-only control individuals did not differ, which indicates that the chemical dispersant alone was not toxic to lobster larvae at the levels tested. Additionally, the measured physiological responses did not differ between WAF and CEWAF, nor were there differences in assessment scores and mortality (Appendix D, Figure D2), suggesting that the addition of Slickgone EW does not modify the effect of conventional heavy crude oil on American lobster larvae (Figure 2). My findings agree with those of Capuzzo and Lancaster (1982), who found that the $\dot{M}O_2$ response of stage I *H. americanus* larvae to naturally dispersed and chemically dispersed (Corexit 9527) crude oil exposure did not differ. My finding of an associated lack of a response in cardiac function to CEWAF exposure or dispersant alone, which has not been previously investigated, is consistent with there being an overall resiliency of oxygen supply and demand to dispersant exposure in larval lobster.

Temperature-Dependency of Physiological Effects of Physically Dispersed Crude Oil Exposure

While $\dot{M}O_2$ and heart rate of larval lobsters were unaffected by physically dispersed crude oil exposure at 12°C, exposure to the highest PAH concentration at 9 and 15°C caused temperature-dependent outcomes. Although we used different parent females for each of the temperature trials, de Jourdan et al. (2022) found low variation in response to oil exposure across parentage in lobsters; in other words, it does not seem likely that the use of larvae from different females in each experiment could explain the variable oil sensitivity between temperatures.

Increased mortality and decreased heart rate in lobster larvae following exposure to crude oil at 15°C

Following a 24-hour exposure to the highest ∑PAH concentration (33.82 µg L^{-1}) at 15°C, 40% of individuals died and there was a significant 13% decrease in heart rate among surviving larvae (Figure 3F). Although not previously investigated in larval crustaceans, combined effects of crude oil exposure and temperature have been reported in larval fish. Pasparakis et al. (2016) measured $\dot{M}O_2$ in hatched mahi-mahi after embryonic exposure to crude oil at 26 and 30°C. They found a concentration-dependent increase in $\dot{M}O_2$ in larvae exposed as embryos to ∑PAHs of 8.3, 21.8, and 49.6 µg L^{-1} at 26°C. Interestingly, after exposure to ∑PAHs of 15.2, 30.4, and 48 µg L^{-1} at 30°C, there was no change in larval $\dot{M}O_2$ at the lowest concentration and an insignificant decrease at the medium concentration relative to the control. At the highest exposure concentration, there was reduced hatching and high mortality, and thus $\dot{M}O_2$ could not be measured

(Pasparakis *et al*., 2016). Although there are differences in study design in that they measured persistent effects of embryonic exposure compared to my direct larval oil exposure, overall, our results are consistent with the same conclusion that there can be additive effects of temperature and crude oil exposure on oxygen supply and demand in early life stages. Although we did not see a significant decrease in $\dot{M}O_2$ at the highest ∑PAH concentration at 15°C, the large mortality and decreased heart rate suggest that, as in larval fishes, the combined effect of high PAH and warming could impair oxygen supply and energy demand in a way that potentially resulted in increased mortality.

The sensitivity of lobster larvae to crude oil exposure at 15°C could be a consequence of an increased allostatic load. Allostasis is the ability for organisms to maintain homeostasis in the face of changing demands (e.g., stress); they can physiologically adjust to respond to an external stressor, in order to maintain normal cellular function (McEwen and Seeman, 1999; Nichols *et al*., 2011; Hook *et al*., 2014). At some point, however, the level of stress will exceed the ability for the organism to compensate, which can lead to rapid mortality (Nichols *et al*., 2011). Elevated temperatures tend to enhance larval performance (e.g., development, growth, swimming ability) due to Arrhenius effects, but beyond optimal temperatures, performance may be impaired (Perrichon *et al*., 2018). For example, aerobic scope increases with increasing temperature up to an optimum temperature, resulting in greater metabolic capacity which can optimize fitness-related performance (Clark *et al*., 2013). Beyond its thermal optimum, aerobic scope is reduced, and thus the metabolic capacity for animals to sustain critical physiological activity can be reduced (Clark *et al*., 2013). The detoxification of PAHs costs energy and an animal's aerobic scope can help sustain this energy demand

(Perrichon *et al*., 2018). However, if aerobic scope is diminished, for example at warmer temperatures, the energy supply to detoxify PAHs may be constrained. Thus, in the present study, it is possible that 15°C was beyond the optimum temperature, resulting in insufficient aerobic scope to cope with exposure to crude oil at a ∑PAH of 33.82 μg L^{-1}, and ultimately causing mortality.

Several studies have investigated thermal limits to performance and survival in American lobster larvae and were reviewed by Quinn (2017). The thermal tolerance range of stage I American lobster larvae, based on lower and upper lethal temperatures (i.e., critical thermal minimum, CT_{min}, and critical thermal maximum, CT_{max}, respectively), was conservatively estimated to be 4-26°C. The optimal range of larval lobster performance (i.e., based on growth) was estimated to be 8-20°C (Quinn, 2017). Specific to larval lobster respiratory function, Small et al. (2015) measured the influence of temperature on metabolic rate in larval European lobster (*Homarus gammarus*) and found that $\dot{M}O_2$ of stage I *H. gammarus* was lower at 21°C than at 17°C, which was coupled with decreased survivorship in stage II individuals. This suggests that at 21°C, individuals had already exceeded their upper thermal limit of metabolic performance, independent of the additional demands of other stressors (Small *et al*., 2015). Environmental stressors can make an animal more sensitive to warming (e.g., reduce CT_{max}) and impair or lessen performance at a given temperature, which could explain why I observed mortality and reduced physiological performance already at 15°C (Todgham and Stillman, 2013).

Capuzzo and Lancaster (1981) observed a reduction in larval lobster $\dot{M}O_2$ after a 96-h exposure to crude oil at 20°C and stage I larvae exhibited the greatest decrease

relative to the other larval stages. This could support that temperature plays a role in the physiological response of lobster larvae to oil exposure. In fact, Laughlin and Lindén (1983) exposed mysid shrimp (*Neomysis integer*) to sublethal concentrations of physically dispersed fuel oil across a temperature range of 6 to 21.5°C and subsequently measured $\dot{M}O_2$. They found that the combined effects of higher temperature and higher petroleum hydrocarbon concentrations produced the greatest effects on $\dot{M}O_2$, while at lower temperatures, mysids had the greatest resistance to petroleum hydrocarbons (Laughlin and Lindén, 1983).

Exposure to the highest PAH concentration at 15°C resulted in larvae with a significantly lower heart rate relative to the controls (Figure 3F). $\dot{M}O_2$ was also lower in the surviving individuals, although not significantly (Figure 3C). These findings are consistent with the notion that these larvae were becoming unable to compensate for the increased stress of oil exposure and were beginning to lose physiological function. Also of note, there was no effect of exposure on f_H, $\dot{M}O_2$, or mortality in individuals at the other tested concentrations at 15°C, or any impairment at cooler temperatures, which provides support that lobster larvae are able to accommodate some stress of oil exposure so long as concentration and/or temperature is modest. Thus, the highest tested concentration at 15°C ($\sum$PAH=33.82 μg L^{-1}) may approximate a temperature-dependent threshold concentration that can be tolerated by lobster larvae. In the future, it would be useful to expose individuals to concentrations at smaller increments in attempt to precisely establish a threshold PAH concentration, as well as investigate exposure at warmer temperatures.

Increased oxygen consumption rate in lobster larvae following exposure to crude oil at 9°C

The observed increase in $\dot{M}O_2$ at 9°C to the highest exposure concentration of crude oil ($\sum$PAH=39.66 μg L^{-1}) may suggest that routine metabolic rate increased to meet energetic demands of oil detoxification (Figure 3B). In other words, larvae exposed to $\sum$PAH of 39.66 μg L^{-1} at 9°C had sufficient aerobic scope to increase their metabolic rate to sustain energy demands without mortality or perturbed heart rate as observed at the highest PAH concentration at 15°C. Because no change in $\dot{M}O_2$ was observed at 12°C, this could indicate that the thermal optima for PAH detoxification is closer to 12°C, and thus detoxification mechanisms were more efficient than at 9°C, but this possibility should be investigated.

Notably, four individuals drove the increase in $\dot{M}O_2$ at 9°C. While inherent interindividual variation exists with any biological trait, the response of these four larvae was likely not simply a reflection of intrinsic non-experimental variability because there was a lack of a similar level of interindividual variation at the other exposure concentrations and control at 9°C. Rather, there may be interindividual variation in the response to exposure to high $\sum$PAH. To resolve this, it would be useful to conduct a future experiment with a longer exposure time (e.g., 36-48 hours) or higher exposure concentrations to observe whether more individuals display an increase in $\dot{M}O_2$.

The increase in $\dot{M}O_2$ observed at 9°C was not associated with a concomitant change in f_H, contrary to my expectation. Intuitively, I expected a correlation between metabolic rate and cardiac performance, whereby hemolymph oxygen delivery would increase or decrease to match energetic demand. Although this is the case in many adult

vertebrates, there is increasing evidence of only a loose, or altogether lack of relationship between cardiac output and oxygen consumption in some invertebrates, along with early life stages of fishes (Barrionuevo and Burggren *et al*., 1999; Bruning *et al*., 2013; Perrichon *et al*., 2017, 2018; Oellermann *et al*., 2020). In larval fish species, it is hypothesized that the diffusion of oxygen through skin and tissues is sufficient to supply oxygen to the tissues, and therefore cardiac regulation can be decoupled from oxygen demands (Pasparakis *et al*., 2016; Perrichon *et al*., 2017). Because I observed an increase in $\dot{M}O_2$ at the highest exposure concentration at 9°C, but no effect on f_H, it may suggest some independence of these two physiological functions in larval lobsters. Although the circulatory system will continue to play a role in supplying metabolic fuel and removing metabolic waste, which varies with metabolic rate, changes in fuel delivery with increased $\dot{M}O_2$ may be sustained by other mechanisms (e.g., increasing the concentration of fuels in the blood). Indeed, it has previously been found that millimeter-sized larval crustaceans are able to supply oxygen to peripheral tissues by diffusion via the exoskeleton, which lowers their dependence on oxygen delivery from the heart (Oellermann *et al*., 2020). Thus, it is possible that lobster larvae had sufficient diffusive oxygen uptake that heart rate did not have to increase to compensate for the increase in oxygen consumption.

Alternatively, oil exposure could have affected cardiac performance in a way not reflected by a change in heart rate. Circulatory oxygen supply to the body is ultimately determined by cardiac output, which is the product of heart rate and stroke volume. Thus, increases in stroke volume can increase cardiac output, and sustain higher $\dot{M}O_2$, in the absence of changes in heart rate. When warmed from 2-22°C, adult *H. americanus*

showed increases in heart rate, whereas stroke volume decreased (Worden *et al.*, 2006). As such, stroke volume regulation of cardiac output in lobster may be more important at cooler temperatures, and it is possible that it played a role in sustaining the higher $\dot{M}O_2$ observed at the highest exposure concentration in my study at 9°C. Overall, measurements in heart rate alone may overlook certain impairments in cardiac performance (McMahon *et al*., 1999). Stroke volume and cardiac output have rarely been measured in crustacean larvae, though Harper and Reiber (1999) developed a protocol for measuring stroke volume in *Palaemontes pugio*, commonly known as grass shrimp. It would be useful in future work to develop a similar method for measuring stroke volume in lobster larvae to further elucidate the cardiac response to oil exposure.

Conclusions

In the present study, I found that the metabolic and cardiac response of stage I American lobster larvae to physically dispersed conventional heavy crude oil was temperature-dependent, with increased metabolic demand at the highest concentration at 9°C, and reduced cardiac performance and increased mortality at the highest concentration at 15°C. Across all experiments, larval lobsters demonstrated metabolic and cardiac resiliency to physically dispersed crude oil exposure at 12°C. Furthermore, the addition of Slickgone EW, measured at 12°C, did not affect the physiological responses to conventional heavy crude oil in lobster larvae. Overall, my findings suggest that lobster larvae were least affected by oil exposure at 12°C, but that warming may exacerbate the effect of exposure to oil. Indeed, my study highlights the importance of conducting toxicological assessments over a range of relevant environmental

temperatures. My findings demonstrate the importance of incorporating the influence of temperature on sublethal physiological effects of crude oil exposure into oil spill risk assessments to prevent underestimations of PAH toxicity in cold-water marine environments. Because sea surface temperatures are rising across the geographic range of American lobster larvae due to climate change, it is especially important that we develop a better understanding of upper limiting temperatures for larval responses to crude oil.

Table 1. Criteria used to assess lobster larvae immediately following the 24 h exposure period in each experiment.

Score	Assessment Criteria
0	*No observable effect*: vigorously swimming, active internal movement *Minor effects*: passive swimming or erratic swimming, positioned on side or back, rigid body position, exopodites/pereiopod beating in coordinated motion
1	*Moribund (mortally affected)*: no swimming activity, twitching, sporadic movement of mouthparts and exopodites/pereiopod, positioned on side or back, faint heartbeat, slight internal organ movement
2	*Dead*: no swimming, no visible heartbeat, change in colouration towards brown/opaqueness

Table 2 (next page). Nominal concentrations (%) of water-accommodated fractions (WAF) and chemically enhanced water-accommodated fractions (CEWAF) of crude oil solutions used for exposure trials and the corresponding sums of polycyclic aromatic hydrocarbons (∑PAH; μg L^{-1}).

	Preparation	Temperature (°C)	[Nominal] (%)	∑PAH (µg L^{-1})
Experiment 1	WAF	12	0	0.00
			10	5.38
			19	10.22
			37	19.90
			72	38.72
Experiment 2	WAF	12	0	0.00
			10	5.38
			19	10.22
			37	19.90
	CEWAF	12	0	0.00
			3.2	22.39
			5.6	31.37
			10	56.02
Experiment 3	WAF	9	0	0.00
			3.2	1.27
			10	3.97
			32	12.69
			100	39.66
	WAF	12	0	0.00
			0.32	0.085
			1	0.27
			3.2	0.85
			10	2.67
			32	8.54
			100	26.70
	WAF	15	0	0.00
			3.2	1.08
			10	3.38
			32	10.82
			100	33.82

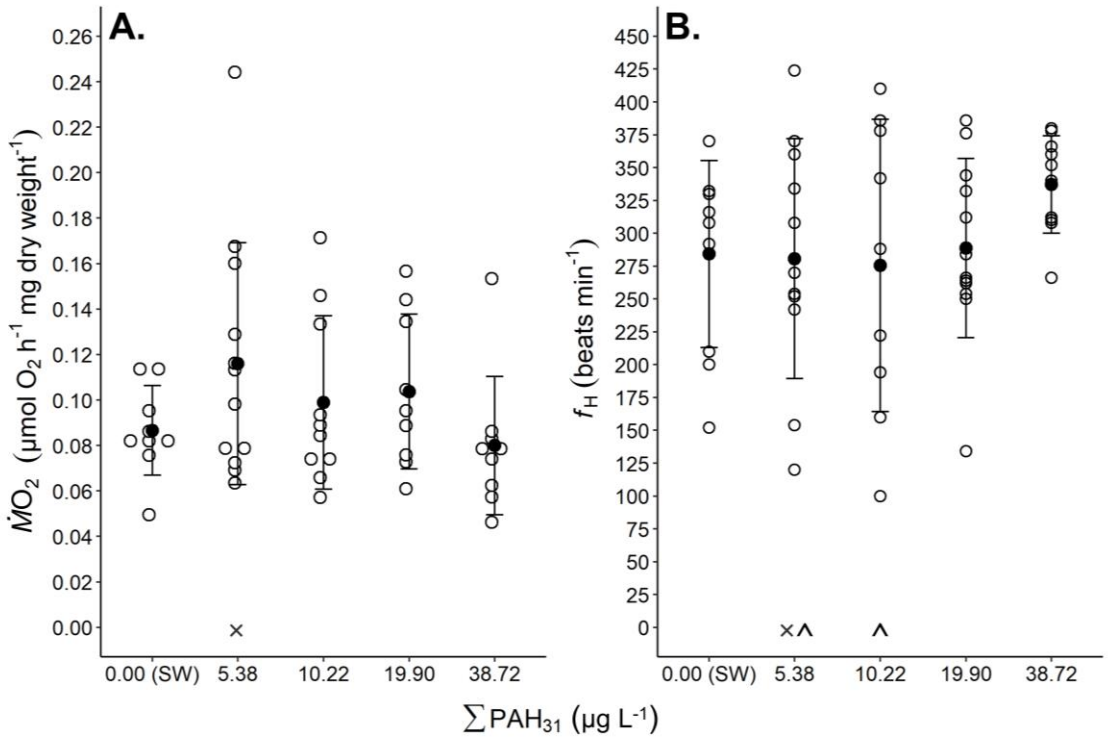

Figure 1. Effects of an acute 24-hour exposure to increasing concentrations of a water-accommodated fraction (WAF) of crude oil on the routine metabolic rate (routine oxygen consumption rate, $\dot{M}O_2$) (panel A) and heart rate (f_H) (panel B) of stage I American lobster larvae (Experiment 1). The WAF was prepared with conventional heavy crude oil using an oil to water ratio of 1:1. Larvae were also exposed to a seawater control (SW). Concentrations are expressed as the sum of total polycyclic aromatic hydrocarbons (PAHs) in each treatment. Closed black circles are means for each treatment (± standard deviation, n=9-12). Open circles represent the $\dot{M}O_2$ or f_H of individual larva within each treatment. "×" represents a mortality during the 24 h exposure period, which was excluded from $\dot{M}O_2$ and f_H analyses. "^" represents a mortality during the minimum one-hour post-respirometry recovery period prior to heart rate measurement, which was

excluded from f_H analyses. One-way generalized linear models and Type II Wald chi-square tests showed no significant effects of WAF exposure on $\dot{M}O_2$ (χ^2 =6.27, df=4, p=0.18) or f_H (χ^2 =3.27, df=4, p=0.51).

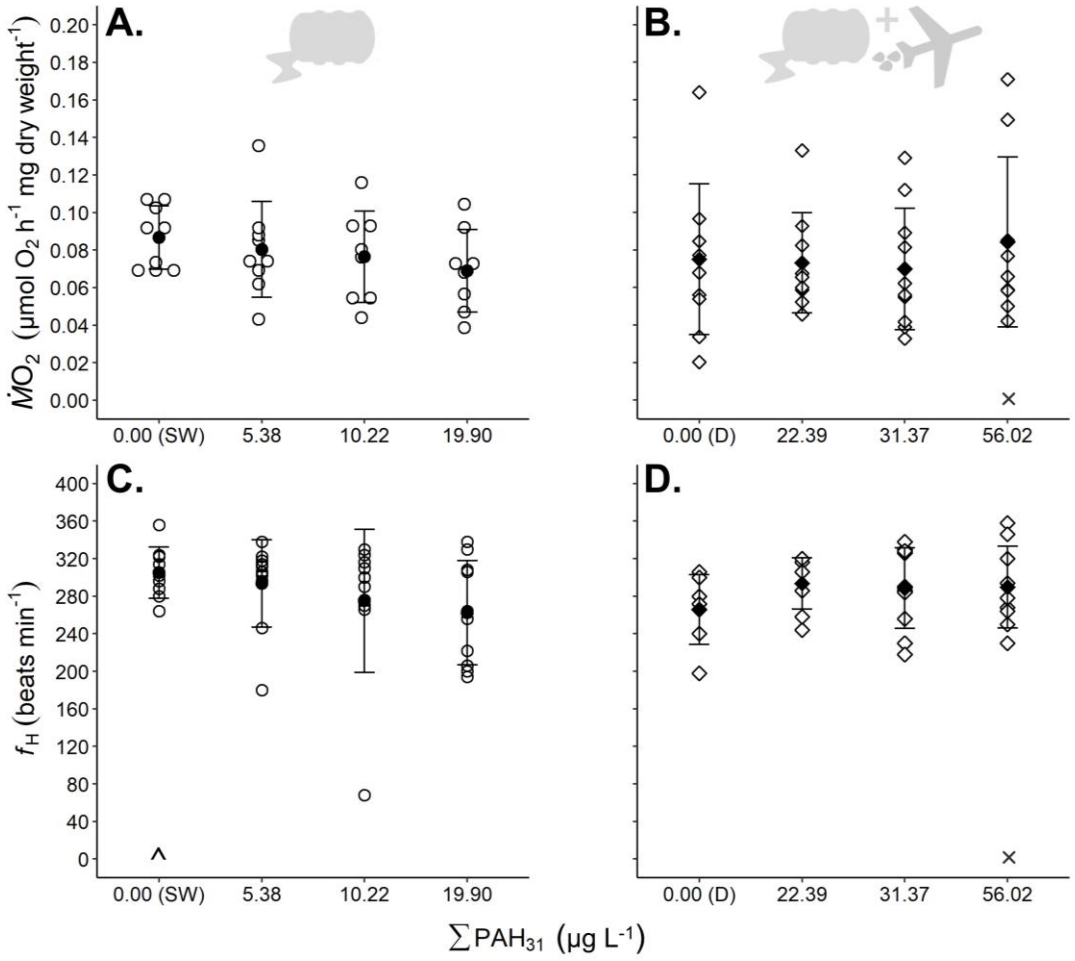

Figure 2. Effects of an acute 24-hour exposure to increasing concentrations of a water-accommodated fraction (WAF; panels A and C) and a chemically enhanced WAF (CEWAF; panels B and D) of crude oil on the routine metabolic rate (routine oxygen consumption rate, $\dot{M}O_2$) (panels A and B) and heart rate (f_H) (panels C and D) of stage I American lobster larvae (Experiment 2). The WAF was prepared with conventional heavy crude oil using an oil to water ratio of 1:1, and the CEWAF was prepared with conventional heavy crude oil and chemical dispersant (Slickgone EW) at a dispersant to water ratio of 1:20. Larvae were also exposed to a seawater control (SW) and a dispersant-only control (D). Concentrations are expressed as the sum of total polycyclic

aromatic hydrocarbons (PAHs) in each treatment. Closed black circles are means for each WAF treatment (± standard deviation, n=8-10) and closed black diamonds are means for each CEWAF treatment (± standard deviation, n=7-10). Open circles and open diamonds represent $\dot{M}O_2$ and f_H measurements of individual larva for each WAF or CEWAF treatment, respectively. "×" represents a mortality during the 24 h exposure period, which was excluded from $\dot{M}O_2$ and f_H analyses. "^" represents a mortality during the minimum one-hour post-respirometry recovery period prior to heart rate measurement, which was excluded from f_H analyses. One-way generalized linear models and Type II Wald chi-square tests showed no significant effects of WAF or CEWAF exposure on $\dot{M}O_2$ (χ^2 =2.72, df=3, p=0.44 and χ^2 =0.77, df=3, p=0.86, respectively) or f_H (χ^2 =2.50, df=3, p=0.47 and χ^2 =2.65, df=3, p=0.45, respectively).

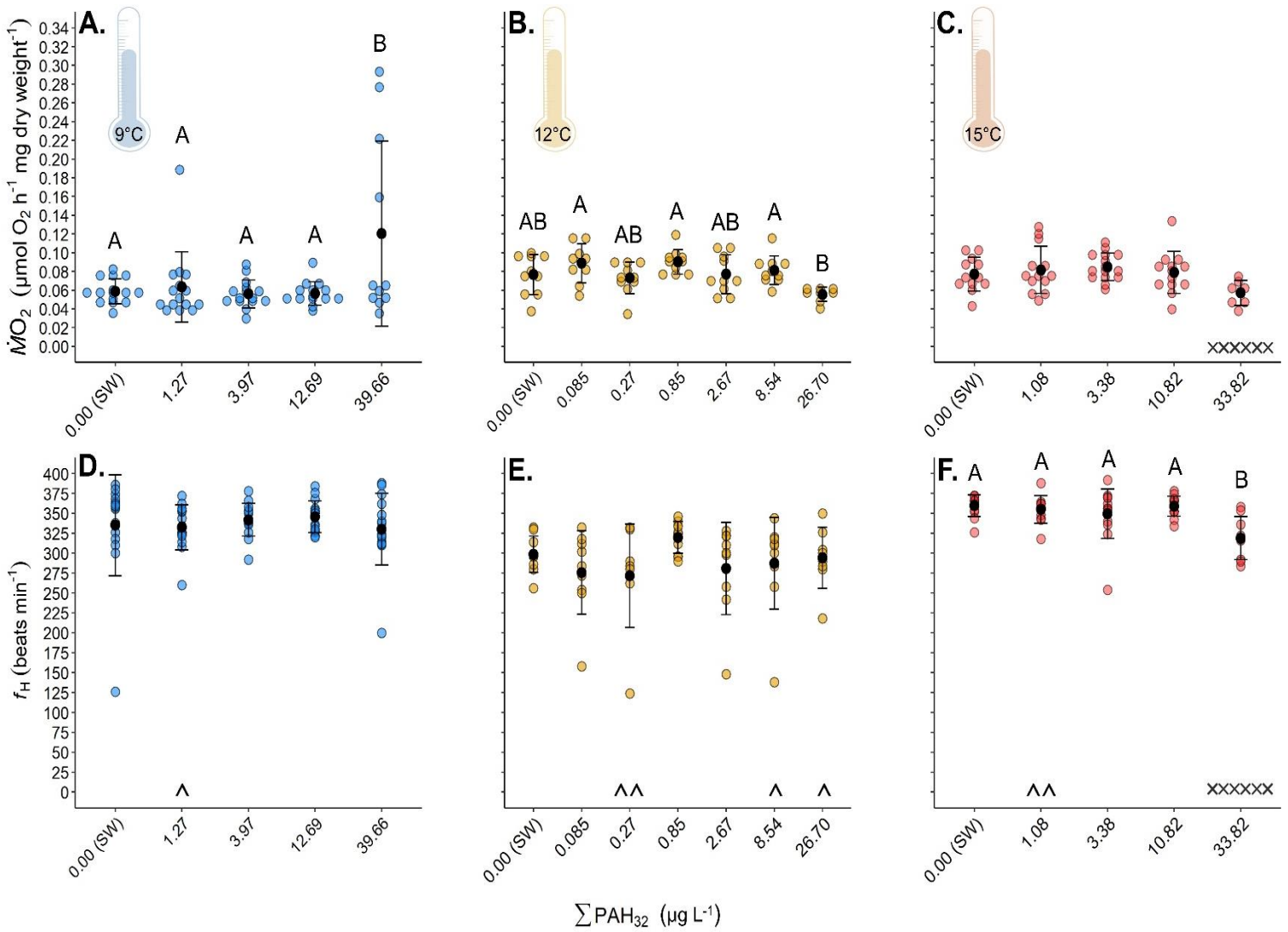
A.
B.
C.
D.
E.
F.
9°C
12°C
15°C
$\dot{M}O_2$ (µmol O_2 h^{-1} mg dry weight^{-1})
f_H (beats min^{-1})
0.00 (SW)
1.27
3.97
12.69
39.66
0.085
0.27
0.85
2.67
8.54
26.70
1.08
3.38
10.82
33.82
$\sum PAH_{32}$ (µg L^{-1})

Figure 3 (previous page). Effects of an acute 24-hour exposure at three environmentally relevant temperatures to increasing concentrations of a water-accommodated fraction (WAF) of crude oil on the routine metabolic rate (routine oxygen consumption rate, $\dot{M}O_2$) (panels A, B, C) and heart rate (f_H) (panels D, E, F) of stage I American lobster larvae (Experiment 3). The WAF was prepared with conventional heavy crude oil using an oil to water ratio of 1:100. Larvae were also exposed to a seawater control (SW). Concentrations are expressed as the sum of total polycyclic aromatic hydrocarbons (PAHs) in each treatment. Closed black circles are means for each treatment (± standard deviation, n=8-15). Blue, yellow, and red circles represent the $\dot{M}O_2$ and f_H of individual larva for each treatment at 9°C (panels A and D), 12°C (panels B and E), and 15°C (panels C and F), respectively. "×" represents a mortality during the 24 h exposure period, which was excluded from $\dot{M}O_2$ and f_H analyses. "^" represents a mortality during the minimum one-hour post-respirometry recovery period prior to heart rate measurement, which was excluded from f_H analyses. A one-way generalized linear model and Type II Wald chi-square test was used at each temperature to assess if there was a significant effect of concentration ($p<0.05$). A Bonferroni post hoc multiple comparison test was carried out when a significant effect of concentration was found. Concentrations sharing the same letter within a temperature are not significantly different.

Chapter 3: General Discussion

The overall goal of my thesis was to investigate how American lobster larvae physiologically responded to petroleum exposure, both alone and in combination with chemical dispersion or temperature. My main finding was that the metabolic rate and heart rate of lobster larvae were generally resilient to physically dispersed crude oil, with the important exception that the response became temperature-dependent at the highest tested concentrations. Additionally, I found that the metabolic rate and heart rate of larval lobster were not affected by the addition of a chemical dispersant.

Relationship Between Oil Loading and Measured PAH Concentrations

Because there was no effect of crude oil exposure on $\dot{M}O_2$ or f_H in Experiments 1 and 2 at an oil loading of 1 g oil/L, the loading was increased to 10 g oil/L for Experiment 3, with the objective of exposing lobster larvae to higher maximum PAH concentrations and establishing a threshold for the onset of $\dot{M}O_2$ or f_H effects. However, despite an increased oil loading for WAF preparations in Experiment 3 vs. Experiment 1 and 2, there was no increase in measured PAH concentrations, which seems counterintuitive. However, similar plateaus in WAF hydrocarbon concentration have previously been reported. Forth et al. (2017a) observed that the concentration and chemical composition of WAFs changed with oil-to-water mixing ratios between 0.01-1 g oil/L, but when mixing ratios were >1 g oil/L, an equilibrium was reached and changes in chemical composition remained largely unnoticeable across different WAF preparations. Hydrocarbon concentrations of WAFs are based primarily on the concentration of the dispersed particulate oil; as the fraction of dispersed oil increases, so

does the frequency of droplet-droplet interactions, which in turn increases the coalescence rate of the particulate oil (Forth *et al*., 2017a, 2017b). Coalescence forms large droplets of oil, and because large droplets have a greater buoyancy than small droplets, they rise quickly to the surface and have less time to lose soluble hydrocarbons, which ultimately reduces the dispersed phase (National Academies of Sciences, Engineering, and Medicine, 2020). Beyond a loading of 1-2 g oil/L, this results in a saturation effect that likely explains the similarity in PAH concentrations observed between our WAFs prepared with 1 and 10 g oil/L (Forth *et al*., 2017a).

Comparison of Measured PAH Concentrations Between WAF and CEWAF

In my study, the measured PAH concentrations in the CEWAF samples were higher than in the WAF samples despite lower nominal concentrations used in the CEWAF preparation. For example, in Experiment 2, the measured PAH concentration for 10% WAF was 5.38 µg L^{-1} ∑PAH, compared to 56.02 µg L^{-1} ∑PAH for 10% CEWAF. This finding is consistent with other studies (Ramachandran *et al*., 2004; Gardiner *et al*., 2013; Lee *et al*., 2013). Dispersants lower the interfacial tension of oil and form small droplets that are driven into the water column; surfactant molecules are present on the surface of the oil droplets, which prevent the oil from re-coalescing (Ramachandran *et al*., 2004; Athas *et al*., 2014). Because smaller oil droplets are slower to surface than larger droplets, such as those formed by coalescence in a WAF, there will be increased dissolution of hydrocarbons into solution (Ramachandran *et al*., 2004; National Academies of Sciences, Engineering, and Medicine, 2020). However, many of these hydrocarbons are suspected to be in the form of colloidal micelles, and thus are not in a

freely dissolved phase. Because dissolved hydrocarbons drive toxicity, PAHs in colloidal micelles are less bioavailable and in a less toxic form to organisms (Fuller *et al*., 2004; Schein *et al*., 2008; Gardiner *et al*., 2013; Redman *et al*., 2016). This could explain why I observed no difference in physiological response or mortality in CEWAF-exposed larvae, despite higher measured PAH concentrations.

Ecological Relevance and Implications of the Study

When assessing the environmental relevance of my findings, it is ultimately important to recognize that laboratory studies cannot perfectly mimic the ecological, chemical, or physical changes that are experienced at an active spill site. For example, the exposure conditions of my study were static (i.e., no renewal of test solutions throughout the 24 h exposure period), which is likely unrepresentative of the changes in exposure concentrations that would be expected in the field, where conditions are dynamic (Bejarano, 2018; Hodson *et al*., 2019). Thus, oil spill response decisions are not made based solely on toxicity data from laboratory studies (such as those from my study); rather, these data are important for risk assessments, response planning, and population level impact models to predict responses based on different oil spill scenarios (Bejarano *et al*., 2014).

To assess the potential impacts of oil spills using laboratory studies, it is important to select exposure concentrations that are environmentally realistic and relevant. In my study, exposure concentrations of physically dispersed conventional heavy crude oil ranged from 0-39.66 µg L^{-1} ($\sum PAH_{31\text{-}32}$). When the Deepwater Horizon blow-out released more than 200,000 tons of crude oil into the Gulf of Mexico, water

samples collected at the site of the spill identified $\sum PAH_{50}$ concentrations ranging from <0.01 to 237 µg L^{-1} at depths of 0-10 m from the floating oil, and up to 7.4 µg L^{-1} at 10-20 m depths (Forth *et al*., 2021). Of these samples, 94.5% had concentrations <5 µg L^{-1}. More relevant to cold-water ecosystems, an oil spill off the coast of Rhode Island released 2,700 tons of No. 2 fuel oil into the Atlantic Ocean, which led to the death of approximately nine million adult lobsters. Water samples collected at the spill site at depths of 2 to 25 m measured ∑PAHs ranging from 13.7-49.7 µg L^{-1} on day four of the spill, and up to 115 µg L^{-1} on day seven (Reddy and Quinn, 1999). Based on these data, the exposure concentrations I used in my study are realistic and relevant to typical PAH concentrations that have been observed during an active spill, including in cold oceans.

The findings of my study can provide conservative toxicological insights and considerations to inform decision-makers of potential implications of an oil spill for larval lobsters. My data suggests that lobster larvae will show resilience to oil spills with acute PAH exposures up to 38.72 µg L^{-1}, at least at cooler temperatures (i.e., 9-12°C). In the summer of 2021, the Gulf of Maine had record high sea surface temperatures, at times exceeding 17°C (Gulf of Maine Research Institute, 2021). Likewise, sea surface temperatures in the Bay of Fundy are steadily increasing and reached up to 15°C in July of 2020 (Hebert *et al*., 2021). Given the spatial and temporal variation experienced by American lobster larvae, and consequently the wide range of possible developmental temperatures, my findings also demonstrate the importance of assessing oil toxicity under a range of relevant environmental conditions. In my study, 40% of larvae died following exposure to the ecologically relevant ∑PAH concentration of 33.82 µg L^{-1} at an environmentally relevant 15°C, and cardiac impairment manifested through reduced heart

rate in surviving individuals. This suggests that increasing temperatures could exacerbate the cost of PAH exposure under an oil spill scenario in the North Atlantic and increase the sensitivity of larval lobsters; thus, my findings demonstrate the importance of considering temperature as a factor in oil spill risk assessments in relevant areas.

Laboratory toxicity data also informs oil spill response plans about the effectiveness of dispersant products relative to each other or other response options, and the potential organismal or ecological effects of dispersant use (e.g., whether the application of a certain dispersant is likely to result in injury or death of marine organisms) (Aurand and Coelho, 2005). My results from Experiment 2 provide relevant data to the latter objective. I found that the addition of Slickgone EW to conventional heavy crude oil did not affect the metabolic rate, heart rate, nor survival of lobster larvae during a 24 h acute exposure. As such, when evaluating the application of this dispersant product in oil spill response planning in a relevant cold-water ecosystem, its lack of effect on lobster larvae at the tested concentrations could support its use. However, the effect of temperature on the response of larval lobsters to chemically dispersed oil has not been studied and there is a need to address this knowledge gap considering that increased temperature exacerbated their response to physically dispersed oil.

To avoid underestimating the sensitivity of lobster larvae to sublethal exposure to physically and chemically dispersed crude oil, it would be beneficial to investigate the resiliency of other endpoints. Although we only observed deleterious effects (i.e., mortality and reduced heart rate) at the highest WAF concentration at 15°C and no effect at the other tested concentrations, it is still possible there were other sublethal effects occurring at these other concentrations that contributed to allostatic load. It would be

useful to increase the exposure time (>24 hours) to assess whether prolonged oil exposure would increase the sensitivity of $\dot{M}O_2$ and/or f_H at these lower concentrations. Though more research is required to address these questions, my results provide novel insight into the potential physiological impacts of oil spills in a non-standard toxicological study organism, which has superlative economic and ecological importance in North America.

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Appendix A. Larval dry weights.

Table A1 (next page). Dry weights of stage I American lobster larvae (<24 h post-release) from each control (SW=seawater, D=dispersant) and exposure group across the three experiments (n=8-15 per treatment). One-way ANOVAs were used to compare larval weights within each experiment. Data are presented as means ± standard deviation.

	Preparation	Nominal concentration (%)	Temperature (°C)	Dry weight (mg)	p-Value
Experiment 1	WAF	Control (SW)	12	1.05 ± 0.070	0.93
		10		1.03 ± 0.064	
		19		1.02 ± 0.080	
		37		1.04 ± 0.11	
		72		1.05 ± 0.088	
Experiment 2	WAF	Control (SW)	12	1.01 ± 0.074	0.99
		10		1.01 ± 0.10	
		19		1.00 ± 0.070	
		37		1.00 ± 0.075	
	CEWAF	Control (D)		1.02 ± 0.010	0.16
		3.2		1.01 ± 0.064	
		5.6		0.95 ± 0.13	
		10		1.04 ± 0.058	
Experiment 3	WAF	Control (SW)	9	1.16 ± 0.050	0.80
		3.2		1.15 ± 0.061	
		10		1.16 ± 0.066	
		32		1.13 ± 0.086	
		100		1.13 ± 0.092	
		Control (SW)	12	0.91± 0.10	0.80
		0.32		0.91± 0.095	
		1		0.94± 0.090	
		3		0.93± 0.045	
		10		0.94± 0.065	
		32		0.91± 0.11	
		100		0.89± 0.070	
		Control (SW)	15	0.90 ± 0.050	0.091
		3.2		0.94 ± 0.061	
		10		0.93 ± 0.063	
		32		0.92 ± 0.060	
		100		0.86 ± 0.10	

Appendix B. Results of validation experiments for heart rate methods.

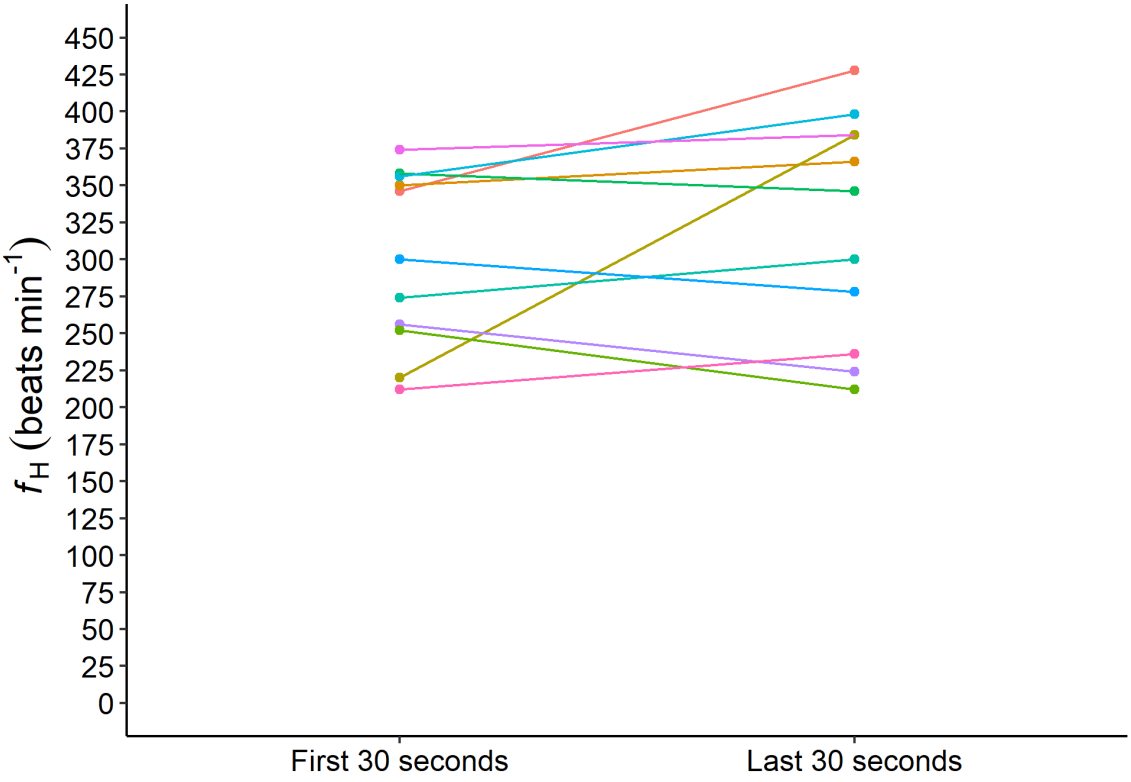

Figure B1. Heart rates of stage I American lobster larvae in seawater at room temperature calculated from the first 30 seconds and last 30 seconds of a five-minute video (n=11). Each coloured line connects the heart rates of an individual larva. Heart rates did not differ significantly between the two-time intervals (paired t-test, p=0.21).

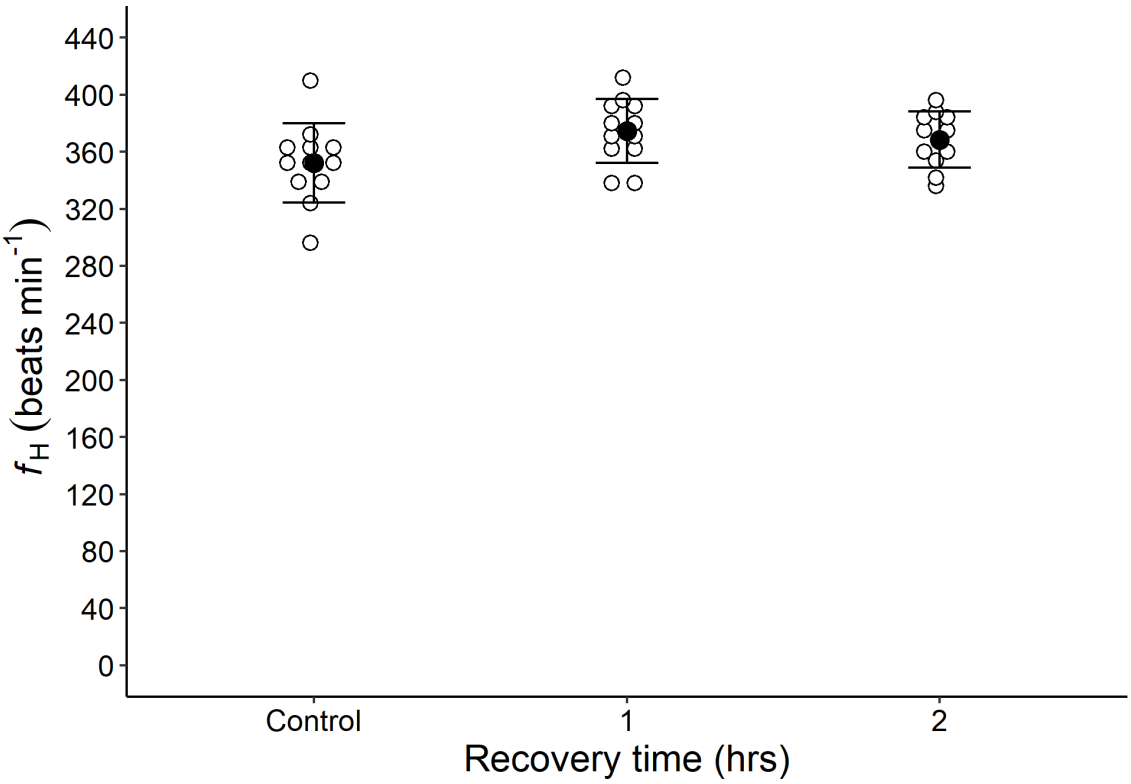

Figure B2. The effect of post-respirometry recovery time on heart rate (f_H) of stage I American lobster larvae. Closed circles are means ± standard deviation (n=11-12) and open circles are the values for individual larvae. Heart rates were recorded on two different groups of larvae recovered in separate scintillation vials containing filtered seawater for one- or two-hours post-respirometry, and a control group simply held in a scintillation vial containing filtered seawater. The f_H of one- and two-hour recovered larvae was not significantly different than control larvae, nor from each other (p=0.071, one-way ANOVA).

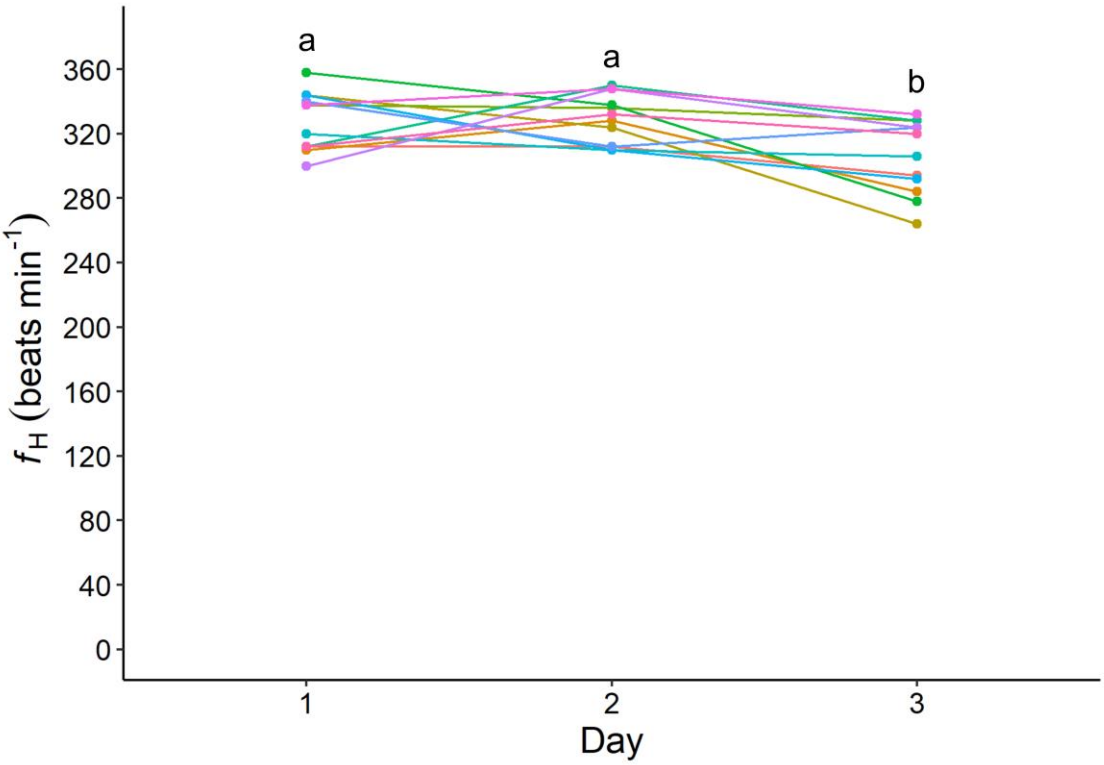

Figure B3. Heart rates of stage I American lobster larvae measured across 3 days (n=10). Day 1 measurements were performed on larvae following one-hour recovery from respirometry, and the measurement was repeated on the same larvae at the same time on the following two days. Each coloured line connects the heart rates of an individual larva. Days sharing the same letter are not significantly different ($p \geq 0.05$, one-way repeated measures ANOVA with Tukey's pairwise comparisons). The coefficient of variation (CoV) across all days was 5.8 ± 3.6% (mean ± standard deviation).

Appendix C. Results for water quality.

Table C1. Post-exposure water quality measurements for each experiment. Each parameter was measured on a pooled sample of three replicates per exposure solution and data are presented as means ± standard deviation.

	Temperature (°C)	**Dissolved oxygen (% air saturation)**	**pH**	**Salinity (psu)**
Experiment 1	12.4 ± 0.3	90.2 ± 1.6	7.8 ± 0.03	31.2 ± 0.1
Experiment 2	12.0 ± 0.4	92.7 ± 0.6	8.0 ± 0.04	31.7 ± 0.2
	8.9 ± 0.2 °C	97.2 ± 0.2%	8.0 ± 0.02	31.0 ± 0.3
Experiment 3	12.1 ± 0.2 °C	98.8 ± 1.4%	7.9 ± 0.03	30.4 ± 0.3
	14.6 ± 0.1 °C	90.9 ± 0.1%	7.8 ± 0.07	30.3 ± 0.05

Appendix D. Larval assessment scores.

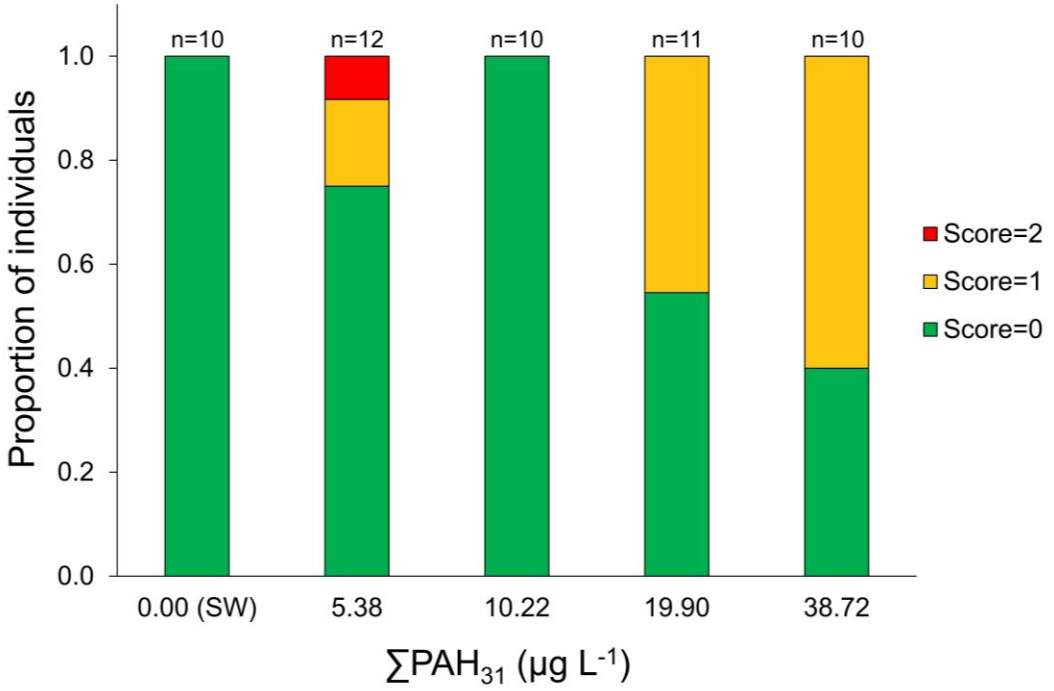

Figure D1. Proportion of stage I American lobster larvae with each assessment score following 24 h exposure to increasing concentrations of a water-accommodated fraction (WAF) of crude oil and a seawater (SW) control at 12°C (Experiment 1). See Table 1 for scoring criteria.

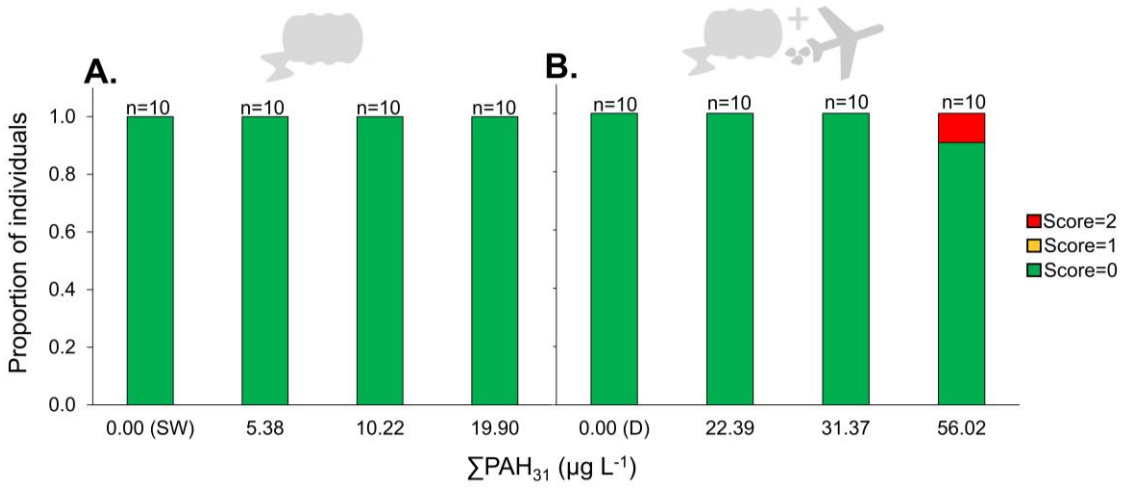

Figure D2. Proportion of stage I American lobster larvae with each assessment score following 24 h exposure to increasing concentrations of a water-accommodated fraction (WAF) of crude oil and a seawater (SW) control (panel A) and a chemically dispersed WAF and a dispersant-only (D) control (panel B) at 12°C (Experiment 2). See Table 1 for scoring criteria.

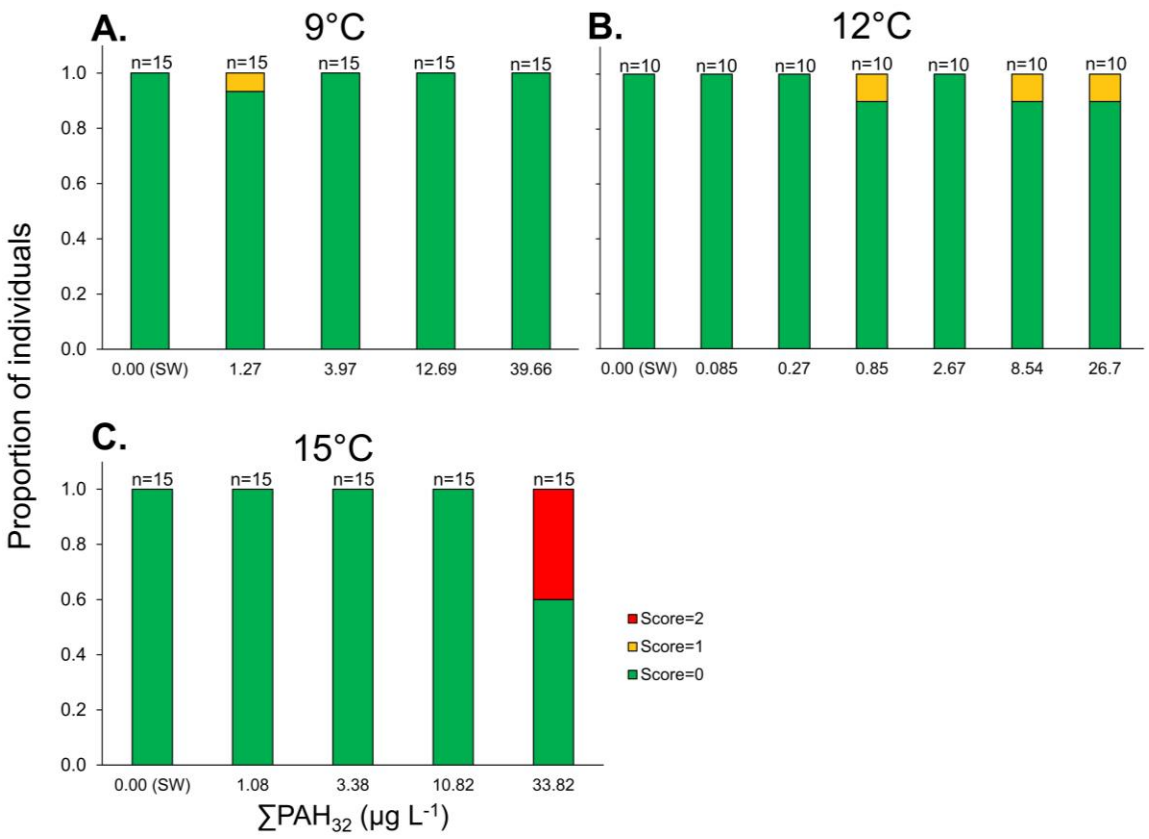

Figure D3. Proportion of stage I American lobster larvae with each assessment score following 24 h exposure to increasing concentrations of a water-accommodated fraction (WAF) of crude oil and a seawater (SW) control at 9°C (panel A), 12°C (panel B) or 15°C (Panel C) (Experiment 3). See Table 1 for scoring criteria.

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Leis, E.M., Easy, R.H., **Scovil, A**., Cone, D.K., Cloutman, D.G., McAllister, C.T., and Robison, H.W. 2021. *Urocleidus sayanus* n. sp. (Monogenoidea: Dactylogyridae) from gills of pirate perch (*Aphredoderus sayanus*). *The Journal of Parasitology* 107(2): 214-221.

Conference Presentations:

Scovil, A., de Jourdan, B., and Speers-Roesch, B. 2021. Assessing the effect of physically and chemically dispersed crude oil on the occurrence of blue-sac disease symptoms in early life stages of Atlantic cod (*Gadus morhua*). (Oral). Canadian Ecotoxicology Workshop, Halifax, Canada.

Scovil, A., Boloori, T., de Jourdan, B., and Speers-Roesch, B. 2021. The effects of physically and chemically dispersed crude oil on the metabolic and cardiac function of larval American lobster (*Homarus americanus*). (Oral). Multi-Partner Research Initiative Student Research Forum, Virtual.

Scovil, A., Boloori, T., de Jourdan, B., and Speers-Roesch, B. 2021. The effects of physically and chemically dispersed crude oil on the metabolic and cardiac function of larval American lobster (*Homarus americanus*). (Poster). Canadian Society of Zoologists, Virtual.

Scovil, A., Boloori, T., de Jourdan, B., and Speers-Roesch, B. 2020. The effects of physically and chemically dispersed crude oil on the metabolic and cardiac function of American lobster larvae (*Homarus americanus*). (Poster). Society of Environmental Toxicology and Chemistry North America, Virtual.