Assessing contamination baselines and contextualizing diluted bitumen effects in marine birds on Canada's Pacific Coast

by

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Abstract

Pacific Canada has a rich avifauna, however pipeline and port expansions, together with a growing human population will increase the quantity of petroleum used and potentially released into the marine environment. Birds are vulnerable to spilled oil, but research has not been synthesized since 1993. I reviewed the individual-level effects of crude oil and refined fuel exposure in avifauna with peer-reviewed articles to provide a critical synthesis of the state of the science and identify data gaps. One major gap was an absence of toxicity data for unconventional crude petroleum, such as oil sands bitumen that dominates Canadian production and will be delivered to the Port of Vancouver in increasing volumes. Breeding birds that become oiled may contaminate the shells of their eggs, and conventional crude oil can cause embryotoxicity at small quantities. To generate needed toxicity data in a sensitive early life stage, I conducted an egg oiling experiment with a major oil sands product, diluted bitumen (dilbit). Results indicated no detectable adverse physiological effects, although xenobiotic biotransformation gene expression and polycyclic aromatic compound (PAC) residue in the embryo confirmed that dilbit penetrates the eggshell and is absorbed by the embryo. Birds rapidly metabolize PACs in petroleum, and to date, oil pollution biomonitoring with invertebrates suitable for such contaminants has little overlap with marine wildlife hotspots. I surveyed oil pollution by measuring PACs and metals in bivalve mussels at bird breeding colonies. PACs were generally lower at remote sites than Vancouver Harbour, with the exception of Triangle Island, a Marine National Wildlife Area. However, the PAC data indicates that pollution sources are likely dominated by combustion rather than spilled petroleum. Measuring contaminant concentrations in tissue to infer whether effects are occurring in wildlife suffers from uncertainty. I studied relationships between whole embryo contaminant concentrations and mRNA transcript expression of toxicologically relevant genes in embryonic liver tissue from a Pacific Ocean seabird. I found several biologically plausible associations between contaminant concentrations and the expression of different genes, particularly for mercury. Accordingly, eggs routinely collected for biomonitoring programs could be used to relate chemical exposure to effects in free-living birds.

Keywords: Oil spill; Ecotoxicology; Aromatic hydrocarbons; Persistent organic pollutant; Mollusc; Trace element

Dedication

To my mother Deborah Phillips King and my father Thomas Eugene King. Thank you for putting me before you own ambitions, sharing your values, stoking my curiosity, and encouraging my education. There is a part of you in any accomplishment of mine, and your influence is undoubtedly reflected in this thesis.

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Preface

Petroleum production trends in Canada are driven by increased exploitation of oil sands bitumen, which is produced at twice the volume of conventional crude and accounts for more than 90% of Canada's oil reserves. Extracted bitumen by itself is too viscous to easily transport like conventional liquid crude petroleums. It is instead transported as any of several types of mixtures. One major formulation for transportation by pipeline is a product named diluted bitumen, also called dilbit, which is bitumen combined with natural gas condensate or naphtha to form a free-flowing liquid. Thus, dilbit is markedly different from well-researched conventional crude oil spilled in the past, and its behavior in the environment and effects on wildlife represent major knowledge gaps, which a 2010 spill in the Kalamzoo River, Michigan revealed. Like conventional crude oil, toxic components of dilbit relevant to wildlife are polycyclic aromatic compounds (PACs) and metals. A recent initiative to increase pipeline capacity from Canada's oil sands production fields to the Westridge Marine Terminal in Burnaby, British Columbia will likely make dilbit the main petroleum shipped from that tanker vessel-loading facility. This increase in regional traffic of tankers with dilbit cargo and the ancillary maritime activity (e.g. tugs, pilot vessels, port activity) is expected to occur within a wider regional context of port facility expansions, intensified vessel traffic, and a growing human population in British Columbia's lower mainland. Collectively, this translates to a potential for increased releases of crude dilbit, refined fuels, and fuel combustion emissions which can release polycyclic aromatic compounds (PACs) and metals into the environment.

The aforementioned industrial activities are located in the coastal waters of Pacific Canada that provide habitat for resident and migratory marine birds, including internationally important breeding colonies. Marine birds are often the wildlife taxon most affected by petroleum releases. Surface slicks compromise the air-sea interface where birds live and forage, and exposure to lingering contaminants can affect birds long after a spill, resulting in toxic syndromes from both external oiling and ingestion. Large spills can kill hundreds of thousands of birds and alter population dynamics for years to decades. The potential for increased risk of a dilbit spill where avifauna constitute an important, yet vulnerable, taxon represents a major challenge for wildlife resource sustainability.

Few, if any, studies to date have examined the ecotoxicological effects of dilbit on birds. The goal of my doctoral thesis research was to study how dilbit and related pollution in the environment might affect marine birds in Pacific Canada. This thesis consists of four chapters where I reviewed the individual level effects of petroleum on birds (Chapter 1), tested avian dilbit toxicity in a sensitive early life stage (Chapter 2), surveyed PACs and metals at bird breeding colonies in Pacific Canada (Chapter 3), and described correlative contaminant-gene expression effects in wild-collected seabird embryos (Chapter 4).

Chapter 1 of this thesis is a comprehensive literature review I conducted to assess the state of the science and synthesize what is known about the individual level effects of petroleum on birds since the last major review in 1993. This highlighted the data gap that, in contrast to conventional crude oils, no previous studies exist to provide toxicity information on exposure to bulk bitumen produced and transported in vast quantities. Because the embryonic life stage is well documented in past studies to be sensitive to crude oil, for instance a few microliters contaminating the eggshell causing mortality, I conducted an egg oiling study with dilbit to assess its toxicity in Chapter 2.

Subsequent chapters turned to monitoring studies to inform what petroleum related contamination may exist in important avian habitats (e.g. PACs, metals) and how chemical contaminant monitoring with seabird eggs might be better linked to effects *in vivo*. Regular pollution monitoring can generate data baselines that can inform comparisons with other areas and subsequent samples. In this way, they can document environmental contamination where suspected or known petroleum releases may occur, such as an oil spill event. However, vertebrates are poor candidates for monitoring PAC and metal pollution even though such pollution threats may be relevant to their populations. I therefore used bivalve mussels collected at bird breeding colonies in Pacific Canada to make spatial comparisons of ambient PAC and metals at bird breeding colonies and make inferences about potential sources in Chapter 3.

Pollution from human petroleum transport and use may exert effects in wildlife in addition to persistent contaminants already in the marine environment which bioaccumulate and are deposited into eggs via maternal transfer. Eggs are a common matrix for routine monitoring of such persistent contaminants in the environment. However, inferring whether adverse effects may occur from measured contaminant

concentrations suffers from uncertainty, and such contaminants may exert effects on sensitive embryos developing in the eggs. Therefore in Chapter 4, I examined hepatic mRNA gene expression with a qPCR gene array in tandem with chemical analysis of persistent organic pollutants and mercury in the embryo carcass to investigate correlation between contaminant burden and biological effects on transcription of toxicity-related genes. This approach can be applied to conduct effects-based biomonitoring with seabird eggs and may have applications in certain oil spill damage assessment scenarios.

The accomplishments that this research represents includes include the first review on petroleum effects in birds in nearly 30 years (Chapter 1), the first avian toxicity study on a major oil sands bitumen product (Chapter 2), the first petroleum contaminant surveys with bivalve mussels across major wildlife hotspots in Pacific Canada (Chapter 3), and among the first and to date most rigorous seabird contaminant biomonitoring studies with paired chemical and gene expression effect measurements (Chapter 4).

Chapter 1.

Effects of petroleum exposure on birds: A review

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1.1. Chapter abstract

Birds are vulnerable to petroleum pollution, and exposure has a range of negative effects resulting from plumage fouling, systemic toxicity, and embryotoxicity. Recent research has not been synthesized since Leighton's 1993 review despite the continued discharge of conventional petroleum, including high-volume oil spills and chronic oil pollution, as well as the emergence of understudied unconventional crude oil types. To address this, we reviewed the individual-level effects of crude oil and refined fuel exposure in avifauna with peer-reviewed articles published 1993–2020 to provide a critical synthesis of the state of the science. We also sought to answer how unconventional crude petroleum effects compare with conventional crude oil. Relevant knowledge gaps and research challenges were identified. The resulting review examines avian exposure to petroleum and synthesizes advances regarding the physical effects of oil hydrocarbons on feather structure and function, as well the toxic effects of inhaled or ingested oil, embryotoxicity, and how exposure affects broader scale endpoints related to behavior, reproduction, and survival. Another outcome of the review was the knowledge gaps and challenges identified. The first finding was a paucity of oil ingestion rate estimates in birds. Characterizing environmentally realistic exposure and ingestion rates is a higher research priority than additional conventional oral dosing experiments. Second, there is an absence of toxicity data for unconventional crude petroleum. Although the effects of air and water contamination in the Canadian oil sands region have received attention, toxicity data for direct exposure to unrefined bitumen produced there in high volumes and other such unconventional oil types are needed. Third, we encountered barriers to the interpretation, replication, broad relevance, and comparability of studies. We therefore propose best practices and promising

technological advancements for researchers. This review consolidates our understanding of petroleum's effects on birds and points a way forward for researchers and resource managers.

1.2. Introduction

The harmful effects of petroleum exposure on birds have long been recognized and have been the subject of concerted research since the 1960s (Hartung, 1963). The resulting literature is voluminous and punctuated by waves of studies following large, high-profile spills such as the *Exxon Valdez*, *Prestige*, and *Deepwater Horizon* disasters (Table 1.1). Nonetheless, the continued economic importance of petroleum, the variable and complex chemical composition of hydrocarbon mixtures, and the economically-driven development of unconventional sources such as oil sands bitumen in Canada (Figure 1.1) suggests that petroleum toxicity to marine birds will remain an important research subject (CAPP, 2017; Green et al., 2017; NEB, 2016a).

Petroleum has a broad range of harmful effects on birds which include the physical fouling of plumage, toxicity of ingested petroleum, and embryotoxicity (Leighton, 1993). Such effects can result from petroleum quantities as small as a few milliliters on the feathers or a few microliters on the eggshell (Leighton, 1993; Morandin and O'Hara, 2016). A number of older reviews in addition to Leighton's (1993) provide useful perspectives on avian petroleum toxicity and a wealth of references, as do a number of more recent reviews on closely related topics (Table 1.2). Despite that, a comprehensive summary of known toxicological effects of petroleum on birds has not been published since Leighton's (1993) seminal review 27 years ago, where he concluded:

> It is difficult to draw general conclusions from the diverse, and often conflicting, results of the many studies of the effects of ingested petroleum oils. This difficulty makes the attempt all the more important,

After the intervening decades of research, environmental scientists and resource managers would benefit from an updated overview of the effects of petroleum on birds.

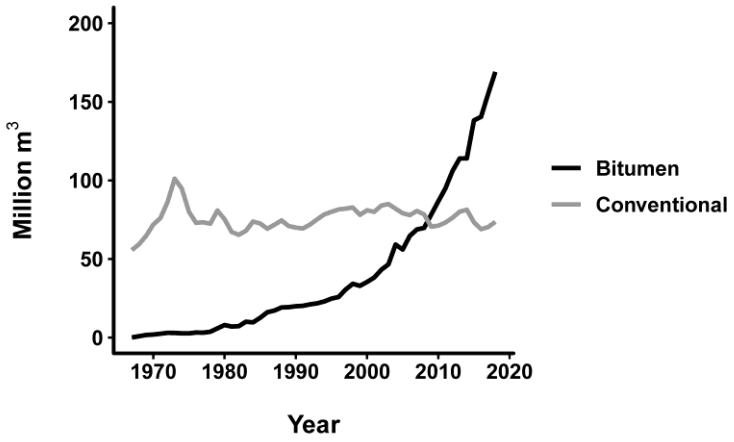

Figure 1.1 Annual production (million m^3) of conventional crude petroleum (grey) and bituminous oil sands crude petroleum (black) in Canada from 1967 to 2018. Data source: Canadian Association of Petroleum Producers (CAPP, 2020).

This literature review provides a critical narrative synthesis of petroleum pollution in the environment and avian exposure (Section 1.3), the immediate harmful effects of crude and refined petroleum on avifauna (Section 1.4), longer-term and ecologically mediated effects that may emerge (Section 1.5), and finally an exploration of knowledge gaps, challenges, and how to address them (Section 1.6). On those respective themes we specifically synthesize exposure rate estimates (Section 1.3.3), indications of exposure (Section 1.3.4), and effects. Immediate effects include the physical effects of oil on feathers (Section 1.4.1), the toxic effects of oils (Section 1.4.2), the consequences of both, and embryotoxicity (Section 1.4.3). Emergent consequences at the individual level relate to behavior (Section 1.5.1), reproduction (Section 1.5.2), and survival (Section 1.5.3). The highest priority knowledge gaps for future research to address include, first, a paucity of environmentally relevant exposure data (Section 1.6.1), and secondly, a need for toxicity data for unconventional petroleum such as oil sands bitumen and other unconventional oil types (Section 1.6.2). Lastly, we identify specific research challenges and common shortcomings regarding study design and reporting for which we propose solutions to improve research (Section 1.6.3).

1.2.1. Methods

To review the effects of crude and refined petroleum exposure in avifauna, including all manner of study designs and individual level endpoints up to survival and

Table 1.1 Scientific highlights from research conducted since 1993 on the effects of petroleum exposure on birds.

Topic	Highlight	Reference(s)	Spill[a]
Reproduction	Reproduction study on the breeding grounds of an oil-impacted population	(Field et al., 1993)	*EV*
Immunotoxicity	Avian immunotoxicity demonstrated for a PAC[b]	(Trust et al., 1994)	Lab
Electrolytes & Reproduction	Reduced circulating calcium and eggshell thinning from ingested oil	(Stubblefield et al., 1995c)	*EV*
Reproductive hormones	Reproductive hormone effects in penguins with crude oil-contaminated plumage	(Fowler et al., 1995)	NI
Thermal and metabolic balance	Evidence of metabolic starvation in a wild seabird	(Oka and Okuyama, 2000)	NI
Behavior	Feeding and maintenance activity effects in birds rehabilitated after becoming oiled	(Anderson et al., 2000)	*UP*
Long-term survival	Decreased survival in birds overwintering in oil spill-impacted areas	(Esler et al., 2000b)	*EV*
Reproduction	Low quality chick diet in oil spill-impacted areas	(Golet et al., 2002)	*EV*
Reproduction	Reproductive success reduced by poor prey availability after oil spill	(Velando et al., 2005b)	*P*
Survival & Reproduction	Age- and sex-specific oil spill mortality projected to impair population recovery	(Martínez-Abraín et al., 2006)	*P*
Metabolism	Decreased blood glucose in oil-fed wild birds	(Alonso-Alvarez et al., 2007b)	*P*
Neurotoxicity	Depressed brain acetylcholinesterase activity in wild birds oiled at sea	(Oropesa et al., 2007)	*P*
Lungs & Immunotoxicity	Immunotoxicity from inhaled BTEX[c]	(Olsgard et al., 2008)	Lab
Behavior	Habitat use affected in oil spill-impacted area	(Banks et al., 2008)	*SE*
Reproduction & Survival	Evidence of increased cost of reproduction in oiled and rehabilitated birds	(Wolfaardt et al., 2008a)	*AS*
Feather structure	µm thin oil-on-water films disrupted seabird feather microstructure	(O'Hara and Morandin, 2010)	Lab
Oxidative balance & Reproduction	Ingested oil affected plasma antioxidant balance and carotenoid-based sexual ornamentation	(Pérez et al., 2010a)	*P*
Metabolism	Reduced hepatic fatty acid oxidation in embryos dosed with PACs[b] *in ovo*	(Westman et al., 2013)	Lab
Stress hormones	Dietary oil exposure decreased corticosterone secretion in response to an acute stressor	(Lattin et al., 2014)	*DH*
Exposure	EROD[d] activity indicated exposure to residual oil up to 22 years post-spill	(Esler et al., 2017)	*EV*
Flight performance	Migratory fight performance effects from feather oiling in a long-distance migrant	(Maggini et al., 2017c)	*DH*
Gastrointestinal damage	Experimental evidence of gastrointestinal damage from ingested oil	(Harr et al., 2017b, 2017a)	*DH*
Oxidative balance	Ingested oil affected hepatic oxidative balance	(Pritsos et al., 2017)	*DH*

Haemotoxicity	Hemolytic injury in spill-impacted wild birds	(Fallon et al., 2017)	*DH*
Cardiotoxicity	Cardiac function affected in oiled birds	(Harr et al., 2017c)	*DH*
Exposure	*Cyp1a5*[e] gene expression showed oil exposure in a passerine	(Perez-Umphrey et al., 2018)	*DH*
Lungs	Inhaled BTEX[c] induced pulmonary CYP1A[f] protein	(Dubansky et al., 2018)	*DH*
Migration	Ingested PACs affected pre-migratory mass gain dynamics in a long-distance migrant	(Bianchini and Morrissey, 2018a)	Lab
Metabolism	Targeted metabolomic analysis showed effects on fatty acid and amino acid metabolism from external oiling	(Dorr et al., 2019)	*DH*

[a] Oil spill events: *Exxon Valdez* (*EV*) crude oil spill, *Unocal pipeline* (*UP*) crude oil spill, *Prestige* (*P*) fuel oil spill, *Sea Empress* (*SE*) crude oil spill, *Apollo Sea* (*AS*) heavy fuel oil spill, *Deepwater Horizon* (*DH*) crude oil spill, Not identified (NI), or laboratory exposure (Lab)

[b] polycyclic aromatic compound (PAC)

[c] Volatile monoaromatics that include benzene, toluene, ethylbenzene, and xylene (BTEX)

[d] ethoxyresorufin-O-deethylase enzyme (EROD) activity, a biomarker of planar hydrocarbon exposure

[e] The second of two main avian cytochrome P450 1A genes (*Cyp1a5*), inducible by planar hydrocarbon exposure

[f] cytochrome P450 1A protein CYP1A, a biomarker of planar hydrocarbon exposure

reproduction, we conducted a systematized review (Grant and Booth, 2009). We searched for research articles in two databases, Web of Science and Academic Search Premiere. We iteratively optimized search terms until searches returned the maximum number of relevant hits that could reasonably be reviewed (hundreds to <3000 hits). The presence of highly relevant and often cited works among the search results were judged to be a good indicator of search terms that worked well. After optimization, we searched for peer-reviewed English language journal articles published 1993–2020 (inclusive) returned with the search terms by topic (petroleum OR fuel OR hydrocarbon* OR “oil spill” OR bitumen OR “crude oil”) AND (avian OR *bird* OR *fowl*). A search on 1 April 2020 yielded 2290 hits in Web of Science and 1574 hits in Academic Search Premiere. We downloaded these search results, combined hits from the two databases, removed duplicates, and scored hits for relevance based on title and abstract as unrelated (zero), not relevant (one), somewhat relevant (two), and very relevant (three). We read and reviewed all articles with a score of two or three (83 and 101 articles, respectively). From the articles reviewed, we added relevant citations therein that met the above criteria and reviewed these as well (i.e. snowball method; 30 articles). While no peer-reviewed articles from this search were screened out a priori based on quality criteria, any quality issues germane to the interpretation of results are dealt with in our narrative review and the findings given more or less weight according to the quality of the study in our

synthesis. Where interpreting recent findings (since 1993) required consulting older articles and weighing their evidence, we primarily used sources in Leighton's (1993) review and cite them.

Petroleum doses are reported in units that differ from one study to another. To facilitate comparison among studies, we provide doses in milliliters per kilogram body weight (ml kg^{-1} bw), or for multiday and dietary studies in milliliters per kilogram body weight per day (ml kg^{-1} bw d^{-1}). Those rates are given on a body mass basis, so unfamiliar readers may more intuitively visualize the amount of petroleum in relation to a typical duck (e.g. *Anas platyrhynchos*) weighing approximately one kilogram where we discuss exposure rates. In many cases, conversions and estimates were necessary. In order to estimate the doses (as in Hartung, 1995), we based our conversions on several factors: petroleum dose per day, petroleum density, and bird body mass as given in the study. If not reported, we substituted the average body mass from the Birds of the World online database (Cornell Lab of Ornithology, 2020), petroleum density of 0.9 grams per milliliter, and a feeding rate of 10% body weight per day following Hartung (1995). Where external oiling was reported, we provide the percent surface area (SA) of the bird affected, and, where important to the context, the amount of petroleum in milliliters per kilogram body weight (ml kg^{-1} bw) if possible.

Table 1.2 Peer-reviewed journal review articles since 1993 related to the topic of petroleum effects and toxicity in avifauna.

Content relevant to petroleum effects on avifauna	References
Petroleum pollution effects on birds of the northeast Pacific Ocean	(Burger and Fry, 1993)
Toxic and physical effects of petroleum on birds	(Leighton, 1993)
Thermoregulatory and metabolic effects of petroleum, as well as dispersants, and bird cleaning	(Jenssen, 1994)[a]
The fate and effects of the *Exxon Valdez* oil spill on birds	(Hartung, 1995; Stubblefield et al., 1995a; Wiens, 1995)[a]
Effects of contaminants on avian reproduction, including petroleum	(Fry, 1995)[a]
Possible mechanisms for petroleum immunotoxicity in birds	(Briggs et al., 1996, Briggs et al., 1997)[a]
Beached bird surveys as a tool for monitoring chronic oil pollution	(Camphuysen and Heubeck, 2001)[b]

Content relevant to petroleum effects on avifauna	References
Avian endocrine disruption from contaminants, including petroleum	(Scanes and McNabb, 2003)[a]
Avian immunotoxicity from contaminants, including petroleum	(Fairbrother et al., 2004)
Effects of PACs[c] in avifauna	(Albers, 2006)[a]
Oil spill effects and human intervention in two species of marine birds	(Wolfaardt et al., 2009)[a]
Ecological and physiological effects of petroleum relevant to terrestrial vertebrates affected by shore-cast crude petroleum from the *Deepwater Horizon* oil spill	(Bergeon Burns et al., 2014)[b]
Avian toxicity of oil sands process-affected water	(Beck et al., 2015)[a]
Aquatic toxicity of diluted bitumen	(Dew et al., 2015)
Thyroid toxicity associated with petroleum in mammals and fish	(Fowles et al., 2016)[a]
Risks and effects of operational oil and gas discharge to marine birds	(Morandin and O'Hara, 2016)[a]
Avian toxicity studies conducted under the *Deepwater Horizon* Natural Resource Damage Assessment process	(Bursian et al., 2017a)
Wildlife population effects of the *Exxon Valdez* spill, their cause, and recovery status	(Esler et al., 2018)[a]

[a] Returned by database search (see methods).

[b] Cited by article in database search (see methods).

[c] Polycyclic aromatic compounds

1.3. Petroleum in the environment and avian exposure

1.3.1. Petroleum is a broad class of complex hydrocarbon mixtures

The fact that petroleum encompasses a broad and heterogeneous class of hydrocarbon-based substances, typically mixtures, cannot be overemphasized. It is their shared chemical and physical properties that make types of petroleum comparable in a general sense, yet particular differences in properties and composition affect movement, fate, and toxicity in the environment (reviewed in Dupuis and Ucan-Marin, 2015; Lee et al., 2015). Crude oil is refined into various fractions and transformed into a wide array of products. This review primarily considers information about crude oil and refined fuels which are produced, transported, and used in the highest volumes, and therefore, most

often discharged into the environment. For crude oil, physical and chemical properties vary by geological origin, production methods, and in some cases upgrading methods to create a more easily marketed or transported material. Fuels and especially crude oils typically contain thousands of compounds, but from a compositional perspective, the four main chemical classes of hydrocarbons in petroleum are saturates, aromatics, resins, and asphaltenes. From a toxicity perspective, the major classes of concern in vertebrates are naphthenic acids, metals, aliphatics, and aromatics, the latter being most associated with toxicity.

The aromatic fraction of petroleum is primarily implicated in avian toxicity (Albers, 2006; Gentes et al., 2007b; reviewed in Leighton, 1993; Walters et al., 1987). Aromatics include monoaromatic compounds like benzene, toluene, ethylbenzene, and xylene (BTEX), as well as polycyclic aromatic compounds (PACs) such as parent polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs (alkyl-PAHs), and heterocycles of nitrogen, oxygen, and sulfur. Though petroleum studies have traditionally focused on parent PAHs, we subsume PAHs, alkyl-PAHs, and aromatic heterocycles within the more inclusive term PAC (reviewed in Achten and Andersson, 2015; Andersson and Achten, 2015). Not only must petroleum be considered as a complex mixture, but once released into the environment its composition is changed over time by interactions with air, water, temperature, light, and microbial activity, a process called weathering.

1.3.2. Oil sources, weathering, fate, and risk to birds

Human activity is a major source of petroleum to the environment. Roughly half the petroleum entering the marine environment comes from natural seeps on the seafloor, the other half from human sources related to oil and gas production, petroleum transportation, and consumption (Kvenvolden and Cooper, 2003; NRC, 2003). Accidents and conflict can result in large-scale, often well-publicized, spills from pipelines (Dew et al., 2015; McCrary et al., 2003), train tank cars (Dupuis and Ucan-Marin, 2015), wells (Bursian et al., 2017a; Evans et al., 1993), shipwrecks, especially tanker wrecks (Burger and Fry, 1993; Wolfaardt et al., 2009), and salvage (Camphuysen and Leopold, 2004) which impact birds. Though they seldom receive public attention, relatively small but regular petroleum discharges, known as chronic oil pollution, are documented in marine waters and can be an important source of avian mortality as well (Boersma, 1986; Camphuysen and Heubeck, 2001; García-Borboroglu et al., 2006; O'Hara and Morgan,

2006; Wiese and Robertson, 2004; Wilhelm et al., 2009). Sources of chronic petroleum discharge include oil and gas production (McCrary et al., 2003; Morandin and O'Hara, 2016; Rattner et al., 1995; Ronconi et al., 2015), vessel traffic and shipping, especially oil tanker shipping (tank cleaning particularly, see Hampton et al., 2003b; Bertazzon et al., 2014; Camphuysen, 2010; Dahlmann et al., 1994; García-Borboroglu et al., 2006; Lucas and MacGregor, 2006; O'Hara et al., 2009; O'Hara and Morgan, 2006; Wiese and Ryan, 2003), and submerged wrecks (Hampton et al., 2003a; Henkel et al., 2014b). Even where chronic pollution is low, natural seeps can constitute an import source of crude oil to birds (Henkel et al., 2014b).

At local and regional scales, the amount and dominant sources of chronic oil pollution likely vary. Tankers often carry loads of crude oil or refined petroleum products, whereas fuel oil (often called bunker fuel) normally powers larger ships; smaller vessels like fishing and pleasure craft use refined fuels such as diesel and gasoline (reviewed in Burger and Fry, 1993). A single shipwreck can release different petroleum types such as heavy fuel oil, diesel, and lubricating oil in various amounts (Crawford et al., 2000).

For crude oil that enters the environment, types and sources are increasingly associated with the production of petroleum reserves considered unconventional. Unconventional is a general term that refers to crude petroleum reservoirs of a less desirable quality or requiring a departure from conventional drilling methods to extract from geological formations (Zee Ma, 2016). Examples of unconventional oil resources are heavy crude oil, shale oil, and oil sands. The Canadian oil sands are the third largest known oil reserve in the world (NRC, 2017). It serves as a useful example of an unconventional bituminous crude oil resource that has been exploited for many years, has a massive footprint and production volume (Figure 1.1), and has been studied for its impact to wildlife (reviewed in Beck et al., 2015; Dew et al., 2015). The properties of unconventional crude oil products differ from one another and conventional crude oil, and, therefore, their behavior in the environment and toxicity is potentially different.

When petroleum enters the environment, its composition and movement is dynamic over time as the oil weathers and is carried by water. Petroleum's hydrophobic nature and the adhesive properties of certain oils when weathered cause it to adsorb or adhere to solid surfaces. Albers (2003) and Lee et al. (2015) reviewed the transport and fate of petroleum in aqueous environments, which is highly relevant to birds which float

on, swim in, and dive through water (Figure 1.2). Initially, unweathered oil is less dense than water and under most conditions floats as a film on the water surface. The action of wind, waves, and surface currents spreads and thins the oil. During the first hours to days following release, low molecular weight compounds evaporate into the air and soluble components dissolve into the water (reviewed in Morandin and O'Hara, 2016). Sunlight photoxidizes petroleum and microbes actively biodegrade it. Together, these processes change the composition, texture, and density of oil over time, and importantly change the types of toxic compounds present through their loss, concentration (e.g. PACs) (Stubblefield et al., 1995a), or degradation. Energy from wind or waves, or the presence of suspended sediments, may entrain floating oil below the surface to form an oil-in-water emulsion. The fraction of petroleum that does not evaporate or dissolve typically sinks or is washed up on beaches. During these processes, from discharge to eventual fate, petroleum slicks (>3 μm) and sheens (≤3 μm) (Morandin and O'Hara, 2016) on the water's surface are considered most hazardous to avifauna in the short-term, while sediment-bound petroleum is thought to be the source of long-term exposure to spilled oil after the surface film dissipates (Day et al., 1995; reviewed in Esler et al., 2018; Paruk et al., 2016; Perez-Umphrey et al., 2018).

Petroleum discharge into the environment tends to occur where there is intensive maritime or industrial activity (e.g. oil sands), and areas of high petroleum spill risk that overlap with critical avian habitat is where birds are most at risk of exposure (Bertazzon et al., 2014; reviewed in Burger and Fry, 1993; Fox et al., 2016; Morandin and O'Hara, 2016). Oil spill volume correlates weakly with the number of oiled birds observed, and for spills of comparable volume, associated mortality estimates can vary enormously (Burger and Fry, 1993; Hampton et al., 2003b). This is because the proximity of a spill to feeding or breeding habitat, the type of oil, amount, time of year, duration, and the composition of the avian community can all affect the number and kinds of birds affected (Camphuysen, 2011; Day et al., 1995; Esler et al., 2018; Wiens, 1995). Oil spill management response and intervention to protect wildlife can also be a factor (e.g. Wolfaardt et al., 2009). Wildlife and spill response professionals have sought to score the vulnerability of individual bird species based on ecological factors and conservation concerns (Chilvers and Battley, 2019; King and Sanger, 1979; Romero et al., 2018; Williams et al., 1995), although ecological factors (e.g. feeding characteristics, migration) may not always predict oil spill impacts such as on habitat use for a given species (Day

et al., 1995). Several studies using beached bird survey and aerial surveillance data point to regional decreases in mortality from chronic oil pollution at sea from improvements in environmental regulation, monitoring, and enforcement (reviewed in Camphuysen and Heubeck, 2001; Camphuysen, 1998, Camphuysen, 2010, Camphuysen, 2011; Larsen et al., 2007; O'Hara et al., 2009; Stienen et al., 2017; Wilhelm et al., 2009), though gaps and limitations may persist such as oil pollution from offshore shipping and smaller vessels (Bertazzon et al., 2014; Camphuysen, 2010; Hampton et al., 2003b; O'Hara et al., 2009).

Beyond bulk petroleum itself, industrial activity related to petroleum extraction and processing can also discharge petrogenic (i.e. oil source) contaminants into the water and air (Beck et al., 2015; Custer et al., 2000, Custer et al., 2001; King et al., 1987; Morandin and O'Hara, 2016). In particular, unconventional oil is often extracted by surface mining and industrial processes which produce massive volumes of tailings, tailings water, and air emissions. Oil sands production separates bitumen from water and other mineral particles (e.g. sand), generating tailings in the form of sediment and water, referred to as oil sands process-affected material or oil sands process affected-water, respectively (reviewed in Beck et al., 2015). Those terms refer to inherently variable classes of tailings that potentially contain residual bitumen itself or associated toxic compounds concentrated above background levels in the sediment or water phase (PACs, monoaromatics, metals, salts, and naphthenic acids) (reviewed in Beck et al., 2015; Dew et al., 2015). Thus, oil sands industrial activity is a source of airborne petrogenic PACs on a local scale (Cruz-Martinez et al., 2015a; Fernie et al., 2018b; Mundy et al., 2019) and may be a source of mercury and PAC contamination on a broader regional scale (Hebert, 2019; Hebert et al., 2011, Hebert et al., 2013; Paruk et al., 2018). PAC concentrations in environments near oil sands industrial activity may be dominated by alkylated PAC and heterocycle PAC congeners rather than parent PAHs (Cruz-Martinez et al., 2015a; Fernie et al., 2018b; Mundy et al., 2019), so analyses of parent PAH compounds can underestimate exposure.

A common strategy to mitigate petroleum spilled on the water's surface is to apply chemical dispersants. These are chemical formulations (e.g. Corexit 9500, Corexit 9527) designed to act as a combination surfactant and solvent to emulsify oil films into droplets which more readily disperse, dissolve, and evaporate (Dave and Ghaly, 2011; Dupuis and Ucan-Marin, 2015; Fiocco and Lewis, 1999; Lee et al., 2015; McDougall and

DeVink, 2020; Peakall et al., 1987). Dispersants thus alter the fate, and potentially the effects, of spilled oil. For instance, while successful dispersion of a surface slick could prevent oil from fouling a bird's plumage, subsurface organisms on which birds may depend could suffer greater impact from toxicity. Furthermore, dispersants may be applied to large petroleum spills in vast quantities and may modify the effects of oil on birds or have adverse effects of their own, including on feather function and toxicity (Finch et al., 2012; Jenssen, 1994; Peakall et al., 1987; Rocke et al., 1984; Stroski et al., 2019; Whitmer et al., 2018; Wooten et al., 2012). A discursive consideration of the effects of common dispersants and oil-dispersant-mixtures that may result from their use is beyond the scope of this review and given elsewhere (e.g. Jenssen, 1994; McDougall and DeVink, 2020; Peakall et al., 1987), so we focus on effects attributed primarily to petroleum.

1.3.3. Exposure routes, rates, and duration

Birds can be exposed (Figure 1.2) directly to bulk petroleum via external oiling and feeding, and petroleum-derived contaminants may be inhaled or trace amounts ingested with soil, sediment, water, or prey. External oiling may cause harmful physical effects. It occurs when birds come into contact with petroleum (e.g. floating on the water surface) and it adsorbs to their feathers in amounts that may range from trace amounts to completely covering the body. Petroleum has seldom (Cunningham et al., 2017) been documented to irritate the skin of birds, and there is little evidence that dermal exposure alone is appreciably toxic, although eye lesions may result from contact with oils (reviewed in Hartung, 1995; Fiorello et al., 2016). Importantly, external petroleum contamination on the feathers may be aspirated (Camphuysen and Leopold, 2004; reviewed in Hartung, 1995) or ingested while preening oil-fouled feathers (Hartung, 1963). Even birds without visible oil on the plumage may ingest oil (Balseiro et al., 2005; Fallon et al., 2017), which may be toxic. Birds may consume petroleum-contaminated grit (King and Bendell-Young, 2000), water (reviewed in Beck et al., 2015), and prey such as invertebrates, fish, and oiled birds (Bonisoli-Alquati et al., 2016; reviewed in Burger and Fry, 1993; Cunningham et al., 2017; reviewed in Esler et al., 2018; Horak et al., 2017; Perez-Umphrey et al., 2018; Rattner et al., 1995; Seegar et al., 2015; Velando et al., 2010; Zuberogoitia et al., 2006). Although it has usually been assumed that the inhalation risk of volatile petroleum hydrocarbons (e.g. BTEX) is minor because of their

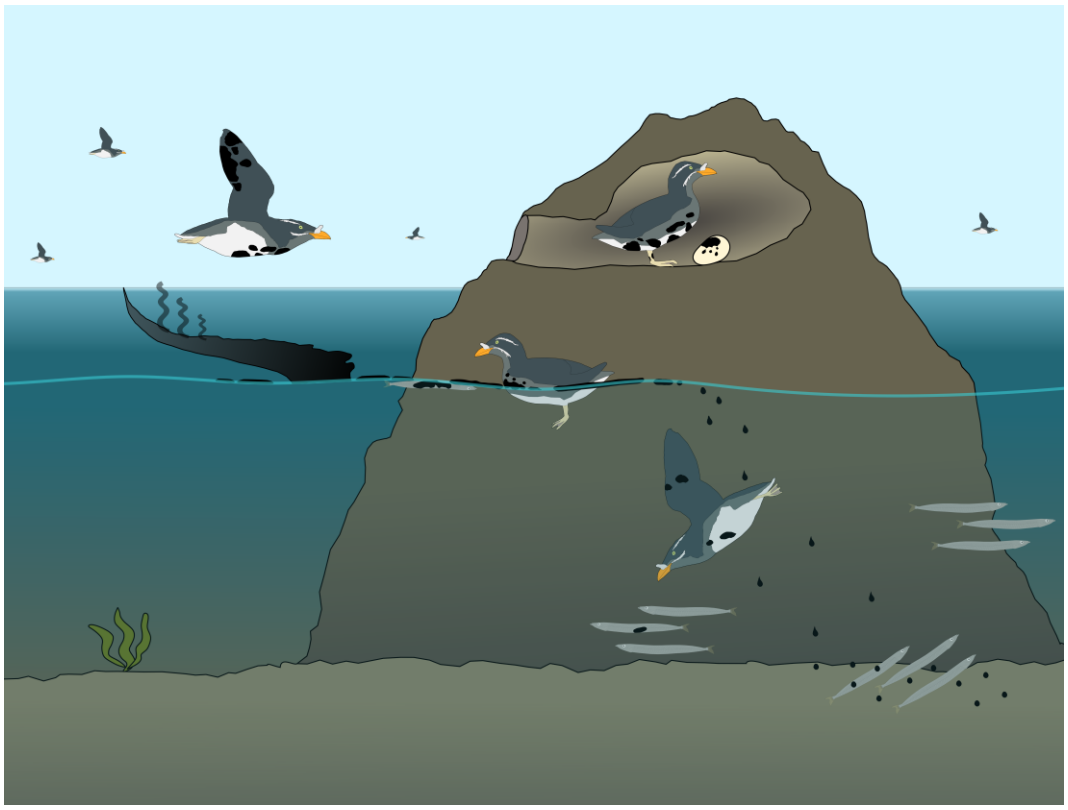

Figure 1.2 **Illustrated here in a marine bird, the most important effects of spilled petroleum on birds can include embryotoxicity in oiled eggs, compromised function of oiled feathers for flight performance, swimming performance, buoyancy, and thermoregulation, ingestion of petroleum via contaminated feathers and prey, and inhalation of volatile petroleum components. Indirectly, reduced prey availability can affect habitat use and chick provisioning.**

rapid volatilization, mixing, and dilution in the air (reviewed in Hartung, 1995), recent studies suggest that airborne concentrations associated with oil sands industrial activity and crude oil spills at sea may cause toxic effects (Cruz-Martinez et al., 2015a, Cruz-Martinez et al., 2015b; Dubansky et al., 2018; Olsgard et al., 2008, Olsgard et al., 2009). Dietary exposure to atmospherically deposited petrogenic PACs may be an important exposure route, though the potential for such comparatively low concentrations to cause adverse effects remains unclear (Cruz-Martinez et al., 2015a; Fernie et al., 2018b; Mundy et al., 2019).

There is little information on the extent and rate that birds may actually ingest petroleum, though available estimates suggest rates on the order of several ml kg^{-1} bw d^{-1} are possible if birds are both oiled themselves and feeding on contaminated prey. Hartung's (1963) studies in waterfowl indicated that captive American black ducks (*Anas*

rubripes) oiled with 0.8 to 16.6 ml kg^{-1} bw of a radiolabeled carbon and mineral oil mixture removed half of the application by preening within 8 days, most of which was ingested. Assuming complete ingestion, the maximum rate of ingestion when preening is most rapid over the first day after exposure is 3.32 ml kg^{-1} bw d^{-1} (online article supplementary data Exposure Estimate 1). Hartung (1995) later used hydrocarbon residue in bivalve mussels to estimate that ducks feeding on the most contaminated mussels during the height of the *Exxon Valdez* spill ingested 0.14 ml kg^{-1} bw d^{-1} (online article supplementary data Exposure Estimate 2). It thus seems reasonable that a duck with moderately oiled plumage and feeding on heavily contaminated invertebrates might ingest up to 4 ml kg^{-1} bw d^{-1} over several days. Alternatively, avoidance of food which is evidently oil-contaminated by means of olfaction, taste, or experience of prior intoxication could mitigate dietary exposure to oil. Results here are mixed. Mallards (*Anas platyrhynchos*) given crude oil in the diet did not avoid up to 10% (w/w) petroleum in feed (9.5 ml kg^{-1} bw d^{-1}) (reviewed in Stubblefield et al., 1995a), although laughing gulls (*Leucophaeus atricilla*) given fish injected with weathered crude oil in this range were observed to reject the food if they sensed the oil (Horak et al., 2017). Unfortunately, little has been done to quantify environmentally realistic petroleum ingestion rates beyond Hartung's (1963, 1995).

The most severe risk of exposure to petroleum spilled into the environment occurs in the days and weeks immediately after a large spill, but low-level exposure to lingering hydrocarbons may continue in some cases from many months up to several decades (reviewed in Esler et al., 2018; Perez-Umphrey et al., 2018; Velando et al., 2010). Exposure to residual crude oil hydrocarbons in sediment is documented to occur even up to 2 to 20 years after the initial spill, for instance, likely through invertebrate prey (Esler et al., 2010, Esler et al., 2011, Esler et al., 2017, Esler et al., 2018; Golet et al., 2002; Trust et al., 2000; Velando et al., 2010). Storms may resuspend petroleum buried in sediment from past spills (Paruk et al., 2016; Perez-Umphrey et al., 2018). Petroleum hydrocarbon exposure, especially in the absence of visibly oiled birds, is inferred from the analysis of chemical residue in tissue or biomarkers.

1.3.4. Indicating exposure and effects: tissue residue concentrations and biomarkers

Petroleum exposed birds may not always appear to be oiled, and the chemical analysis of petroleum compounds in avian tissues is important for understanding rates of exposure and kinetics within the body, and for inferring thresholds of potential harm. Ingested petroleum passes rapidly through the gastrointestinal tract, is incompletely absorbed, and rapidly metabolized by biotransformation systems within the body (Dean et al., 2017b; reviewed in Hartung, 1995). Absorption and kinetics *in vivo* vary widely among the constituent compounds in petroleum (Lawler et al., 1978a, Lawler et al., 1978b; Pérez et al., 2008). The amount of tissue residue data available has improved over recent years (reviewed in Leighton, 1993).

PACs are both implicated in petroleum toxicity and often detectable in the tissue of oil exposed birds. Wild birds may be regularly exposed to some background of pyrogenic- (i.e. combustion) (Custer et al., 2001; Vidal et al., 2011) and petrogenic- (i.e. oils) source PACs (Kayal and Connell, 1995; Pereira et al., 2009). Carbon isotope analysis (^{14}C) may help ascribe petrogenic origin to trace hydrocarbon contaminants found in tissue (Bonisoli-Alquati et al., 2016). Ingestion of oil can cause exposure to concentrated PACs on top of some shifting baseline of more chronic, low-level exposure in the diet (Custer et al., 2000; King et al., 1987; Michot et al., 1994; Miles et al., 2007; Willie et al., 2017). PAC analyses have historically focused on parent PAHs, though the recognition of the environmental and toxicological importance of alkyl-PAH and heterocycle congeners is increasingly recognized (reviewed in Achten and Andersson, 2015; Cruz-Martinez et al., 2015a; Fernie et al., 2018a; Mundy et al., 2019). Oiled seabirds can have quantifiable PAC concentrations in plasma and liver (Troisi et al., 2006, Troisi et al., 2007, Troisi et al., 2016; Troisi and Borjesson, 2005), and elevated petrogenic PAC residue has indicated exposure to trace petroleum contamination from oil spills at sea (Alonso-Alvarez et al., 2007b [erythrocytes]; Paruk et al., 2016 [plasma]; Pérez et al., 2008 [erythrocytes]; Seegar et al., 2015 [erythrocytes]; Zuberogoitia et al., 2006 [eggs]), produced water (Rattner et al., 1995 [stomach contents]), oil sands industrial activity (Fernie et al., 2018a [muscle and feces]; Gurney et al., 2005 [bile]; Paruk et al., 2018 [plasma]), and chronic oil pollution (Custer et al., 2000; King et al., 1987 [carcass]). Data from correlative, controlled dosing, and treatment-reference area studies provide evidence that elevated petrogenic PAC concentrations in tissue can

cause biological effects on various endpoints including growth and metabolism, as well as circulating metabolites, erythrocytes, and thyroid hormones (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Fernie et al., 2018a, Fernie et al., 2019; Gurney et al., 2005; Pérez et al., 2010a; Troisi et al., 2007, Troisi et al., 2016). Beyond the brief summary above of petrogenic PAC residue and the effects of exposure, a more complete discussion of PAH residue in birds is found elsewhere (Albers, 2006).

Crude petroleum, some fuels, and waste oils may have high concentrations of potentially toxic metals such as arsenic, cadmium, copper, chromium, lead, manganese, mercury, selenium, vanadium and others, though compared to PAC tissue residue there is little data linking metal concentrations in tissue to petroleum exposure or any resulting adverse health effects. Most available studies since 1993 show little evidence of elevated metal residue associated with petroleum contamination in the environment or direct exposure (Burger et al., 2008; Godwin et al., 2016; Kammerer et al., 2004; Mochizuki et al., 2013; Pérez-López et al., 2006; Sanpera et al., 2008). However, the feathers of birds from areas affected by the Exxon Valdez crude oil spill 15 years earlier showed elevated cadmium and manganese in one of three species studied (Burger et al., 2007, Burger et al., 2008). An analogous study at bird breeding colonies impacted by the Prestige fuel oil spill examined the feathers of chicks from two species and showed a declining trend in copper and lead concentrations over subsequent years from putatively spill-caused increases (Moreno et al., 2011). A screening of metal concentrations in the blood of mallards (*Anas platyrhynchos*) experimentally exposed to oil sands process-affected water and exhibiting subclinical blood biochemistry and endocrine (thyroid and corticosterone) effects had higher vanadium than controls and sex-specific differences in molybdenum (Beck et al., 2014). As yet however, there is no evidence linking trace oil sands contaminant exposure *in situ*, or other bulk petroleum exposure, to elevated concentrations of vanadium or molybdenum in liver or kidney (Godwin et al., 2016; Kammerer et al., 2004; Mochizuki et al., 2013). As with hydrocarbons, the concentrations of metals will vary among different petroleum types, and inter-species differences among birds from the same areas are apparent (Burger et al., 2008; Moreno et al., 2011).

The most sensitive and widely used biomarkers of petroleum hydrocarbon exposure are measures of the cytochrome P450 monooxygenase (CYP) system. This is an assemblage of enzyme isoforms concentrated and typically measured in the liver but

also found in other tissues. The CYP enzyme system forms the primary phase I biotransformation system for a wide range of xenobiotics, including monoaromatic (e.g. BTEX) and PAC compounds found in petroleum (Alexander et al., 2017; Brunström et al., 1991; Cruz-Martinez et al., 2015a, Cruz-Martinez et al., 2015b; Dubansky et al., 2018; Gentes et al., 2007b; Head and Kennedy, 2007; Newman, 2010; Walters et al., 1987). CYP1A induction via the aryl hydrocarbon receptor is typically used as a biomarker of exposure in birds. The two avian CYP1A enzyme isoforms are CYP1A4 and CYP1A5 (Alexander et al., 2017). CYP1A induction is quantified by mRNA relative abundance (*cyp1a4* and *cyp1a5* gene expression), protein abundance (CYP1A immunoassay), and, most commonly, enzyme activity (alkoxyresorufin-O-deethylase activity). Those CYP1A biomarkers have provided evidence for exposure to petroleum hydrocarbons from large spills at sea (Esler et al., 2010, Esler et al., 2011, Esler et al., 2017; Golet et al., 2002; Perez-Umphrey et al., 2018; Trust et al., 2000; Velando et al., 2010), chronic petroleum pollution at sea (Miles et al., 2007; Willie et al., 2017), oil sands industrial activity (Cruz-Martinez et al., 2015a; Gentes et al., 2006; Smits et al., 2000), and laboratory inhalation studies with environmentally realistic volatile compound concentrations in air (Cruz-Martinez et al., 2015b; Dubansky et al., 2018). CYP1A system biomarkers have also been used extensively to characterize exposure in experiments (Alexander et al., 2017; Bianchini and Morrissey, 2018a; Gurney et al., 2005; Prichard et al., 1997; Trust et al., 1994). While elevated CYP1A enzyme activity itself is not necessarily deleterious (Leighton, 1993; Newman, 2010), studies have linked ethoxyresorufin-O-deethylase activity (EROD) evidence of petroleum exposure to decreased survival in free-living birds (Esler et al., 2000b; Gentes et al., 2006; Iverson and Esler, 2010; Velando et al., 2010). Other CYP enzyme systems like CYP3A may interact with PACs as well (Mundy et al., 2019).

Other biomarkers of oxidative stress and damage may be useful, though they are less specific to petroleum exposure, and possibly affected by infection, nutrition status, physical activity (e.g. migration, reproduction), age, and other factors (reviewed in Costantini, 2008; Skrip and McWilliams, 2016). The best studied and apparently sensitive example is the occurrence of oxidatively denatured hemoglobin (Heinz bodies) within erythrocytes (Fallon et al., 2013, Fallon et al., 2017; Harr et al., 2017a; Horak et al., 2017; Leighton, 1986; see Section 1.4.2). This or other markers of oxidative stress and damage (Section 1.4.2) found in tissue or plasma may indicate petroleum exposure

if corroborated by data to support ingested oil as the cause or strong circumstantial evidence, such as a known oil spill having impacted avian habitat.

1.4. Physical and toxic effects of petroleum exposure

The short-term negative impacts to birds from petroleum may be categorized as: 1) the physical effects of contaminated plumage, 2) the toxicological effects of petroleum that is ingested or inhaled, and 3) for contaminated eggs, potential embryotoxicity (reviewed in Leighton, 1993). Birds contacting bulk petroleum in the environment often face plumage fouling and ingestion simultaneously. The biological response *in vivo* (Figure 1.3) includes immediate effects, a cascade of responses to compensate and overcome the challenges posed by oil exposure or, if these are unsuccessful, a terminal

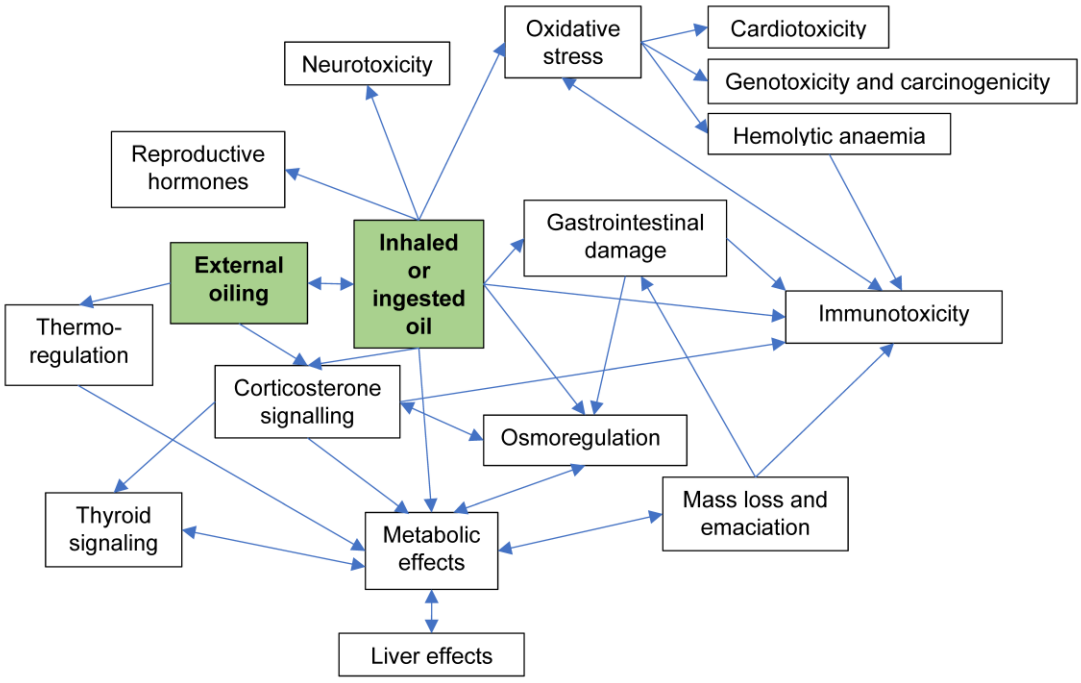

Figure 1.3 Conceptual diagram of petroleum exposure's (green shaded boxes) effects (white boxes) on avian biology.

decline until death. Heavily oiled birds may succumb rapidly to the physical effects of oiling, while birds surviving the initial encounter will face sustained challenges from compromised plumage and potential toxicity.

1.4.1. Plumage fouling: the primary physical effect of petroleum

Birds are often the wildlife most visibly impacted by oil spill events, because as Leighton's (1993) terse synopsis makes clear, “oil floats on water and birds have feathers.” Feathers provide a lightweight and streamlined surface, water repellence, and trap air close to the skin, thus enabling flight, swimming, diving, buoyancy, and thermoregulation. Petroleum readily adsorbs to feathers, compromising these properties critical to their function in air and water (Figure 1.4), and is thus likely the primary negative effect on birds rather than toxicity (see Section 1.5.3). This section deals with feather structure, how the structure of individual feathers is compromised by petroleum, and implications for thermoregulation, metabolism, locomotion, and recovery.

Disruption of feather structure

Feathers' light weight and water repellence results from their intricate, hydrophobic microstructure. From the central midline rachis, barbs branch off, and branching off from these, microscopic hooked barbules on the distal edge and smooth barbules on the proximal edge (Figure 1.4). The barbules on the leading edge of a barb interlock with the barbules on the trailing edge of the neighboring barb to form a zippered matrix on a microscopic scale. This microstructural matrix opposes the surface tension of water and the hydrophobic, lipid-coated keratin they are comprised of resists water's polarity (O'Hara and Morandin, 2010; Stephenson, 1997). Barbules from adjacent feathers similarly interlock to form a contiguous, water repellent layer. However, because of its hydrophobic, oleophilic properties, this feather structure is extremely vulnerable to contact with other oils.

Petroleum compromises the function of the feather structure by lowering the surface tension of water while simultaneously adsorbing to the hydrophobic feather matrix, causing the interlocking barbules to become clogged and their supporting barbs to collapse (Fig. 4) (Jenssen, 1994; Morandin and O'Hara, 2016; O'Hara and Morandin, 2010; Stephenson, 1997). Decreased water surface tension and collapsed microstructure means that water can enter the gaps in the otherwise impenetrable feather matrix and displace the air that provides insulation and buoyancy (Jenssen, 1994; Morandin and O'Hara, 2016). The amount of oil adsorbed onto the feather sufficient to disrupt its structure is small. Collapse and clumping of neighboring barbules

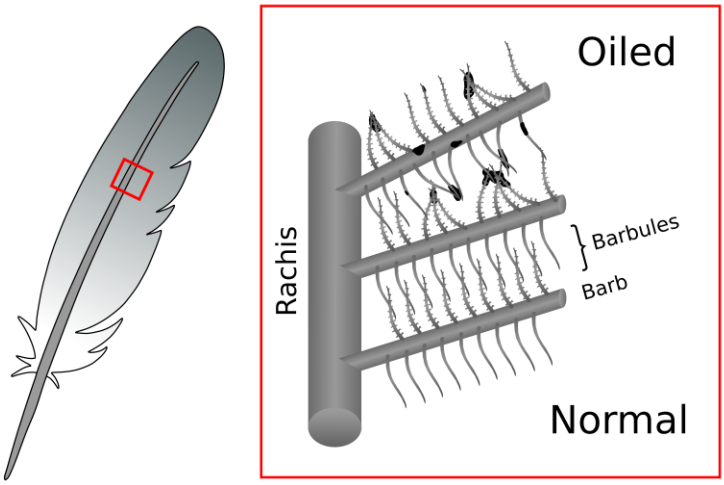

Figure 1.4 **Feather function is achieved by its lightweight, hydrophobic keratin structure which interacts with the surface tension of water. From the central rachis, barbs branch off. Branching off from these are microscopic hooked barbules on the distal edge and smooth barbules on the proximal edge. These interlock with barbules on the neighboring barbs. Petroleum is also hydrophobic and adsorbs to these structures, fouling them even at trace amounts. For instance, films of oil on the water surface <3 µm may clog barbule hooklets and cause neighboring barbules to adhere. Increasing amounts of oil progressively disrupt this hierarchical structural matrix to the point that barbs become disarranged with heavy fouling.**

(quantified as amalgamation index) has been demonstrated for a majority of diverse marine species when feathers contact petroleum-on-seawater films as thin as 3 µm or less (Fritt-Rasmussen et al., 2016; Matcott et al., 2019; O'Hara and Morandin, 2010). Structural compromise can be apparent even when the amount of oil contaminating the feather is undetectable on a mass basis (several milligrams) (e.g. O'Hara and Morandin, 2010), though the threshold of effect varies somewhat among petroleum types and species. In addition to surface films, exposure to oil droplets suspended in water are also relevant to diving birds. A dive tank study with common murres (*Uria aalge*) showed effects on feather microstructure at crude oil-in-water concentrations of 0.2 ml l^{-1} and significant gross observations of plumage wetting at 2.0 ml l^{-1} (Whitmer et al., 2018). As opposed to barb amalgamation indices from manual counts, computer image analysis may prove useful for quantifying feather microstructure damage (Bigger et al., 2017). Studies to date have examined petroleum effects on individual feather barbs; it is not known if the barbule connections between feathers is affected to a comparable degree. In addition to petroleum itself, spill response approaches such as using chemical dispersants or burning oil slicks can affect feather structure and function through the

same mechanisms by generating oil-dispersant-water mixtures and hydrophobic burn residue, respectively (Fritt-Rasmussen et al., 2016; reviewed in Jenssen, 1994; Whitmer et al., 2018).

The finding that even trace amounts of oil can disrupt feather structure is an important revelation, because visible amounts of oil on the plumage are known to affect thermal balance at a few milliliters or more, and heavy oiling can be fatal (Hartung, 1967; reviewed in Jenssen, 1994). Heavy oiling can collapse plumage to such an extent that a bird may drown or be effectively immobilized (Golightly et al., 2002; reviewed in Leighton, 1993), but contact with small amounts of oil may have more subtle effects. A bird swimming through a 1 to 3 µm thick oil film could pick up 1 ml of oil per 0.3 to 1.0 m^2 of surface swum, and surface sheens at offshore oil and gas platforms are associated with produced water discharges containing total hydrocarbon concentrations (≤0.15 ml l^{-1}) that approach thresholds for feather structure effects (0.2 ml l^{-1}) (Morandin and O'Hara, 2016; Whitmer et al., 2018). Thus, even petroleum that has thinned or become diluted to low concentrations at sea may still affect feathers. Larger amounts of petroleum ranging from several to tens of milliliters are associated with effects on energy balance in a dose-dependent fashion (reviewed in Jenssen, 1994; Oka and Okuyama, 2000; Whitmer et al., 2018). The highly adsorbent properties of feathers and spread of oil by preening suggests that the relationship between the volume of petroleum adsorbed and how much surface area (SA) of the bird appears oiled is indirect, but for the sake of example we calculate (see Section 1.2.1) from external dosing studies that 20% SA corresponds to contacting 5 to 10 ml kg^{-1} bw (Cunningham et al., 2017; Dannemiller et al., 2019; Maggini et al., 2017a, Maggini et al., 2017c). This is consistent with the amount of petroleum contaminating dead, moderately oiled birds in winter (e.g. 6.4 ml kg^{-1} bw) (Hartung, 1963), so it seems reasonable that a bird encountering a thin film of oil could foul its plumage sufficiently to affect thermoregulation and metabolism under cold conditions (Hartung, 1967; also see Jenssen, 1994). Effect thresholds, or how much oil, remains an open question for most taxa. At present, quantitative links between the amount of oil contaminating feathers and organism energy balance do exist (reviewed in Jenssen, 1994), and the development of a general, unified model linking exposure, feather function, thermal balance, and metabolism would be invaluable. Feather type, age from molting, species, and petroleum type appear to be important factors (Fritt-Rasmussen et al., 2016; O'Hara and Morandin, 2010; Stephenson, 1997).

Thermal and metabolic balance effects

Because thermoregulation and metabolism are tightly coupled, reduced thermal insulation by oil-compromised plumage can increase heat loss and subsequently metabolic rate to compensate, which can be costly (Jauniaux et al., 1998; reviewed in Jenssen, 1994). Here we summarize Jenssen's (1994) review on the subject. Conductive heat loss is greater at lower temperatures, at higher surface area to volume ratios (i.e. smaller body size), and in water as opposed to air. To compensate for heat loss, metabolic rate increases up to a point, but if the rate of heat loss exceeds metabolic heat production, then hypothermia results. If hypothermia is avoided, elevated metabolic rate must be maintained by depleting lipid reserves or feeding more.

Should oiled birds at sea not succumb to drowning or hypothermia from heavy oiling, lesser amounts of oil may cause starvation from the increased metabolic cost of thermoregulation, or to use Hartung's (1967) term, accelerated starvation (reviewed in Jenssen, 1994; Leighton, 1993). For instance, Oka and Okuyama (2000) calculated that an oiled rhinoceros auklet (*Cerorhinca monocerata*), if unable to keep feeding, would starve to death in 1.2 days at sea during winter at mid-latitudes. Recent studies of birds oiled at sea showed decreased body mass, emaciation, muscle atrophy, and depleted lipid reserves such as liver and subcutis, usually accompanied by empty stomachs (Balseiro et al., 2005; Fowler et al., 1995; Gandini et al., 1994; Jauniaux et al., 1997; Oka and Okuyama, 2000; Simpson and Fisher, 2017). Such postmortem findings are consistent with starvation in seabirds (Jauniaux et al., 1998). Data from oil spills and wildlife rescue operations support the link between low body mass or body condition and near-term morbidity and mortality (Anderson et al., 2000; Camphuysen and Leopold, 2004; Duerr et al., 2016; Gartrell et al., 2019; reviewed in Jenssen, 1994; Parsons et al., 2018; Sharp, 1996; Sievwright et al., 2019a). Paradoxically, seabirds with only light or moderate oil fouling appear to starve at sea, whereas more heavily oiled individuals that are forced ashore by completely compromised plumage are more likely to survive cleaning and rehabilitation (Camphuysen and Leopold, 2004; Duerr et al., 2016; Gartrell et al., 2019; Henkel et al., 2014b; Jauniaux et al., 1997).

Recent experiments where birds were externally exposed to moderate amounts of weathered crude oil show some results consistent with the large body of evidence for metabolic effects in externally oiled wild birds caused in part by altered thermal balance.

Maggini et al. (2017a) found decreased body mass in western sandpipers (*Calidris mauri*) 3.5 d after a single 10 ml kg^{-1} bw (20% SA) external exposure, and during a cold temperature challenge those birds lost less fat mass and more lean mass than other treatments, perhaps because fat reserves were already depleted. However, no difference in metabolic rate was detected. While no body weight effects were seen in externally oiled (32 to 45 ml kg^{-1} bw, 14 to 40% SA) ring-billed gulls *(Larus delawarensis*) after several weeks (Dannemiller et al., 2019), double-crested cormorants (*Phalacrocorax auritus*) repeatedly oiled with 8 ml kg^{-1} bw (cumulatively 46 ml kg^{-1} bw, 20% SA) increased their food consumption compared to the control group and gained weight at a faster rate over the three week trial, likely due to increased energetic cost of thermoregulation in the oil-covered birds (Cunningham et al., 2017). The experimental effects on body mass, body composition, and food consumption at moderate, e.g. 20% SA, amounts of external oil exposure are less pronounced than the dramatic emaciation seen in oiled wild birds, presumably related to the easy access to dry land, food, and warmth (e.g. heat lamps) in captivity. Furthermore, besides the plausible effects on thermal balance from oiled plumage, the above metabolic endpoints can also be affected by the toxicity of ingested oil (see Section 1.4.2) and, as in those experiments mentioned, separating physical effects of oil on feathers from toxicity is often impossible.

Flight and swimming performance

Feathers enable bird species to move efficiently through water and, more uniquely, air by optimizing streamlining, buoyancy, and lift, but moderate amounts of oil impair flight (e.g. 20% SA) and swimming performance. Of course heavily oiled birds may be effectively immobilized by plumage collapse or the stickiness of viscous crude oils (Golightly et al., 2002), but comparatively small amounts of oil may cause birds to work harder to fly by reducing lift and thrust, increasing wing loading and drag, and making feathers susceptible to stress damage (Maggini et al., 2017c; Perez et al., 2017a). Experiments in Western sandpipers (*Calidris mauri*) and homing pigeons (*Columba livia*) with weathered crude oil (20 to 30% SA) on their feathers indicated impaired flight performance, increased energy expenditure, decreased body mass, and reduced take-off speeds (Maggini et al., 2017b, Maggini et al., 2017c; Perez et al., 2017a, Perez et al., 2017b, Perez et al., 2017c). Analogous to oil-compromised flight performance, plumage oiling may hinder swimming and diving performance by increasing hydrodynamic drag or affecting buoyancy. Adélie penguins (*Pygoscelis*

adeliae) lightly oiled with vegetable oil, similar in properties to mineral oils, may have had to work harder to swim in one experiment (Culik et al., 1991). Lower swim speeds, elevated energy expenditure evident from increased heart and metabolic rates, and an eagerness to exit the water were observed. While there are few recent studies on swimming and dive performance with oiled plumage, the common co-occurrence of emaciation and empty stomachs in dead, oiled seabirds may support a reduced ability to capture prey necessary to overcome increased metabolic costs (Gandini et al., 1994; Oka and Okuyama, 2000; Simpson and Fisher, 2017).

Recovery by preening

Birds that survive with oil-fouled plumage appear to be able to remove visible traces of oil over several weeks by preening, however there is little information on the extent and speed with which function is restored. Observations from experimentally oiled shorebirds in captivity and observational studies in wild gulls and terns agree that birds were able to remove visible oil within about 2 to 10 weeks (Burger and Tsipoura, 1998; Camphuysen, 2011; Maggini et al., 2017c). Molting does not necessarily eliminate the oil, as it can transfer from the old feathers to the new (Kerley et al., 1985). It is widely recognized from work in rescued birds that when the plumage is cleaned of contaminating oil and allowed to dry, water repellence and insulation is restored (reviewed in Jenssen, 1994). One recent report shows that diving and foraging behavior in cleaned and rehabilitated little blue penguins (*Eudyptula minor*) was normal (Chilvers et al., 2015), suggesting a return to regular swimming and diving performance if contaminating oil is preened out or humans intervene. However, for birds that survive unaided by humans, the extent and time required for preening to fully restore feather structure and function has received relatively little attention, especially given the effects of trace amounts of oil on feather microstructure (Fritt-Rasmussen et al., 2016; Matcott et al., 2019; O'Hara and Morandin, 2010).

1.4.2. The primary effects of systemic petroleum toxicity

Petroleum toxicity is characterized as systemic (Leighton, 1993), affecting the structure and function of multiple organs, systems, and processes within the body (Figure 1.3). While such physiological systems are not usually limited to a singular

location or function, this section summarizes the proximate effects of petroleum toxicity, to the extent that they are known, on target tissues, organs, and body systems.

Inhalation and the lungs

Aspirated petroleum causes lipid pneumonia (Hartung and Hunt, 1966; reviewed in Leighton, 1993) and volatile components of petroleum which are inhaled plausibly affect the lungs (Dubansky et al., 2018; reviewed in Hartung, 1995). Pathological examination of avian lung and air sac tissue has not indicated obvious damage (Balseiro et al., 2005; Fry and Lowenstine, 1985; Simpson and Fisher, 2017; Szaro et al., 1978). Only recently have studies examined the toxicological effects of airborne petroleum contaminants in more detail. Inhaled volatile hydrocarbons, including monoaromatics (BTEX), from weathered crude oil increased CYP1A protein expression in lung epithelia (Dubansky et al., 2018). These phase I biotransformation enzymes may metabolically activate aromatic compounds as they pass through the epithelia, and studies on airborne petroleum contaminants point to effects on immune function, liver, thyroid, and stress response (Cruz-Martinez et al., 2015a, Cruz-Martinez et al., 2015b; Dubansky et al., 2018; Fernie et al., 2016; Olsgard et al., 2008, Olsgard et al., 2009).

Gastrointestinal damage

Ingested petroleum passes through the gastrointestinal tract, and evidence of damage there has been mixed, yet recent experimental work supports petroleum ingestion as a potential cause of gastrointestinal injury. Gastrointestinal lesions are common in dead marine birds suspected to have succumbed to oil exposure at sea (Balseiro et al., 2005; Gandini et al., 1994; Jauniaux et al., 1997, Jauniaux et al., 1998; Simpson and Fisher, 2017), but the same lesions are also common where starvation is the cause of death and no oil exposure is suspected (Jauniaux et al., 1998). Past reviews have arrived at diverging conclusions as to whether gastrointestinal damage and function is a primary effect of petroleum ingestion (Burger and Fry, 1993; Leighton, 1993). Common lesions include inflammation, petechiae, hemorrhage, and ulceration (Balseiro et al., 2005; Gandini et al., 1994; Jauniaux et al., 1997, Jauniaux et al., 1998; Simpson and Fisher, 2017). The presence of oil-like substances in the affected intestinal lumen hints at a direct cause of such lesions in some cases (Balseiro et al., 2005). Experiments with double-crested cormorants (*Phalacrocorax auritus*) exposed orally (5 or 10 ml kg^{-1} bw d^{-1}) or externally (20% SA every 3 d) to weathered crude oil for several

weeks passed bloody excreta and oral doses caused enteric lesions and intestinal inflammation (Cunningham et al., 2017; Harr et al., 2017a, Harr et al., 2017b). Some older dosing studies also indicate hemorrhage (Hartung and Hunt, 1966) and histopathological changes (edema) (Miller et al., 1978). The presence of gastrointestinal injury even with access to abundant food supports the case for some mechanism of gastrointestinal injury from ingested oil independent of acute starvation and tissue catabolism (Cunningham et al., 2017). Coagulopathy observed in some dosing studies could help explain gastrointestinal hemorrhaging (Cunningham et al., 2017; Dubansky et al., 2018; Harr et al., 2017b). The serious implications of intestinal epithelia damage to nutrient absorption and immune function was reviewed by Briggs et al. (1997), and the former is certainly consistent with observations of malnutrition and dehydration in oil-stricken birds (Balseiro et al., 2005; Duerr et al., 2016; Troisi et al., 2007).

Osmoregulation and excretion

The intestines, kidneys, and in some species, salt glands, are essential to osmotic balance in birds, and there is some evidence that petroleum is harmful to all of these organs (reviewed in Leighton, 1993). Osmotic stress could potentially be fatal in the presence of other stressors (reviewed in Burger and Fry, 1993). However, Leighton (1993) highlights the difficulty in separating primary and secondary toxic effects on osmoregulation, as well as the difficulty in drawing general conclusions from past studies, especially given the link between the corticosteroid stress axis and osmoregulation. Nevertheless, changes to circulating electrolyte or nitrogenous waste concentrations or changes in the kidney resulting from experimental petroleum exposure are consistent with dehydration observed among birds oiled at sea (Balseiro et al., 2005; Duerr et al., 2016; Troisi et al., 2007), supporting some nonspecific link between petroleum toxicity and osmoregulation as a whole.

There has not been consensus among reviews on whether the kidney is a major target of ingested oils (Briggs et al., 1996; Hartung, 1995), and opinions remain mixed. There is evidence from older studies of kidney lesions or damage in oil-exposed birds (e.g. Fry and Lowenstine, 1985; Hartung and Hunt, 1966; Szaro et al., 1978) and developing chicken embryos in oiled eggs (Couillard and Leighton, 1990a, Couillard and Leighton, 1990b). More recent experimental work demonstrated increased kidney size following weathered crude oil exposure for several weeks (5 to 10 ml kg^{-1} bw d^{-1} orally

or 20% SA every 3 d externally) in double-crested cormorants (*Phalacrocorax auritus*) and laughing gulls (*Leucophaeus atricilla*), as well as histopathological lesions consistent with chronic irritation and inflammation in orally dosed groups (Harr et al., 2017b; Horak et al., 2017). In contrast, no histological changes were found in the kidneys of juvenile mallards (*Anas platyrhynchos*) in a similar experiment (9.5 ml kg^{-1} bw d^{-1}) (Stubblefield et al., 1995b). Increases in urea or uric acid consistent with decreased kidney function have been documented in the plasma or serum of double-crested cormorants experimentally exposed to weathered crude oil (Dean et al., 2017a, Dean et al., 2017b), although besides kidney function, metabolic changes (e.g. protein catabolism) or hydration status can also affect plasma urea and uric acid (Balseiro et al., 2005; Styles and Phalen, 1998).

Like changes to concentrations of circulating waste products, electrolytes such as calcium can be clinically useful in toxicological trials, but have done little to resolve the physiological mechanisms at work or their relation to toxic pathologies. Electrolyte effects associated with oil exposure vary among species, exposure route, duration, or dose, and likely some combination of these. As in the past, recent experimental results reporting circulating electrolytes (e.g. Ca^{2+}, Na^{+}, K^{+}, Cl^{-}, HCO_3^{-}) differ from one study to the next and are difficult to interpret collectively (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Beck et al., 2014; Bursian et al., 2017b; Dannemiller et al., 2019; Dean et al., 2017a, Dean et al., 2017b; Golet et al., 2002; Maggini et al., 2017a; Newman et al., 2000; Prichard et al., 1997; Seiser et al., 2000). Effects on Ca^{2+} are common, though divergent, possibly age- and sex-dependent, and linked to eggshell thinning (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Dean et al., 2017a; Golet et al., 2002; reviewed in Leighton, 1993; Seiser et al., 2000; Stubblefield et al., 1995c). Effects on circulating Ca^{2+} and eggshell thinning together with effects on the expression of genes related to intracellular Ca^{2+} regulation (*rgn*) following *in vitro* hepatocyte exposures to PACs (Crump et al., 2017) suggests a direct link between petroleum toxicity and calcium homeostasis.

Metabolic effects and the liver

Metabolic effects from the toxicity of ingested petroleum are suggested by dysregulated body mass or food intake at the whole organism level, gross or histological changes at the organ level, and molecular level effects. Generalizations about the

metabolic pathways affected and the mechanisms at work are complicated by myriad factors, including differences in species, dose, duration, oil composition, sampling, and the fact that metabolites participate not in discrete systems, but in multiple linked pathways to generate energy with available substrates and homeostatic mechanisms. However, the weight of evidence points to effects on glucose, protein, and fatty acid metabolism. The liver plays a central role in these effects. It is the first major organ in contact with substances brought into circulation via intestinal absorption, and is critical to metabolism, and especially biotransformation. Effects on the liver are common, yet evidence of actual damage is limited. Instead, petroleum appears to result in more subtle effects on homeostatic metabolic mechanisms.

Gross and histological effects

Recent studies reporting widespread effects on body mass, feeding, or growth, together with older studies (reviewed in Leighton, 1993) indicating changes to food consumption or decreased growth rates, suggest metabolic effects from ingested oil. Mass loss has resulted from oral dosing in studies with weathered crude oil and fuel oil (Bursian et al., 2017b; Dean et al., 2017b; Horak et al., 2017; Lee et al., 2012). Pre-migratory mass gain dynamics have been affected in an oral dosing study with PACs (Bianchini and Morrissey, 2018a), and field studies indicate negative associations between blood PAC burdens and body condition or body mass (Alonso-Alvarez et al., 2007a; Paruk et al., 2016), as well as sediment PAC concentrations and pre-migratory fattening (Bianchini and Morrissey, 2018b). Although significant mass loss was not associated with lethal toxicity (5 to 10 ml kg^{-1} bw d^{-1}) in double-crested cormorants (*Phalacrocorax auritus*) dosed orally with large amounts of weathered crude oil, that study did indicate an association between reduced feed intake and mortality (Cunningham et al., 2017). Paradoxically, avoiding oiled food items can restrict food intake sufficiently that survival is better among birds ingesting higher cumulative doses of oil (Horak et al., 2017). PAC exposure has resulted in growth effects (size or mass over time) in young birds (Gurney et al., 2005; Trust et al., 1994), though not in one dosing study with weathered crude oil (Prichard et al., 1997).

The liver and pancreas are organs important to digestion and metabolic regulation which gross and histological examinations reveal to be structurally altered or damaged by ingested petroleum. Dosing studies commonly report increases in liver

mass in birds ingesting crude oil or fuel oil over several days or weeks (Dean et al., 2017b; Harr et al., 2017b; Horak et al., 2017; Lee et al., 2012; Stubblefield et al., 1995c). Metabolic starvation from oiled plumage can have the opposite effect on liver mass (Oka and Okuyama, 2000), and decreased liver mass is sometimes reported in wild birds in association with petrogenic PAC exposure in the environment (Cruz-Martinez et al., 2015a; Rattner et al., 1995). It is, therefore, not surprising that reports of increased liver mass tend to come from captive dosing studies. Increased liver mass is not synonymous with toxic injury or damage per se. It can be caused simply by glucose or lipid accumulation, but also biotransformation activity (Cattley and Cullen, 2018). Recent dosing studies have helped to rule out pathological lesions or oxidative damage as the cause of increased liver weight in adult birds ingesting weathered crude oil (Bursian et al., 2017b; Dean et al., 2017b; Harr et al., 2017b; Pritsos et al., 2017; Stubblefield et al., 1995c). Liver failure reported in oiled and rescued birds (Burger and Fry, 1993) has not been supported by the large number of peer-reviewed petroleum dosing studies with adult birds. In fact, pathological signs of hepatic damage (e.g. necrosis, neoplasia, or inflammation) rarely result from petroleum exposure, including where increased liver mass is evident, and the few reports of hepatic damage are associated with fuels rather than crude oil (Cattley and Cullen, 2018; Fry and Lowenstine, 1985; Hartung and Hunt, 1966; Stubblefield et al., 1995b). However, young birds may be more sensitive, as liver necrosis has been identified as an important lesion in embryos (Couillard and Leighton, 1990a, Couillard and Leighton, 1991a; Westman et al., 2013, Westman et al., 2014) and nestlings (Leighton, 1986; Szaro et al., 1978). For pancreatic tissue, in contrast to liver, there has been evidence of cellular death in adult double-crested cormorants (*Phalacrocorax auritus*) ingesting 5 to 10 ml kg^{-1} bw d^{-1} weathered crude oil (Harr et al., 2017b).

Biochemical effects

Biochemical or molecular data can signal shifts in metabolic processes, and while findings vary appreciably among studies, the most salient and consistent results among avian petroleum studies suggest toxic effects related to glucose homeostasis, protein catabolism, fatty acid oxidation, and bile acid metabolism. In particular, a number of studies report metabolite concentrations in the plasma or serum fraction of the blood in birds, though their scattered findings are a challenge to interpret (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Bianchini and Morrissey, 2018a, Bianchini and

Morrissey, 2018b; Bursian et al., 2017b; Dean et al., 2017a, Dean et al., 2017b; Duerr et al., 2016; Gartrell et al., 2019; Golet et al., 2002; Horak et al., 2017; Lattin et al., 2014; Newman et al., 2000; Parsons et al., 2018; Prichard et al., 1997; Seiser et al., 2000; Stubblefield et al., 1995b, Stubblefield et al., 1995c). Decreased blood glucose concentrations were found in three species of birds in oral dosing experiments and wild individuals in oil spill-impacted areas, and glucose has been shown to be negatively correlated with ∑PACs in erythrocytes (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Dean et al., 2017a, Dean et al., 2017b; Golet et al., 2002). Protein metabolism effects, most likely skeletal muscle catabolism, may exist in birds orally exposed to petroleum, as suggested by titers of circulating total protein, creatinine, creatine kinase, urea, uric acid, and 3-methyl histidine in blood (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Bianchini and Morrissey, 2018a; Dean et al., 2017a, Dean et al., 2017b; Horak et al., 2017). Besides glucose and protein metabolism, increased fatty acid oxidation may occur, as has been shown in the hepatic tissue of avian embryos exposed to PACs (Westman et al., 2013, Westman et al., 2014). The toxic metabolic effects of any ingested petroleum may be further complicated in externally oiled birds because of their potentially increased energetic costs of thermoregulation and locomotion (Anderson et al., 2000; Cunningham et al., 2017; Dean et al., 2017a; Dorr et al., 2019; Maggini et al., 2017a; Perez et al., 2017b). Nonetheless, metabolic effects observed in externally oiled birds are consistent with the effects of ingested petroleum described so far: disturbance to glucose homeostasis, muscle catabolism, and increased fatty acid oxidation unexpected in non-fasting piscivorous birds (Dean et al., 2017a; Dorr et al., 2019; Maggini et al., 2017a). Indeed, both glucose and protein dysregulation are associated with mortality in oil-exposed birds undergoing rehabilitation (Anderson et al., 2000; Gartrell et al., 2019). Furthermore, the expression of genes linked to glucose and fatty acid homeostasis are affected by PAC exposure, as shown by *in vitro* avian embryo hepatocyte studies (Crump et al., 2017; Mundy et al., 2019). Altered glucocorticoid receptor density in liver and muscle resulting from chronic, dietary exposure to weathered crude oil has been shown, and might play a role in glucose and fatty acid metabolism, at least in stressed animals (Lattin et al., 2014; Lattin and Romero, 2014). There is some evidence that exposure to weathered crude oil or dissolved oil contaminants can affect bile acid metabolism (Beck et al., 2014; Bianchini and Morrissey, 2018a; Dorr et al., 2019; Harr et al., 2017b; Mundy et al., 2019), though data are few and their implications less clear.

Clinical enzyme biomarkers in the plasma or serum have often been used to screen for hepatic and bile system damage or metabolic shifts, but these results vary so much that their interpretation from past studies, utility as a robust and sensitive biomarker, and merit for continued use is questionable. Clinical enzyme assays are used for pathological diagnosis, and biomarkers of hepatocyte damage include alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and aspartate aminotransferase (AST), while alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) are considered markers of biliary damage (Cattley and Cullen, 2018). Among these however, the degree of specificity to hepatic or biliary damage, validation in avian species, and relevant clinical baselines vary and are lacking in most cases (Cattley and Cullen, 2018; Harr, 2002). Among all petroleum related studies, the presence and direction of significant biomarker effects vary to such an extent that their collective interpretation appears impossible, and data connecting effects to signs of hepatic or biliary damage is scant (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Bianchini and Morrissey, 2018a; Bursian et al., 2017b; Dean et al., 2017a, Dean et al., 2017b; Golet et al., 2002; Kertész and Hlubik, 2002; Newman et al., 2000; Prichard et al., 1997; Seiser et al., 2000; Stubblefield et al., 1995b, Stubblefield et al., 1995c; Trust et al., 1994; Yamato et al., 1996). Even similar oral dosing studies with the same toxicant within the same species fail to produce consistent effects (Dean et al., 2017a, Dean et al., 2017b). Clinical enzyme biomarker effects appear to vary by time, age, sex, effects on extrahepatic tissue (e.g. skeletal muscle), and environmental factors, complicating their interpretation (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b; Dean et al., 2017a, Dean et al., 2017b; Dorr et al., 2019; Horak et al., 2017; Newman et al., 2000; Prichard et al., 1997; Seiser et al., 2000; Trust et al., 1994). Only a handful of petroleum related studies observe correlations between individual clinical enzyme biomarkers and other liver metabolism effects which seem individually robust (Alonso-Alvarez et al., 2007a, Alonso-Alvarez et al., 2007b [AST and blood glucose]; Bianchini and Morrissey, 2018a [blood GGT, but not AST, and hepatic EROD]; Golet et al., 2002 [blood AST, LDH, and hepatic EROD]; Hartung and Hunt, 1966 [AST and hepatic dye clearance]). While one such study links diesel fuel ingestion to elevated AST and degenerative changes in liver tissue (Hartung and Hunt, 1966), the fact remains that substantial increases in markers like AST and ALT can occur without consistent histopathological indications of damage in the liver (Trust et al., 1994).

Because the liver plays a key physiological role in both metabolism and detoxification, there is a theoretical coupling between these two roles such that the biotransformation of petroleum hydrocarbons might also modulate baseline metabolic processes, although avian work establishing clear connections between the two remains scarce. As discussed previously, hepatic CYP1A enzyme system biotransformation activity is a reliable and sensitive indicator of exposure to aromatic petroleum compounds. It is not clear that hepatic CYP1A enzyme activity alone is deleterious (Leighton, 1993; Newman, 2010), although increased biotransformation activity logically comes at some cost to routine metabolism in the form of energy allocation, endogenous CYP1A enzyme functions (e.g. steroid hormone metabolism) (Lu et al., 2020), or the generation of reactive oxygenated species as by-products (Couillard and Leighton, 1993). PACs have been shown to affect hepatic clearance in dye assays (Hartung and Hunt, 1966; Patton and Dieter, 1980), though the extent and mechanisms by which petroleum hydrocarbon biotransformation activity is mechanistically coupled with broader metabolic effects in hepatic tissue or beyond remains unclear.

Oxidative balance and damage

Reactive oxygen species (ROS) that may damage cellular biomolecules arise as inherent by-products of metabolism (e.g. superoxide) or, in the case of petroleum exposure, foreign compounds and their metabolites. Like other vertebrates, birds have complex antioxidant systems consisting of a host of enzyme and non-enzyme antioxidants to quench ROS and thereby prevent oxidative damage to biomolecules such as lipids, proteins, and DNA (reviewed in Costantini, 2008; Skrip and McWilliams, 2016). PACs in petroleum are substrates for CYP biotransformation enzyme systems and may be transformed into potentially damaging ROS, which must be countered (i.e. balanced) by antioxidant systems if damage is to be prevented (Couillard and Leighton, 1993; Skrip and McWilliams, 2016; Xue and Warshawsky, 2005). Gene expression studies using *in vitro* hepatocyte exposures to PACs show modulated transcript abundance of genes that play a role in antioxidant defense system pathways (Crump et al., 2017; Mundy et al., 2019), which substantiates a mechanistic link between PACs and maintaining oxidative balance.

Although oxidative damage to erythrocytes has long been recognized as an important mechanism of petroleum toxicity (Leighton et al., 1983), only recently have

studies begun to provide more data on oxidative balance and markers of damage. Dosing studies demonstrated effects of petroleum on oxidative balance and damage in avian species following the *Deepwater Horizon* crude oil spill and *Prestige* fuel oil spill. Across the five species studied, all exhibited effects on some antioxidant defense endpoint (total antioxidant capacity or some component thereof, e.g. glutathione) in at least one exposure scenario, and in many cases some signs of oxidative damage (Bursian et al., 2017b; Dean et al., 2017b; Horak et al., 2017; Maggini et al., 2017a; Pérez et al., 2010a; Pritsos et al., 2017). These effects included titers of antioxidant defenses in liver tissue as a result of acute oral exposure (i.e. one to several days) (Dean et al., 2017b), acute external exposure (Maggini et al., 2017a), and subacute (i.e. several weeks) oral exposure (Bursian et al., 2017b; Dean et al., 2017b; Horak et al., 2017) to *Deepwater Horizon* weathered crude oil. Acute exposure to *Prestige* fuel oil similarly produced plasma antioxidant defense titers (Pérez et al., 2010a). Among these studies, evidence of oxidative damage in the form lipid peroxidation markers in liver and plasma was weak (Bursian et al., 2017b; Maggini et al., 2017a; Pérez et al., 2010a; Pritsos et al., 2017) but red bill spot, a carotenoid-based and antioxidant-related sexual ornament, was smaller in gulls (Pérez et al., 2010a), and pathological signs of oxidative damage to erythrocytes and cardiac tissue were seen at high doses associated with mortality (5 to 10 ml kg^{-1} bw d^{-1}) (Harr et al., 2017a, Harr et al., 2017b; Horak et al., 2017).

Hemotoxicity

Oxidative damage to red blood cells and hemolysis is often a direct effect of petroleum toxicity, and the pathological condition that can result is hemolytic anemia (reviewed in Leighton, 1993). Leighton et al. (Leighton, 1985a, Leighton, 1985b, Leighton, 1986; Leighton et al., 1983) first described the model for petroleum-induced hemolytic anemia by exposing nestling birds to crude oil and showing large reductions in hematocrit after high doses (10 to 20 ml kg^{-1} bw d^{-1}) administered for several days. This response to ingested petroleum is characterized by oxidative damage by ROS metabolites of petroleum hydrocarbons (Couillard and Leighton, 1993) to erythrocyte hemoglobin, subsequent erythrocyte lysis, and an ensuing regenerative response. Evidence of these processes are oxidatively denatured hemoglobin (Heinz bodies), best observed with electron microscopy (Fallon et al., 2017; Harr et al., 2017a; Horak et al., 2017; Leighton, 1985a; although see Fallon et al., 2013), a transient decrease in

hematocrit and hemoglobin, effects on blood antioxidant titers, effects on iron binding capacity (e.g. ferritin) (Fallon et al., 2017; Lee et al., 2012; Troisi et al., 2007), accumulation of erythrocyte material (hemosiderin) mainly in the liver, spleen, or kidney where damaged red cells are phagocytized (Balseiro et al., 2005; Fry and Lowenstine, 1985; Kammerer et al., 2004; Khan and Nag, 1993; Newman et al., 2000; Simpson, 1971; Yamato et al., 1996), and elevated reticulocyte counts from erythropoiesis. The latter regenerative response may alternatively result in elevated hematocrit (Alonso-Alvarez et al., 2007a; Fowler et al., 1995; Newman et al., 2000), decreased blood leukocyte counts, and histological effects in bone marrow where erythropoiesis occurs (reviewed in Briggs et al., 1996; Leighton, 1986). Such effects are consistent with, but not limited to petroleum exposure. While many studies report hematocrit modulations, definitive evidence of petroleum-induced hemolytic injury should be confirmed by 1) clinical anemia, meaningful reductions in hematocrit or hemoglobin (e.g. compared to reference intervals or controls), or a regenerative response with 2) evidence of hemolysis and/or oxidative damage to red blood cells. The potential consequence of hemolytic anemia clearly includes reduced aerobic capacity, and anemia is associated with near-term mortality and morbidity (Duerr et al., 2016; Gartrell et al., 2019; Newman et al., 2000; Parsons et al., 2018; Sharp, 1996; Sievwright et al., 2019a). The next paragraphs synthesize new information related to petroleum hemotoxicity with a combination of evidence from oiled birds brought into rehabilitation operations, measurements made in wild birds, and dosing experiments, which collectively allow for an improved understanding of hemolytic injury.

Since 1993, several studies of oiled birds brought into wildlife rehabilitation have provided useful evidence of hematological damage from oil exposure. Hematological injury has been suggested in fuel oil-exposed white-winged scoters (*Melanitta fusca*) and crude oil-exposed American coots (*Fulica americana*) (Newman et al., 2000; Yamato et al., 1996). Large clinical datasets from wildlife rehabilitation centers have found no direct association between oiling status and low hematocrit upon admission in African penguins (*Spheniscus demersus*) and common murres (*Uria aalge*), but data for the latter suggested a threshold effect such that at low body masses, oiled birds may have lower hematocrit than unoiled rescued birds of the same body mass (Duerr et al., 2016; Parsons et al., 2018). In common murres impacted by various unidentified sources of petroleum, Heinz bodies and plasma ferritin, a protein important for oxidant defense, iron

storage, and inflammatory response, were both significantly and strongly correlated with ∑PACs in plasma (Troisi et al., 2007).

Effects consistent with the mechanism for hemolytic injury and anemia have been shown in wild birds inhabiting oil spill-impacted areas. This includes seven species in areas where fuel oil or crude oil was spilled in recent months, resulting in pronounced incidence or degree of anemia (Fallon et al., 2017; Nisbet et al., 2013, Nisbet et al., 2015; Walton et al., 1997). Remarkably, these effects were found in populations with little or no visible oil on the plumage (Fallon et al., 2017; Nisbet et al., 2013). In contrast to anemia, observations of increased hematocrit made in oiled Magellanic penguins (*Spheniscus magellanicus*) may have been an erythropoietic response to hemolysis from crude oil ingestion (Fowler et al., 1995). Available data suggest that hematological parameters return to normal as petroleum disappears from the environment (Alonso-Alvarez et al., 2007a; Nisbet et al., 2013). However, petroleum persisting in sediment may be an important source of prolonged hematological effects (Paruk et al., 2016; Walton et al., 1997). An association between PACs detected in plasma and lower hematocrit were described in common loons (*Gavia immer*) wintering in crude-oil impacted habitat years after the initial spill (Paruk et al., 2016). Altogether, there is good evidence that effects consistent with hemolysis and reticulosis occur in wild birds exposed to oil despite the complexities of real-world scenarios and inter-species variability.

Experimental evidence for oil-induced hemolytic injury in adult birds is mixed, however. Standard hematological endpoints show little to no response in a majority of recent oral dosing experiments with crude oil in five species (up to 2.4 to 20 ml kg^{-1} bw d^{-1}, with high doses of 5 ml kg^{-1} bw d^{-1} or more in most studies) (Bursian et al., 2017b; Dean et al., 2017b; Horak et al., 2017; Newman et al., 1999; Stubblefield et al., 1995b, Stubblefield et al., 1995c). Signs of hemolytic anemia in adult birds exposed experimentally to petroleum are limited to fuel oil exposures in mallard ducks (*Anas platyrhynchos*) (Hartung and Hunt, 1966; Lee et al., 2012) and weathered crude oil exposure in ring-billed gulls (*Larus delawarensis*) (Dannemiller et al., 2019) and double-crested cormorants (*Phalacrocorax auritus*) (Dean et al., 2017b; Harr et al., 2017a) at similar oral doses (2 to 20 ml kg^{-1} bw d^{-1}) or external applications (14 to 40% SA) over multiple days. In the oral exposure with cormorants, a pronounced hemolytic anemia was associated with mortality and morbidity at 5 to 10 ml kg^{-1} bw d^{-1} over several weeks

(Harr et al., 2017a, Harr et al., 2017b). The wide-ranging results reported in these studies do more to highlight the multifactorial nature of hemolytic injury than actually resolve its complexities. It is clear that anemia frequently occurs in wild birds when brought into captivity (Dannemiller et al., 2019; Newman et al., 1999, Newman et al., 2000). Alternatively, increased food availability and reduced energetic demands in captivity may modulate antioxidant capacity and vulnerability to oxidative damage (reviewed in Skrip and McWilliams, 2016). Finally, inherent inter-species differences exist under similar exposure conditions (Dean et al., 2017b; Fallon et al., 2017).

Young birds may be more sensitive to hemolytic anemia than adults. The strong experimental evidence for hemolytic anemia in birds comes almost entirely from nestlings and juveniles dosed with conventional crude oil (reviewed in Leighton, 1993). In the wild, yellow-legged gull (*Larus michahellis*) chicks with lower hematocrit were at breeding colonies affected by the *Prestige* wreck which spilled predominantly fuel oil rich in naphthalene, a strong oxidant (Alonso-Alvarez et al., 2007a; Couillard and Leighton, 1993; Pérez et al., 2008). In addition, ∑PAC concentration in the erythrocytes was positively correlated with hematocrit although chicks were in similar in body condition, which suggested an erythropoietic cause (Alonso-Alvarez et al., 2007a). In contrast, no such hematocrit effect was found in adults from oiled areas, nor in a later dosing study with adults (Alonso-Alvarez et al., 2007b). The apparent resilience of breeding adult gulls to hematocrit effects in the face of direct naphthalene exposure suggests that adults may be less sensitive to oxidant injury by metabolically activated oil hydrocarbons than nestlings. Analogously, a difference in sensitivity between life-stages is apparent with single-PAC exposures of 7,12-dimethylbenz[a]anthracene in European starlings (*Sturnus vulgaris*), where reduced hemoglobin was found in nestlings given 100 mg kg^{-1} bw, but not adults (Trust et al., 1994). Furthermore, variation associated with PAC exposure in wild loons was age class dependent (Paruk et al., 2016). Finally, there is limited evidence that naphthenic acids in petroleum, despite their apparently low toxicity, may induce hepatic erythropoiesis in nestlings (Gentes et al., 2007b).

While the preceding paragraphs have synthesized the prevailing aspects of petroleum hemotoxicity, it is admittedly difficult to reconcile the often divergent findings related to hemolytic injury from petroleum exposure, a fact that has been noted in past reviews (Fry, 1995; Hartung, 1995). For instance, indicators of hemolytic anemia are conspicuously absent from the large datasets of hematological indices taken upon the

admission of oiled birds to rehabilitation centers (Duerr et al., 2016; Newman et al., 1999; Parsons et al., 2018). That and the few documented hemolytic effects in species which forage by pursuing prey underwater, with the exception of double-crested cormorants (*Phalacrocorax auritus*), merits further examination (Newman et al., 1999; Parsons et al., 2018; Walton et al., 1997), especially in light of the inherent interspecies variability already identified (Dean et al., 2017b; Fallon et al., 2017). Pursuit divers might be more resilient to hemolytic injury, due for instance to high blood hemoglobin concentrations (Minias, 2020). Furthermore, hemorrhage (Balseiro et al., 2005), inflammatory response (Lee et al., 2012), body condition, starvation, and dehydration (Duerr et al., 2016) associated with petroleum exposure may affect hematocrit and hemoglobin in birds exposed to oil in the wild, as well as additional artefacts of captivity mentioned earlier. To improve our understanding of hemolytic injury, sensitive and robust methods are warranted for quantifying oxidative damage to erythrocytes and hemoglobin (see Fallon et al., 2013; Harr et al., 2017a; Skrip and McWilliams, 2016).

Cardiotoxicity

Recent studies have linked cardiac tissue damage to prolonged oil exposure. In double-crested cormorants (*Phalacrocorax auritus*) and laughing gulls (*Leucophaeus atricilla*) given weathered crude oil orally (5 or 10 ml kg^{-1} bw d^{-1}) for several weeks, the heart musculature appeared flaccid upon necropsy (Harr et al., 2017b; Horak et al., 2017). Gross pathology revealed heart lesions in those cormorants as well as externally oiled cormorants (20% SA area every 3 d) (Harr et al., 2017b). Despite such changes in appearance, heart mass is not greatly affected by crude oil or fuel oil exposure in birds (Harr et al., 2017b; Lee et al., 2012). However, echocardiography in the externally oiled cormorants revealed changes to cardiac structure and function that included arrhythmias and qualitative observations of labored breathing (Harr et al., 2017c). Cardiac findings were associated with an increase in plasma Ca^{2+} as well, which is important to muscle contraction (Harr et al., 2017c). Harr et al. (2017b) suggested that the high concentration of mitochondria in the cardiac muscle makes it vulnerable to oxidative damage caused by ingested petroleum. Damage to the membranes of myocardiocytes releases the calcium protein complex troponin I, which may be a useful biomarker for assaying cardiac damage from oil exposure (Daigneault et al., 2017). As with effects of hemolytic anemia on blood oxygen carrying capacity, cardiac output has clear implications for aerobic capacity.

Neurotoxicity

A limited number of studies suggest neurotoxic effects associated with refined fuel ingestion in birds, though this subject represents a knowledge gap. Hartung and Hunt (1966) observed signs of neurotoxicity in mallards (*Anas platyrhynchos*) given diesel fuel, including incoordination, ataxia, tremors, and pupil constriction. Effects on brain mass are unexpected even during substantial mass loss from toxicity (Cattley and Cullen, 2018), and the brain is less often included in reports of pathological examinations. However, one study did report decreased brain mass in mallards with substantial oral doses of fuel oil over 5 d (Lee et al., 2012), although another showed no histological findings in the brain of juvenile mallards given weathered crude oil in the diet over two weeks (Stubblefield et al., 1995b). Substantial decreases in acetylcholinesterase activity in the plasma and brain of birds exposed to diesel fuel and fuel oil, respectively, points to neurotoxic effects on acetylcholinesterase activity (Hartung and Hunt, 1966; Oropesa et al., 2007). The authors of those studies speculate that organic phosphates or PACs were responsible.

Immunotoxicity

Birds ingesting petroleum may exhibit signs of immunotoxicity, due at least in part to effects on cell-mediated immunity (reviewed in Briggs et al., 1996, Briggs et al., 1997; Fairbrother et al., 2004; Leighton, 1993). Early studies concluded that changes to lymphoid organs and lymphocytes in those tissues, changes in circulating leukocytes, and reduced resistance to pathogens are evidence of immunotoxic effects. Recent experimental work and field studies support effects on the lymphatic organs and T-lymphocyte response, and low survival among birds that are cleaned of oil contamination, rehabilitated, and released suggests prolonged immunosuppression (Briggs et al., 1996; Newman et al., 2000; Sharp, 1996).

Changes in lymphoid organs and reduced T-lymphocyte inflammatory response suggest that some aspect of petroleum toxicity affects these, though not other common measures of immunity. Spleen mass was reduced in birds orally dosed with certain crude or fuel oil types over multiple days or weeks, which may relate to lymphoid blastogenesis (Holmes et al., 1979; Horak et al., 2017; Lee et al., 2012; Rocke et al., 1984; Stubblefield et al., 1995b, Stubblefield et al., 1995c). Rather than decreased spleen mass, thymus and bursa of Fabricius (a lymphoepithelial organ in birds important

for B-lymphocyte maturation) mass in nestlings may be decreased by PAC exposure (Cruz-Martinez et al., 2015a; Holmes et al., 1979; Leighton, 1986; Stubblefield et al., 1995b; Trust et al., 1994). Counts of leukocytes circulating in blood are often reported, and significant effects on total circulating leukocytes or lymphocytes in particular have accompanied hemolytic injury (Dean et al., 2017a, Dean et al., 2017b; Harr et al., 2017a; Leighton, 1986; Newman et al., 2000; Nisbet et al., 2015), but are not necessarily altered by petroleum exposure (Beck et al., 2014; Bursian et al., 2017b; Dannemiller et al., 2019; Dean et al., 2017b; Horak et al., 2017; Maggini et al., 2017a; Newman et al., 1999; Seiser et al., 2000; Smits and Williams, 1999). However, abundance of circulating leukocytes may be hormetic, as is the case with inhaled monoaromatics (Olsgard et al., 2009). The inflammatory response of T-lymphocytes may be measured with mitogen injection assays. These have showed some mixed evidence of decreased T-lymphocyte inflammatory response associated with exposure to bulk petroleum or petrogenic oil sands contaminants, though antibody production was not affected (Cruz-Martinez et al., 2015a, Cruz-Martinez et al., 2015b; Harms et al., 2010; Olsgard et al., 2008; Smits et al., 2000; Smits and Williams, 1999; Troisi, 2013). Signs of inflammation in tissue among oil exposed birds are often attributed to toxicity (Harr et al., 2017b; Newman et al., 2000), and inflammatory response markers (e.g. acute phase proteins) in circulating serum and plasma can indicate immunological and inflammatory status (Fairbrother et al., 2004), but petroleum ingestion affects inflammatory response markers in a manner dissimilar to bacterial infection, and markers like haptoglobin appear to be ineffective indicators of exposure (Fallon et al., 2017; Lee et al., 2012; Prichard et al., 1997; Troisi et al., 2007).

Overall, there is ample evidence to support effects on cell-mediated immunity via lymphocytes and the lymphatic organs, and while the cause and functional relevance of this remains unknown, parallels between those findings and the immunotoxic effects of PAC exposure implicates that class of compounds in petroleum immunotoxicity. A study in European starlings (*Sturnus vulgaris*) with an immunosuppressive PAC congener, 7,12-dimethylbenz[a]anthracene, reported that oral exposure in nestlings caused effects on cell-mediated immunity similar to petroleum ingestion, including effects on viable spleen cell counts, bursal growth reduction, decreased lymphocyte proliferation (blastogenesis), and decreased macrophage phagocytosis (Trust et al., 1994). The known effects of PACs in fish and mammals on their lymphocytes, phagocytes, and non-specific cytotoxic cells, which are conserved across taxa, further suggests effects on

cell-mediated immunity (Reynaud and Deschaux, 2006). Some likely mechanisms of reduced cell-mediated immunity include: reduced leukopoiesis to overcome anemia caused by petroleum ingestion, damage to gut mucosa and epithelia, altered metabolism and synthesis of acute phase inflammatory proteins, and effects secondary to impaired nutrient absorption and stress (Briggs et al., 1996, Briggs et al., 1997; Dean et al., 2017a; Dorr et al., 2019; Harr et al., 2017a, Harr et al., 2017b; Leighton, 1986; Reynaud and Deschaux, 2006). Each of those factors could cause or contribute to immunosuppression, because the immune system is non-localized and complex. The potential for long-term immunosuppression encourages research into its mechanisms and consequences, as survival may be affected. Claims of decreased phagocytosis require further investigation, and tools more sensitive than organ weights, histopathology, and cell counts could provide valuable data (Briggs et al., 1996, Briggs et al., 1997).

Genotoxicity and carcinogenicity

The potential for petroleum exposure to damage DNA or cause cancer in birds has received very little research attention even though many PACs that may be in petroleum are genotoxic. Metabolically activated PACs or related reactive oxygen species may damage DNA, potentially causing DNA mutations or cancer (reviewed in Cooke et al., 2003; Xue and Warshawsky, 2005). To date, avian studies have not reported cancer or investigated genetic damage but have showed changes to DNA structure that may relate to gene regulation. These include effects on histone acetylation markers, global DNA methylation, and methylation of specific genes following exposure to petroleum, PAC mixtures, and individual PACs, respectively (Brandenburg and Head, 2018; Dorr et al., 2019; Toochaei et al., 2019). Hartung (1995) reviewed the carcinogenicity of spilled crude oil to wildlife briefly. Crude oil has not been shown to be carcinogenic in mammals. Should cancer tumors occur in birds as a result of petroleum exposure, the probability of detecting them in individuals is low. This is because chemically caused tumors tend to occur during the latter part of an animal's lifespan after prolonged latency, yet wildlife populations tend to have proportionally few old individuals and experimental exposures are brief. As Hartung (1995) cautiously concluded about birds impacted by *Exxon Valdez* crude oil, there is an absence of evidence for carcinogenicity from crude oil, though cancer would be hard to detect. At present, genotoxicity even in comparatively long-lived humans affected by oil spills is not well

understood (Laffon et al., 2016). Available assays for signs of DNA damage from ingested or inhaled petroleum PACs in birds could be informative.

Endocrine effects

Ingested contaminants can interfere with hormone signaling by affecting the secretion, synthesis, or catabolism of hormones, or affecting circulating hormones or target tissues (Scanes and McNabb, 2003). Petroleum can affect reproductive hormones, corticosterone signaling which may affect metabolism and the physiological stress response, and thyroid hormone signaling important to metabolism and growth. Because the role of hormone signaling includes controlling metabolism and stress responses necessary for daily survival, but also responses to life-threatening challenges like oil-fouled plumage, dysregulation of these systems from toxicity could be consequential to survival and reproduction.

Reproductive hormones

Several hormones regulate avian reproduction from copulation, to ovulation, to parental care, and early studies indicate numerous effects of petroleum on circulating hormones, fertility, and reproductive performance (reviewed in Leighton, 1993; Scanes and McNabb, 2003). Ducks fed 3 to 5 ml kg^{-1} bw d^{-1} crude oil in the diet showed decreased circulating progesterone, estradiol, estrone, prolactin, and luteinizing hormone levels and perturbations to their normal cycles that resulted in reduced reproductive performance, including laying, fertility, and behavioral changes (Cavanaugh et al., 1983; Cavanaugh and Holmes, 1982, Cavanaugh and Holmes, 1987; Harvey et al., 1981). More recent research extends these findings to oiled birds in the wild. A study in wild Magellanic penguins (*Spheniscus magellanicus*) with spilled crude oil on their plumage (20% SA) had low circulating androgens in both sexes and decreased luteinizing hormone and estradiol in females, which together with increased corticosterone and greater mass loss in females, may have been related to low rates of laying (Fowler et al., 1995).

Stress hormones

Several reviews all point to adrenal gland and corticosterone effects from oil exposure (Burger and Fry, 1993; Hartung, 1995; Jenssen, 1994; Leighton, 1993; Scanes and McNabb, 2003), which more recent studies support. Corticosterone is the circulating

glucocorticoid hormone in birds controlled by the hypothalamic-pituitary-adrenal (HPA) endocrine signaling axis which regulates energy in the body via glucose and fatty acid metabolism.

The adrenal gland that secretes corticosterone is particularly vulnerable to circulating toxins (reviewed in Hinson and Raven, 2006), and recent work substantiates evidence showing that this gland is a target of petroleum toxicity. As in older studies, petroleum exposure was associated with enlarged adrenal glands or interrenal cell hypertrophy (Bursian et al., 2017b; Simpson and Fisher, 2017). Though the incidence of adrenal lesions among exposed study organisms may be infrequent, perhaps apparent in only a few individuals, dose-dependent increases in the severity of lesions in western sandpipers (*Calidris mauri*) at doses where few other effects were observed (1 to 5 ml kg^{-1} bw d^{-1}) suggests that adrenal damage may be a primary toxic effect of ingested hydrocarbons, and not simply a secondary response to physiological stress from systemic toxicity (Bursian et al., 2017b).

Prolonged petroleum exposure is associated with effects on circulating baseline corticosterone in plasma, and long-term exposure may affect the acute (i.e. elevated) corticosterone stress response itself. There is evidence of elevated baseline plasma corticosterone in female Magellanic penguins (*Spheniscus magellanicus*) externally oiled (20% SA) for three weeks (Fowler et al., 1995), as well as adult female mallards (*Anas platyrhynchos*) repeatedly ingesting oil-sands process affected water over several weeks (Beck et al., 2014). Airborne oil sands contaminants inhaled repeatedly for several weeks can also affect baseline corticosterone concentrations, although diverging effects between treatments and species within the same experiment were apparent (Cruz-Martinez et al., 2015b). Those studies highlight both the potential for age and sex effects on circulating baseline corticosterone, and the potential for a hormetic response with respect to dose, time, or species. A hormetic response is likely given the homeostatic mechanisms of the HPA system, as well as data from older avian petroleum toxicity studies (reviewed in Busch and Hayward, 2009; Hartung, 1995; Hinson and Raven, 2006; Leighton, 1993). Corticosterone increases above baseline cycles trigger physiological and behavioral responses (i.e. acute stress response) to help overcome energy-demanding, unexpected challenges that arise (reviewed in Busch and Hayward, 2009). The acute stress response (corticosterone measured immediately after an acute stimulus) was depressed in house sparrows (*Passer domesticus*) after four weeks of

being fed a diet containing 1 ml kg^{-1} bw d^{-1} weathered crude oil (Lattin et al., 2014), a change associated with altered glucocorticoid receptor density in fat and liver (Lattin and Romero, 2014). No long-lasting effect on stress response was evident in little blue penguins (*Eudyptula minor*) three years after they were oiled, rehabilitated, and released (Chilvers et al., 2016).

The above studies, by examining circulating corticosterone titers in petroleum-exposure scenarios, and in particular the response to acute stress, add to older studies which showed 1) transient increases in circulating corticosterone with acute petroleum ingestion, 2) reduced adrenal corticosterone secretion, and 3) effects on baseline circulating corticosterone, often decreases, in birds ingesting petroleum over several days to months (Burger and Fry, 1993; Hartung, 1995; Jenssen, 1994; Leighton, 1993; Scanes and McNabb, 2003). Effects on corticosterone could affect baseline metabolism, immune function, or ability to overcome stressors successfully (reviewed in Briggs et al., 1997; Busch and Hayward, 2009), as well as behavior perhaps (reviewed in Hartung, 1995). Further targeted inquiry into the various components of the HPA axis and cross-talk with biotransformation systems could be useful for untangling the often diverging findings on baseline circulating corticosterone levels and how they may relate to metabolism, while additional study of the cause and consequence of decreased acute stress response would be insightful (e.g. Gorsline and Holmes, 1981, Gorsline and Holmes, 1982; Lattin et al., 2014; Wang et al., 2009). Although feather corticosterone is increasingly used in avian studies of glucocorticoids and stress, it has not proven a useful indicator of oil exposure to date (Cruz-Martinez et al., 2015a; Lattin et al., 2014).

Thyroid hormones

Petroleum contaminant exposure may affect avian thyroid hormone signaling. Thyroid hormones participate in hypothalamic-pituitary-thyroid (HPT) signaling and are critical to growth, development, metabolism, and thermoregulation, including interaction with the HPA and hypothalamic-pituitary-growth hormone (HPGH) axes (reviewed in Decuypere et al., 2005; Scanes and McNabb, 2003). Study of toxic effects on the HPT has mainly focused on the thyroid gland and concentrations of important hormones. Those include thyroid stimulating hormone (TSH) which is secreted by the pituitary gland and regulates thyroperoxidase-mediated synthesis of triiodothyronine (T3) and tetraiodothyronine (T4) in thyroid follicular cells for secretion into plasma. Fowles et al.

(2016) reviewed the potential for thyroid toxicity from exposure to raw and refined petroleum products with a combination of data from regulatory tests in mammals, human epidemiology, and wildlife studies. While most exposures showed low risk of thyroid toxicity, there were some exceptions, and certain PAC congeners present modulated *in vitro* mammalian thyroperoxidase activity. Until recently avian studies were few (reviewed in Leighton, 1993), and though the newly available information is difficult to interpret collectively, the weight of evidence indicates that thyroid toxicity from petroleum exposure cannot be ruled out.

A combination of effects on thyroid gland follicles, hormone concentrations, enzyme activity, and gene expression suggests a possible link between petroleum contaminant inhalation or ingestion and thyroid toxicity, though external oiling also modulates thyroid endpoints. Of the few studies that report on the thyroid gland in petroleum-exposed birds, no effect on thyroid mass was found following oral dosing with fuel oil in mallards (*Anas platyrhynchos*) over several days (Lee et al., 2012), nor histological lesions following oral dosing with weathered crude oil in double-crested cormorants (*Phalacrocorax auritus*) over several weeks (Harr et al., 2017b). In the latter experiment however, an external dosing trial (20% SA) yielded thyroid gland follicular hyperplasia. That finding, together with evidence of lower TSH and body mass associated with higher plasma ∑PAC concentrations in wild common murres (*Uria aalg*e) found oiled (Troisi et al., 2016), suggests that thyroid signaling effects may relate more to metabolic changes or stress than oral toxicity (Dorr et al., 2019; Harr et al., 2017b). The remainder of thyroid endpoint data relates to oil sands contamination of air and water. These laboratory and field studies with three taxa show effects on thyroid gland follicles, hormone concentrations in the thyroid gland or plasma (T3, T4, and T3 to T4 ratio), and hepatic enzyme activity converting T4 to T3 (Beck et al., 2014; Fernie et al., 2016, Fernie et al., 2019; Gentes et al., 2007a), although the endpoints affected and the direction of change vary considerably among studies. Age- and sex-specific effects on plasma hormone concentrations may exist (Beck et al., 2014). Finally, thyroid signaling effects are further supported by changes in the transcription of thyroid hormone related genes (e.g. *thrsp*) in embryo hepatocytes exposed to environmentally relevant PAC mixtures (Crump et al., 2017; Mundy et al., 2019). Additional thyroid gland and hormone studies that are more robust to potentially confounding environmental (site, year) and biological factors (hormesis, age, sex) are needed.

1.4.3. Embryotoxicity

Incubating birds that become oiled may contaminate their eggs (Albers, 1980; Parnell et al., 1984), and oil that seeps through the eggshell can cause embryotoxicity. Leighton addressed the embryotoxicity of petroleum oils extensively (Leighton, 1993). In short, microliter quantities of petroleum applied to the eggshell exterior caused embryo mortality in several taxa, for instance 3 to 19 µl egg^{-1} of various petroleum types in one study with chicken (Couillard and Leighton, 1991a). Such estimates may be conservative because mortality at hatching can be high (Finch et al., 2011; Franci et al., 2018), but studies often evaluate pre-hatch mortality for logistical and ethical considerations. Embryos in later stages of development are less sensitive to oiling (Couillard and Leighton, 1991b; Lewis and Malecki, 1984), although egg neglect from the temporary abandonment of oiled, incubating parents is more consequential (Butler et al., 1988). *In situ*, embryos would be exposed to oil that has experienced some degree of weathering. Substantially weathered oil is often less toxic to embryos than unweathered oil (Finch et al., 2011; Macko and King, 1980; Stubblefield et al., 1995c; Szaro et al., 1980). For example, weathered oil was 5- to 20-fold less toxic in one experiment (reviewed in Stubblefield et al., 1995a), probably due to a combination of chemical and physical changes (e.g. evaporation of lower molecular weight compounds and resulting increase in viscosity) (reviewed in Albers, 2006). Oil dispersant formulations can modulate embryotoxicity as well (Finch et al., 2012; Wooten et al., 2012).

Aromatic hydrocarbons that permeate the eggshell and their metabolites are responsible for the most toxic effects of petroleum *in ovo* (reviewed in Albers, 2006; Ellenton, 1982; Hoffman, 1978, Hoffman, 1979; Lee et al., 1986; Walters et al., 1987). Recent studies further characterize embryonic exposure from petroleum in the environment. Oiled eggs can absorb detectable three to five ring PACs (Goodchild et al., 2020), and volatile aromatics (e.g. BTEX) can penetrate the eggshell to induce CYP1A protein expression (Dubansky et al., 2018). Because PACs are readily biotransformed *in vivo*, whether the maternal transfer of ingested PACs into the egg can reach concentrations that are embryotoxic, as is the case for external oil contamination of eggs, remains unclear. One laboratory study showed that only minor amounts (<0.1%) of ingested three to five ring parent PACs are deposited into eggs, though hydroxylated metabolites often responsible for PAC toxicity were present in higher amounts (Fournier et al., 2010). In wild birds breeding in oil spill-impacted areas, petrogenic PAC residue

maternally transferred into the eggs is variable, though may reach considerable concentrations, as is shown in peregrine falcons (*Falco peregrinus*) (e.g. ∑PAC 21 to 461 ng g^{-1} ww) (Shore et al., 1999; Vidal et al., 2011; Zuberogoitia et al., 2006). Besides PACs, metals may also play a role in embryotoxicity, though fewer data related to petroleum exposure are available (Hoffman, 1979; Kertész and Hlubik, 2002). As the avian embryo is a common model for PAC toxicity, work is needed to put this laboratory data in a context of environmental relevance. Empirical measurements or estimates of the amounts of oil transferred to and absorbed by the eggs of incubating birds would be extremely valuable.

The harmful effects of petroleum exposure on embryos include growth, deformity, generalized edema, decreased fatty acid metabolism and necrosis in the liver, and changes to the spleen, kidney, and heart (Couillard and Leighton, 1989, Couillard and Leighton, 1990a, Couillard and Leighton, 1990b, Couillard and Leighton, 1991a, Couillard and Leighton, 1991b; reviewed in Leighton, 1993; Westman et al., 2013, Westman et al., 2014). Accumulating evidence points to aryl hydrocarbon receptor (AhR) agonism by PACs as an important mechanism of toxicity. Additionally, the physical blockage of gas exchange through the eggshell by oil covering sufficient surface area can cause hypoxia, which might explain findings of heart distention and decreased heart rate and metabolic rate, but cardiotoxicity could also be a factor (Couillard and Leighton, 1989, Couillard and Leighton, 1990a, Couillard and Leighton, 1990b; Goodchild et al., 2020; Harr et al., 2017b; Horak et al., 2017; Simpson and Fisher, 2017; Stubblefield et al., 1995c). If so, cardiotoxicity would be consistent with the adverse outcome pathway proposed for AhR-mediated mortality in early life stage organisms (Farhat and Kennedy, 2019). PACs are agonists of this receptor and its activation, measured as EROD activity, correlates with mortality (Franci et al., 2018). Related observations of generalized edema, increased heart mass, and potential effects on blood osmolarity support AhR-mediated mortality (Couillard and Leighton, 1989, Couillard and Leighton, 1990b; Dubansky et al., 2018). PACs injected into the egg are also shown to affect embryonic fatty acid metabolism and cause lesions similar to petroleum (Westman et al., 2013, Westman et al., 2014). Hemolytic injury, which can occur when petroleum is ingested at later life stages (Section 1.4.2) has not been documented in embryos (Dubansky et al., 2018; Kertész and Hlubik, 2002).

1.5. Emergent effects of petroleum exposure

Even where the molecular and physiological effects of petroleum exposure are not obvious or apparent, both external oiling and ingestion can result in effects on the individual that manifest in complex ways and on protracted timescales. Such emergent effects of oil contamination are evident in avian behavior, especially related to foraging and feeding, reproduction, and ultimately survival. In turn, emergent effects of petroleum contamination combined with acute mortality can contribute to population-level effects.

1.5.1. Behavior

External oiling or oil ingestion can affect the amount and type of certain routine behavioral activities, such as maintenance, feeding, and rest. Externally oiled birds attempt to remove petroleum from their feathers by preening or feather plucking (Camphuysen, 2011; Cunningham et al., 2017; Harr et al., 2017a; Hartung, 1963), and birds may spend more time preening and bathing, even after visible traces of petroleum are removed (Anderson et al., 2000; Burger and Tsipoura, 1998). Aggression, avoidance, and the overall amount of daily activity may be affected (Burger and Tsipoura, 1998; reviewed in Hartung, 1995). As a result, time spent resting or feeding may increase or decrease and lethargy is common, particularly in moribund birds (Anderson et al., 2000; Burger and Tsipoura, 1998; Cunningham et al., 2017; Holmes et al., 1979; reviewed in Leighton, 1993). Those observations come mainly from studies in captive animals. Such behavioral effects may occur in the absence of detectable adverse physiological effects. Accordingly, behavioral endpoints should be included in research studies. Tracking data (radio telemetry and GPS) from wild brown pelicans (*Pelecanus occidentalis*) that were oiled, rehabilitated, and released indicated that movement and habitat use were affected by some aspect of oil exposure or rehabilitation (Anderson et al., 1996; Lamb et al., 2018). One such study suggested that pelicans were perhaps choosing more profitable foraging areas over breeding grounds (Lamb et al., 2018). It is logical that feeding, resting, and maintenance behaviors, as well as aggression and risk-taking, would be affected by external oiling or oil ingestion and the ensuing stress or toxicity. Of course, in wild animals, ecological context mediates the broader behavioral consequences of petroleum exposure.

The ecological traits of a species can amplify or minimize the energetic costs of living with oil-fouled plumage; how those costs may vary among taxa that differ in traits such as feeding, roosting, and migrating has been observed and discussed extensively (Altwegg et al., 2008; Gartrell et al., 2019; reviewed in Hartung, 1995; Henkel et al., 2012; Henkel et al., 2014a; reviewed in Jenssen, 1994; Troisi, 2013; reviewed in Wiens, 1995; reviewed in Wolfaardt et al., 2009). Because of high conductive heat loss in water, reliance on marine or aquatic habitat greatly increases a species' vulnerability to the effects of oil-compromised plumage on thermal balance. Besides habitat, other ecological factors like ambient temperature and body size also govern thermal balance. To illustrate this with an example, the species most vulnerable to oiled plumage would be diving birds that occupy cold-water, pelagic habitats, roost on the water's surface, have a small body size, and have limited fat reserves (Altwegg et al., 2008; Gartrell et al., 2019; reviewed in Hartung, 1995; reviewed in Jenssen, 1994; Oka and Okuyama, 2000; Troisi, 2013; reviewed in Wiens, 1995; reviewed in Wolfaardt et al., 2009). By contrast, the least vulnerable birds would be those with larger body size resident in warm, terrestrial habitats. If oiled at sea, more terrestrial species may readily escape to land (Camphuysen, 2011; Munilla and Velando, 2010), whereas more pelagic species do so reluctantly (Camphuysen and Leopold, 2004; Duerr et al., 2016; Gartrell et al., 2019; Henkel et al., 2014b; Jauniaux et al., 1997). Obligate marine or aquatic birds are particularly affected by plumage oiling and may readily succumb to hypothermia or starvation (Jenssen, 1994). Beyond increased metabolic cost of thermoregulation, increased cost of transport for animals with oiled plumage would disproportionately affect birds where swimming or flight performance is critical to foraging, prey capture, predator escape, long-distance migration, or provisioning offspring (Culik et al., 1991; Maggini et al., 2017b, Maggini et al., 2017c; Perez et al., 2017a, Perez et al., 2017b).

Petroleum contamination can affect prey availability and where birds forage. Long-term ecological effects can result from large petroleum spills (reviewed in Esler et al., 2018). Although the cause of trophic shifts could be multifactorial and hard to identify, they could include top-down effects or decreased prey populations. Changes in prey availability (Moreno et al., 2013; Sanpera et al., 2008; Velando et al., 2005b) and habitat use (Banks et al., 2008; Day et al., 1995; reviewed in Esler and Bowman, 2000a, Esler et al., 2018) have been documented. Disturbance from clean-up activity may also impact habitat use (Burger, 1997; Henkel et al., 2014a). Decreased prey availability or

quality have been linked to reproductive effects (Golet et al., 2002; Moreno et al., 2013; Sanpera et al., 2008; Velando et al., 2005a, Velando et al., 2005b). Where birds themselves are exposed to oil, maintaining caloric intake with adequate prey seems important to overcoming the metabolic cost of oiled plumage and toxic effects (Esler et al., 2000b; Hartung, 1967; Horak et al., 2017; reviewed in Jenssen, 1994).

1.5.2. Reproduction

Mating and nesting success depend on attendance, demographics, and behavior at the breeding grounds, but petroleum exposure may adversely affect those processes either before or during breeding. Migration to breeding grounds or specific colonies is a life-history characteristic typical of birds. The physical and toxic effects of oil exposure at overwintering areas or en route (Esler et al., 2011, Esler et al., 2017; Gibson et al., 2017; Henkel et al., 2014a; Rattner et al., 1995; Seegar et al., 2015; Timoney and Ronconi, 2010) could affect the successful completion of migration or timely arrival in good condition for breeding, especially in long-distance migrants like shorebirds (Bianchini and Morrissey, 2018a, Bianchini and Morrissey, 2018b; Burger, 1997; Burger and Tsipoura, 1998; Bursian et al., 2017a; Henkel et al., 2012; Maggini et al., 2017a, Maggini et al., 2017b, Maggini et al., 2017c). The breeding grounds of petroleum-impacted populations may have fewer attending birds. Delay, absenteeism, emigration, and death affect pairing, nesting, and mate- and nest-site fidelity (Anderson et al., 1996; Camphuysen, 2011; Field et al., 1993; Walton et al., 1997; reviewed in Wiens, 1995; Wolfaardt et al., 2008c). Those may be influenced by other factors as well, including non-even sex ratio (Martínez-Abraín et al., 2006), or effects on physiologically-dependent sexual ornaments, such as the carotenoid-based red spot common to many species of gull, which can be affected by petroleum ingestion (Jaques et al., 2019; Pérez et al., 2010a, Pérez et al., 2010b). Petroleum exposure may affect breeding over multiple seasons; even experienced breeders may not breed consistently or at all (Anderson et al., 1996; Lamb et al., 2018; Wolfaardt et al., 2008a, Wolfaardt et al., 2008b, Wolfaardt et al., 2008c). Furthermore, oiling appears to increase the cost of reproduction, as exposed African penguins (*Spheniscus demersus*) that bred had lower survival than oiled non-breeders and unoiled birds (Wolfaardt et al., 2008a, Wolfaardt et al., 2008b). This research suggests an effect of oiling on fitness, but available long-term breeding behavior data on spill-impacted individuals exists only in penguins (Giese et al.,

2000; Sievwright et al., 2019b; Wolfaardt et al., 2008a, Wolfaardt et al., 2008b). More research in this area is needed, and modeling approaches, for instance, could be useful to predict effects on fitness. Also, more studies linking petroleum exposure at overwintering areas or on migration with effects at distant breeding grounds would be informative (e.g. Field et al., 1993).

For birds that do engage in breeding activity, the success of any breeding attempt will depend on successful laying and incubation, but adverse effects on those have been documented in association with spilled oil or exposed parents. Observational effects recorded among wild birds in petroleum-impacted areas include changes in clutch initiation timing, clutch size, egg abandonment, changes to eggshell volume and thickness, and reduced hatching success (Azkona et al., 2006; Velando et al., 2005b; Vidal and Domínguez, 2015; Zuberogoitia et al., 2006). Those recent findings are consistent with experimental evidence of effects on egg production, yolk deposition, eggshell thickness, fertility, egg rejection, abandonment, and hatching success (reviewed in Leighton, 1993). Eggshell thinning effects have been replicated in one more recent experiment at an environmentally relevant exposure rate with weathered crude oil (2.4 ml kg^{-1} bw d^{-1}), an effect concurrent with decreased blood Ca^{2+} in that experiment, as well a similar Ca^{2+} effect in laying wild female yellow-legged gulls (*Larus michahellis*) given small doses (0.04 ml kg^{-1} bw d^{-1}) of fuel oil (Alonso-Alvarez et al., 2007b; Stubblefield et al., 1995a, Stubblefield et al., 1995c). Hatching success is mediated in part by parental care, though embryotoxicity (Section 1.4.3) can be a factor.

The most ecologically informative measure of reproductive success is the number of recruited offspring, and indirect proxies for this, the number and condition of fledged offspring, can be reduced by oil contamination in the environment (reviewed in Leighton, 1993). Widespread absenteeism of oil-affected parents in the immediate aftermath of a spill may cause mass chick mortality (Crawford et al., 2000). Chick rearing effects (i.e. condition, fledging) may result from reductions in the amount or quality of prey with which parents provision chicks and can persist many years after a spill (Barros et al., 2014; Golet et al., 2002; Moreno et al., 2013; Sanpera et al., 2008; Trivelpiece et al., 1984; Velando et al., 2005a, Velando et al., 2005b). Studies have indicated slower chick growth (Andres, 1999) and reduced chick condition and fledging success (Azkona et al., 2006; Bergeon Burns et al., 2014; Golet et al., 2002; Velando et al., 2005a, Velando et al., 2005b) in petroleum-impacted populations. Oiled and rehabilitated

penguins have also shown decreased fledging success (Barham et al., 2007; Giese et al., 2000; Wolfaardt et al., 2008c), a longer fledging period (Barham et al., 2007), and lighter chicks at fledging (Giese et al., 2000). In those penguins, the sex of the oil-affected mate of the pair affected fledgling growth rate or overall success rate, and both parents being affected by oil was additive (Giese et al., 2000; Wolfaardt et al., 2008c). While lower food intake alone can explain growth and fledging effects, there is good evidence that oil ingestion by chicks reduced growth in some cases (Gurney et al., 2005; reviewed in Leighton, 1993), and industrial PAC contamination in the environment has been associated with non-predation nestling mortality (Bustnes, 2013; Gentes et al., 2006), suggesting that toxicity could be involved in effects on chicks.

Research indicates considerable variation in the severity and type of reproductive impacts on breeding success, even among apparently similar oil spill scenarios (reviewed in Wolfaardt et al., 2009). Reproductive effects are not always present or apparent following major oil spills (Altwegg et al., 2008; Andres, 1999; Burger, 2018; Camphuysen, 2011; Sievwright et al., 2019b) and in areas with considerable petroleum industry activity such as the oil sands (Cruz-Martinez et al., 2015a; Fernie et al., 2018b; Godwin et al., 2019). Breeding phenology and degree of contamination are important, and bird species which can forage over larger areas or on more diverse types of prey may be more resilient to toxic or ecological effects in petroleum-impacted habitats (Andres, 1999).

1.5.3. Survival

Information on how the degree of petroleum exposure relates to the ultimate fate of free-living individuals remains incomplete, but available examples highlight the importance of ecological factors and show potentially decreased survival from external oiling or ingestion of residual hydrocarbons after a spill. Of course, mass avian mortality, especially of marine taxa, during the initial days and weeks of large oil spills have been well-documented (e.g. reviewed in Burger and Fry, 1993; Bursian et al., 2017a; Crawford et al., 2000; Esler et al., 2018). Many studies report the total number of individuals killed, though these are estimates and subject to debate. For birds that escape oiling from floating petroleum during the initial phases of a spill, exposure to residual oil hydrocarbons at impacted areas may persist for many years (e.g. 2 to 20) (Esler et al., 2010, Esler et al., 2011, Esler et al., 2017, Esler et al., 2018; Golet et al., 2002; Trust et

al., 2000; Velando et al., 2010) and can decrease survival in those areas (Esler et al., 2000b; Iverson and Esler, 2010). Few studies report on the survival of living birds shown to be exposed to oil in the wild, and still fewer report survival where exposure is quantified in some way.

As discussed earlier, external oiling can be catastrophic for heavily oiled birds, though smaller amounts of petroleum may still be immediately life threatening by primarily affecting thermal balance (Section 1.4.1). Lethal thresholds for thermal balance effects are complicated by the particularities of environmental conditions and species, and work on this is still needed. It is known that the amount of oil found on dead birds may vary widely, completely saturating the feathers of some individuals, while on other individuals insubstantial amounts of oil (e.g. 6.3 to 21.5 ml kg−1 bw; 10% SA and above) are found on birds showing signs of death consistent with thermal dysregulation, though toxicity may contribute (Balseiro et al., 2005; Fry and Lowenstine, 1985; Hartung, 1963; Oka and Okuyama, 2000). Even if birds initially survive with only minor portions of their plumage affected by oil, long-term survival may still be decreased. In African penguins (*Spheniscus demersus*) with heavy fuel oil contaminating their feathers, those with 5% SA oiled or more were significantly less likely to be re-sighted in the next five years in a dose-dependent fashion, and none of the birds with oil penetrating to the skin were re-sighted (Wolfaardt et al., 2008b). Those observations may reasonably support the widespread assumption that survival is decreased from even light oiling and that heavy oiling is fatal in pelagic marine taxa. Yet, available data for more terrestrial species shows that survival in visibly oiled gulls and plovers is not lower than normal (Camphuysen, 2011; Gibson et al., 2017).

For birds ingesting petroleum by preening or through the diet, the weight of evidence from an abundance of captive oral dosing studies under normal husbandry conditions indicates that birds ingesting realistic amounts (e.g. <5 ml kg^{-1} bw d^{-1}) of petroleum over abbreviated time periods without oil-compromised plumage are likely to survive in the short term (Table 1.3), although ingestion rates remain inadequately characterized (Section 1.6.1). Most studies documented survival following exposure to petroleum amounts as high as 5 to 20 ml kg^{-1} bw d^{-1} for one to seven days (Dean et al., 2017b; Newman et al., 1999; Stubblefield et al., 1995b) or amounts up to 2.4 to 10 ml kg^{-1} bw d^{-1} for several weeks (Bursian et al., 2017b; Stubblefield et al., 1995b, Stubblefield et al., 1995c), but two dosing studies in captive adult birds do point to death

after oral exposure to 5 to 10 ml kg^{-1} bw d^{-1} of crude oil over 6 to 7 or more days (Cunningham et al., 2017; Horak et al., 2017). For birds in general, the rates of ingestion which are actually lethal probably ranges from a few to tens of ml kg^{-1} bw d^{-1} over at least several days for most bird species, a dose-response best characterized as a threshold effect (reviewed in Hartung, 1995). Differences in sensitivity among taxa are evident from multi-species studies using the same petroleum and comparable methodologies (Dean et al., 2017b). This may be due in part to moderate interspecies variation in sensitivity to PACs, as has been documented with embryonic exposures (Crump et al., 2017; Franci et al., 2018; Head et al., 2015; Mundy et al., 2019).

Importantly, the presence of factors such as age, stress, or food availability could affect lethal toxicity thresholds (Holmes et al., 1978; Horak et al., 2017). A number of older studies point to increased toxicity of ingested oils in young birds or in the presence of other stressors. While this information has been mentioned elsewhere (reviewed in Jenssen, 1994; Leighton, 1993), its importance merits emphasis. For instance, in ducks kept in crowded, cold conditions thought to be stressful, the LD50 of single oral doses of refined oils dropped dramatically (e.g. from 24 to 4 ml kg^{-1} bw for diesel fuel) (Hartung and Hunt, 1966). Analogous increases in mortality were apparent in experiments where ducks fed otherwise sublethal amounts of petroleum were stressed with saline water and cold temperatures, or a bacterial challenge (Holmes et al., 1978, Holmes et al., 1979; Rocke et al., 1984). Furthermore, workers at rehabilitation centers have made the anecdotal observation that common murres (*Uria aalge*) with oiled plumage are unable to survive clinically low hematocrit and plasma protein as unoiled murres commonly do, suggesting a decreased resilience to physiological crisis (Duerr et al., 2016). Exposure to low levels of petroleum contamination in the environment may thus lead to decreased survival when birds are faced with physiologically precarious situations (Esler et al., 2000b; Gentes et al., 2006), and we have described previously the ways that petroleum exposure can affect food intake, body mass, thermoregulation, and the corticosterone-mediated acute stress response via the HPA hormone axis. While dosing studies show that birds are likely to survive estimated rates of *in situ* petroleum exposure under normal conditions in captivity, that resilience to environmentally realistic amounts of ingested petroleum may quickly vanish in the presence of environmental stressors, and ingested petroleum may cause or contribute to death in birds that are unable physiologically to handle a stress-inducing, life-threatening challenge.

Table 1.3 **For each of the various body systems affected by petroleum toxicty, we looked through the adult oral dosing studies published since 1993 for lowest observed effect levels for endpoints related to that body system and reports of mortality. This table reports nominal doses.**

Outcome	Experimental endpoint	Species	Oil class	Dose range (ml kg^{-1} bw d^{-1})	Treatment duration (d)	Lowest observed effect level (ml kg^{-1} bw d^{-1})	Lowest lethal dose[a]		Reference
							Dose (ml kg^{-1} bw d^{-1})	Mortality occurrence (d)	
Gastrointestinal damage	Pathological lesions	*Phalacrocorax auritus*	Crude	5–10	21	5	10	7–14[b]	(Harr et al., 2017b)
Osmoregulation and excretion	Increased kidney mass	*Phalacrocorax auritus*	Crude	5–10	21	5	10	7–14[b]	(Harr et al., 2017b)
	Plasma Ca^{2+}	*Larus michahellis*	Fuel	0.04	7	0.04	–	–	(Alonso-Alvarez et al., 2007b)
	Serum Ca^{2+}	*Anas platyrhynchos*	Crude	0.02–2.4	70	2.4	–	[c]	(Stubblefield et al., 1995c)
Metabolic effects and the liver	Body mass	*Leucophaeus atricilla*	Crude	5–10	28	5	5	7–28[b]	(Horak et al., 2017)
		Calidris mauri	Crude	1–5	20	5	–	[b]	(Bursian et al., 2017b)
	Liver mass	*Calidris mauri*	Crude	1–5	20	5	–	[b]	(Bursian et al., 2017b)

Outcome	Experimental endpoint	Species	Oil class	Dose range (ml kg^{-1} bw d^{-1})	Treatment duration (d)	Lowest observed effect level (ml kg^{-1} bw d^{-1})	Lowest lethal dose[a]		Reference
							Dose (ml kg^{-1} bw d^{-1})	Mortality occurrence (d)	
	Plasma glucose	*Larus michahellis*	Fuel	0.04	7	0.04	–	–	(Alonso-Alvarez et al., 2007b)
	Glucocorticoid receptor density	*Passer domesticus*	Crude	1.1	28	1.1	–	–	(Lattin et al., 2014)
Oxidative balance and damage	Plasma vitamin E	*Larus michahellis*	Fuel	0.04	7	0.04	–	–	(Pérez et al., 2010a)
	Hepatic total antioxidant capacity	*Calidris mauri*	Crude	1–5	20	1	–	[b]	(Bursian et al., 2017b)
	Hepatic total, oxidized, and reduced glutathione	*Leucophaeus atricilla*	Crude	5–10	28	10	5	7–28[b]	(Horak et al., 2017)
Hemotoxicity	Decreased hematocrit and reticulosis	*Phalacrocorax auritus*	Crude	5–10	21	5	10	7–14[b]	(Harr et al., 2017b)

Outcome	Experimental endpoint	Species	Oil class	Dose range (ml kg^{-1} bw d^{-1})	Treatment duration (d)	Lowest observed effect level (ml kg^{-1} bw d^{-1})	Lowest lethal dose[a]		Reference
							Dose (ml kg^{-1} bw d^{-1})	Mortality occurrence (d)	
Cardiotoxicity	Gross pathology of cardiac structure	*Phalacrocorax auritus*	Crude	5–10	21	5	10	7–14[b]	(Harr et al., 2017b)
Immunotoxicity	Relative spleen size	*Anas platyrhynchos*	Crude	0.02–2.4	70	2.4	–	[c]	(Stubblefield et al., 1995c)
Endocrine effects	Adrenal interrenal hypertrophy	*Calidris mauri*	Crude	1–5	20	1	–	[b]	(Bursian et al., 2017b)
	Acute corticosterone stress response	*Passer domesticus*	Crude	1.1	28	1.1	–	–	(Lattin et al., 2014)

[a] We considered the lowest lethal dose to be 10 ml kg^{-1} bw d^{-1} for Harr et al. (2017b) because only one out of nine birds died in the 5 ml kg^{-1} bw d^{-1} treatment, inconsistent with the pattern of mortality from toxicity observed with the other birds.

[b] Another similar experiment showed no mortality with doses up to 20 ml kg^{-1} bw d^{-1} for 5 days in this species (Dean et al., 2017b).

[c] Another similar experiment showed no mortality with doses up to 9.5 ml kg^{-1} bw d^{-1} for 14 days in this species (Stubblefield et al., 1995b).

Data on the fate of oiled birds that had been rescued in large numbers, cleaned in rehabilitation centers, and released shows lower long-term survival in some cases that may relate to some aspect of petroleum exposure. During care, oiled birds undergoing rehabilitation do not necessarily fair worse than birds rescued for non-oil related reasons (Gartrell et al., 2019; Montesdeoca et al., 2017; Parsons et al., 2018), though mortality rates may be high (Duerr et al., 2016). Post-release survival in controlled studies (i.e. compared to unoiled wild birds kept temporarily in rehabilitation settings as a control group) was substantially lower over the tracking period in some cases, but no different in other cases (reviewed in Henkel and Ziccardi, 2018; Sharp, 1996; reviewed in Sievwright et al., 2019a; Underhill et al., 1999; reviewed in Wolfaardt et al., 2009). Such considerable variation in post-release survival appears related to species and scenario. Individuals of more resilient species may live out their expected lifespan (reviewed in Wolfaardt et al., 2009), while low post-release survival in other species may relate to petroleum exposure.

Survival often differs by age class or sex in petroleum-impacted bird populations. Sex- and age-specific effects on mortality or survival rates are evident in many studies (Anderson et al., 1996; Azkona et al., 2006; Balseiro et al., 2005; Camphuysen and Leopold, 2004; Duerr et al., 2016; Jauniaux et al., 1997; Martínez-Abraín et al., 2006; Nevins and Carter, 2003; Votier et al., 2008). Those observations often were attributed to different use of habitat. For instance, the carcasses of European shags (*Phalacrocorax aristotelis*) thought to be killed by oil from the Prestige wreck pointed to disproportionately higher mortality among juveniles and females in spill-affected waters compared to adult males that attend the breeding colony earlier to stake and defend nesting territory (Martínez-Abraín et al., 2006). Although sex- or age-specific mortality is usually attributed to differences in habitat use, it is also possible that individuals with smaller body sizes, such as sub-adults or females, may suffer greater effects from exposure to a given amount of petroleum (Camphuysen and Leopold, 2004; Fowler et al., 1995). How such differences in sensitivity among demographic classes relate to survival remain incompletely known despite numerous dosing studies. Mortality-driven demographic shifts may subsequently affect population dynamics.

1.5.4. Populations

Mortality from petroleum spills can reduce bird populations and long-term effects, direct or indirect, may prolong population recovery. Although an examination of mortality estimates and population dynamics over time is beyond the scope of this review, it bears mentioning that the slow intrinsic population growth common to seabirds can put their populations at risk from large mortality events (reviewed in Morandin and O'Hara, 2016). Indeed, the *Exxon Valdez* crude oil spill heavily impacted marine bird populations, which recovered to pre-spill numbers only after several decades, if at all (Esler et al., 2018). Beyond acute mortality among the general population, age- or sex-specific mortality (above paragraph) and sub-lethal effects (Alonso-Alvarez et al., 2007b; Giese et al., 2000; Wolfaardt et al., 2008c), as well as long-term reproductive effects (Barros et al., 2014; Velando et al., 2005a) could each affect population size and structure, and thereby constrain population size or growth. Continued exposure to residual oil or changes in prey availability can be important factors (Barros et al., 2014; Esler et al., 2018; Moreno et al., 2013; Sanpera et al., 2008; Velando et al., 2005a, Velando et al., 2005b).

1.6. Research priorities: bridging knowledge gaps and forging ahead

1.6.1. Where are the exposure estimates?

Despite an abundance of oral toxicity studies, there is a glaring scarcity of estimates for how much petroleum birds that are free to feed and preen actually ingest over time, and more research is profoundly needed here. In this literature review, we found only one paper since 1993 that provided a new estimate of oral ingestion rate. In that work, hydrocarbon residue in bivalve mussels was used to estimate that sea ducks feeding on the most contaminated mussels during the height of the *Exxon Valdez* spill ingested 0.14 ml kg^{-1} bw d^{-1} of crude oil (Hartung, 1995) (online article supplementary data Exposure Estimate 2). A further examination of pre-1993 works yielded only one other primary study estimating ingestion rate. That pioneering and often-cited, though extremely limited, study with three ducks showed that a bird with 16.6 ml kg^{-1} bw of oil on its plumage would consume up to 3.32 ml kg^{-1} bw d^{-1} from preening (Hartung, 1963) (online article supplementary data Exposure Estimate 1). So, even when birds may be

substantially oiled themselves and feeding on contaminated prey, it is hard to imagine ingestion rates far beyond 4 ml kg^{-1} bw d^{-1} or persisting at this level over more than a few days without surreptitious external exposure. Again, data on the duration of exposure expected are also needed. Many conventional oral dosing studies reviewed herein have employed doses, including 'low' doses, well above 4 ml kg^{-1} bw d^{-1}, often over multiple weeks. Until petroleum ingestion rates are more thoroughly characterized, the relevance of toxic effects described at such substantial doses remains uncertain and those studies may be best considered as worst-case scenario examples. Related to the need for ingestion rate data, quantitative data linking surface area of plumage appearing oiled, what observers actually see in the field, to the amount of oil affecting plumage (ml kg^{-1} bw) would be extremely useful for modeling plumage effects, ingestion from preening, and ultimately survival.

An important caveat is that sublethal effects may occur well within the range of environmentally relevant ingestion rates (e.g. 4 ml kg^{-1} bw d^{-1}), as shown by the lowest observed effect levels for endpoints relevant to the body systems affected by petroleum toxicity among the studies we reviewed (Table 1.3). For instance, exposures within this range can cause effects on circulating calcium and eggshell thinning (2.4 ml kg^{-1} bw d^{-1}), plasma glucose (0.04 ml kg^{-1} bw d^{-1}), hepatic antioxidants (1 ml kg^{-1} bw d^{-1}), corticosterone stress response (1.1 ml kg^{-1} bw d^{-1}), and others (Alonso-Alvarez et al., 2007b; Bursian et al., 2017b; Lattin et al., 2014; Stubblefield et al., 1995c).

After nearly 60 years of petroleum dosing studies, the need for better estimates of what constitutes realistic petroleum exposure seems to far outweigh the need for further conventional laboratory oral dosing studies. When one considers that high rates of oral ingestion are only expected in heavily oiled birds, and that the physical and metabolic effects of heavy external oiling alone are disastrous (Hartung, 1967), the use of conventional lethal oral dosing studies and high doses (i.e. 10 to 20 ml) are nearly unjustifiable, at least until their relevance is better demonstrated. Thus, refined and broadly relevant estimates of environmentally realistic ingestion rates, and how concurrent stressors may modify toxicity, are a higher research priority. Related to this, emerging data showing the potential toxicity of airborne petroleum compounds associated with spills at sea or industrial activity (e.g. oil sands) highlights a need for putting air contaminant measurements in an avian exposure context (Cruz-Martinez et

al., 2015a, Cruz-Martinez et al., 2015b; Dubansky et al., 2018; Fernie et al., 2016; Olsgard et al., 2008).

1.6.2. Unconventional crude oil

Unconventional crude petroleum types, though increasingly common, have not been studied for their direct toxicological effects in avifauna, as existing research has focused exclusively on the effects of oil sands process-affected materials and airborne contaminants in the environment near Canadian oil sands mining and processing industrial activity. Oil sands process-affected water has received substantial research attention in particular, work that Beck et al. (2015) has reviewed, concluding that adverse effects, including growth and reproduction, thyroid and stress hormone function, and immune function were weak at best or equivocal and inconsistent between years. Since then, unconventional crude oil research has focused on airborne oil sands industry contaminants that may be inhaled or deposited into aquatic ecosystems. These do show some evidence of toxicity in laboratory and field studies, though field studies with replicate reference sites and years are needed in many cases. *In vitro* hepatocyte exposures with PACs atmospherically deposited to lakes in the oil sands region or found in petroleum coke, a thermal hydrocracking by-product of bitumen upgrading subject to wind erosion, both affect the expression of genes associated with toxicity (Crump et al., 2017; Mundy et al., 2019). Laboratory and field studies in nestling and adult birds report some indications of immune (Cruz-Martinez et al., 2015a, Cruz-Martinez et al., 2015b; Olsgard et al., 2008, Olsgard et al., 2009), thyroid (Fernie et al., 2016, Fernie et al., 2019), and stress hormone (Cruz-Martinez et al., 2015b) effects in association with exposure to airborne or atmospherically deposited oil sands industry contaminants. However, available data shows no consistent reproductive effects in field studies (Cruz-Martinez et al., 2015a; Fernie et al., 2018b; Godwin et al., 2019), and no evidence of decreased survival exists.

Despite the above research suggesting avian toxicity from petrogenic oil sands contaminants in environmental matrices, the toxicity of direct avian exposure to bituminous oil sands crude petroleum and how it may compare to conventional crude petroleum remains unknown. Direct exposure to bulk petroleum occurs when birds such as migratory waterfowl contact residual bitumen in the sediment or floating on the surface water of tailings ponds at industrial sites (reviewed in Beck et al., 2015; Timoney

and Ronconi, 2010). Exposure may occur from accidental spillage along transportation routes as well. At present, the transportation of bituminous petroleum to distant areas and overseas by tanker vessel are projected to increase (NEB, 2016b). As this oil sands example demonstrates, how unconventional petroleum types compare in toxicity to the current understanding of avian petroleum toxicity remains an open question. Yet, it is important to answer in order to adequately assess and manage risk to wildlife resources.

1.6.3. Towards an improved avian model of petroleum effects

Studies on the effects of petroleum exposure on birds are valuable, though technically challenging, and barriers to the replication and broader scientific application of research results abound. There are methodological obstacles related to quantifying exposure, delivering doses, and potential experimental artefacts. Design and reporting deficiencies often limit the interpretability and applicability of a given study. Given these pitfalls, what is the prudent empiricist to do? Fortunately, the available literature contains many good examples and instructive lessons. Careful study design, improved reporting standards, and novel tools and approaches will benefit this area of research, each of which we discuss below. To enhance comparability among studies, we propose a list of minimum information and design criteria for publishing avian petroleum toxicity studies (Table 1.4).

Table 1.4 In order to make experiments replicable and their results more broadly relevant, the following should constitute the minimum information and design criteria for publishing avian petroleum toxicity studies. Some items are essential (E), while others are at least desirable (D).

Design aspect	Criteria	Importance
Toxicant	Type of petroleum and source	E
	Age and storage conditions	D
	Degree of weathering, weathering conditions, percent mass or volume loss	E
	The experimental relevance of weathering	E
	Petroleum density	E
	Petroleum viscosity	D

Design aspect	Criteria	Importance
	Replication of petroleum sample(s) used (e.g. replicate samples of a spilled crude oil, or samples of from multiple production fields and seasons, composited samples, statistical design)	D
Test organism	Species name	E
	Average body mass	E
	Life stage	E
	Controls for sex-specific effects	D
	Pre-experiment baseline data for endpoints of interest	D
	Domestic birds:	
	Husbandry conditions	E
	Strain	D
	Generations in captivity	D
	Any prior exposure to the toxicant	E
	Wild birds:	
	Source population	E
	If brought into captivity, duration and husbandry conditions	E
Oral doses	Administration method (e.g. oil-in-food, gelatin capsule, slurry gavage, neat)	E
	Appropriate controls (e.g. food-grade mineral oil)	E
	Range of at least two or more doses	D
	Dose amounts as g or ml per kg body weight, per day if over multiple days (e.g. g kg^{-1} bw d^{-1} or ml kg^{-1} bw d^{-1})	E
	Report any regurgitation and defecation that could affect exposure	E
	Pre-dose fasting duration	D
	Doses per day	D
	Explanation of environmental relevance of dose	E
External application	Percent surface area oiled and whether this is with folded or stretched wings	D
	Application amounts as g or ml per kg body weight (e.g. g kg^{-1} bw or ml kg^{-1} bw)	E

Design aspect	Criteria	Importance
Test conditions	Any reapplication or cleaning	E
	Temperature	E
	Duration of exposure	E
	Time elapsed from treatment to sampling	E
Embryotoxicity	Average initial fresh egg mass	E
	Dosage on a µl or mg per g egg basis (e.g. µl g^{-1} egg)	E
	Percent egg surface area oiled	D
	Appropriate nontoxic oil control	E
	Whether the embryos could be determined to be alive when the dose was applied	E
	Age at sampling	E
	Hatching success endpoint	D
Endpoints	All endpoints evaluated and their result, even if not significant	E
	Effect sizes	D
	Variability	D
	Power	D
	Context of clinical or literature reference values	D

Among petroleum dosing studies, major barriers to interpreting, replicating, and applying results to other exposure scenarios are caused by insufficient information about or replication of the toxicant. A type of petroleum such as fuel oil or crude oil is more a category than an invariable substance. The composition of, for example, a given crude oil can vary among production fields (e.g. Prudhoe Bay versus South Louisiana), and weathering is important to toxicity (Couillard and Leighton, 1991a; Finch et al., 2011; Macko and King, 1980; Stubblefield et al., 1995a; Szaro et al., 1980). Though common, it is inherently problematic to use a single can of petroleum from a particular source in a battery of laboratory studies to predict the effects of a category of petroleum on an entire species or birds in general. Detailed information about the petroleum, such as what year and season it was produced, and storage conditions are essential (DFO, 2017; Lee et al., 2015). Beyond those details, results are more broadly applicable if the experimental

design accounts for variation in the petroleum's exact composition. That could include using composite petroleum samples, using the petroleum sample as the unit of replication or treatment group, or using statistical models that account for petroleum sample.

Quantifying the amount of petroleum on plumage or that birds ingest has always been a challenge, but can be addressed. Quantifying visible oil on plumage is difficult, subjective, and has no universally recognized standard methodology. Oil scoring methods reported in research papers are seldom comparable, although good examples of scoring methods exist (Camphuysen, 2011; DHNRDA Trustees, 2016), and study skins can be useful (e.g. Cunningham et al., 2017; Maggini et al., 2017b). Oil washed from plumage can be quantified, and methods like Hartung's (1963) would be valuable to further quantify oil ingestion rates. Even in oral exposures, the net amount ingested may be somewhat uncertain because petroleum can be excreted or regurgitated within minutes, making the amount available for absorption less certain (Cunningham et al., 2017; Dean et al., 2017b). Delivery methods can be optimized (e.g. incorporating into food items or slurry) but these, in turn, can affect toxicity. For instance, oil that is administered neat via gavage or in gelatin capsules is more toxic than when incorporated into the diet (reviewed in Hartung, 1995). Employing low doses and routes (e.g. food items) that are environmentally realistic is one obvious solution.

Consistent reporting of critical exposure information like animal weight, oil density, and analytical chemistry methodology enables ready comparison among studies and can facilitate replication of results, meta-analyses, literature reviews, and risk assessments. Petroleum doses are usually given on the basis of grams or milliliters per unit body weight per day (e.g. ml oil kg^{-1} bw d^{-1}). Doses on a body weight basis, or some indication of average animal body mass, are necessary to compare among studies accurately. Like body mass, egg size varies among taxa. Egg oiling studies should measure the initial fresh weight of eggs used and include petroleum application data on a µl g^{-1} egg or mg g^{-1} egg basis, as well as the proportion of egg surface area affected (e.g. Finch et al., 2011). To convert between oil weight and volume, petroleum density is necessary. Dose and density data must be reported in future studies. Lastly, chemical analysis of PAC residue, especially in avian tissue, is extremely valuable. Unfortunately, there is often insufficient analytical information to compare the data between studies. PAC concentration data would be more beneficial to other researchers if only slightly

more information were reported in supplementary files, including target PAC analytes, their detection limits, whether concentrations are given on a wet or dry weight basis, and relevant quality control and quality assurance data. Fortunately, the aforementioned problems found commonly in the literature are easily corrected.

Animal studies are useful, where ethically justifiable, and researchers can benefit from appreciable consistency in the literature regarding experimental conditions and the choice of species used. Some standard toxicity test methods have been successfully adapted to petroleum (Stubblefield et al., 1995a). The current versions of those may be a useful framework (ASTM, 2019; OECD, 1984a, OECD, 1984b, OECD, 2016; USEPA, 1996, USEPA, 2012a, USEPA, 2012b, USEPA, 2015) and we recommend other good experimental designs in the peer-reviewed literature that can serve as a template (e.g. Couillard and Leighton, 1989; Cunningham et al., 2017; Dean et al., 2017b; Kononen et al., 1986; Maggini et al., 2017c; Perez et al., 2017a). Guidelines exist for aquatic petroleum toxicity studies, many of which are applicable to avian research (DFO, 2017). Because petroleum can exert effects via the physical fouling of plumage or blockage of eggshell gas exchange, toxicity studies should use non-toxic control substances with matching physical properties (e.g. vegetable oil) to attempt to separate physical effects of oil from actual toxicity. In terms of test organisms, studies have historically relied on the mallard (*Anas platyrhynchos*), wild caught or domestic, as the laboratory model of choice for petroleum toxicity studies. Wild caught double-crested cormorant (*Phalacrocorax auritus*), and captive-bred zebra finch (*Taeniopygia guttata*) and homing pigeon (*Columba livia*) have also proven to be tractable models (Cunningham et al., 2017; Goodchild et al., 2020; Perez et al., 2017c). While each of these animal models has its respective advantages, studies should make use of species most relevant to the ecological context of the research question at hand.

Experimental artefacts from the handling or captivity of birds may affect results and their interpretability, which should be taken into account. Handling can affect plasma biochemistry related to stress and markers of muscle injury (Chilvers et al., 2016; Dean et al., 2017b; Lattin et al., 2014), and captivity can affect the biology of wild birds, for instance causing anemia (Burco et al., 2014; Cunningham et al., 2017; Dannemiller et al., 2019; Dean et al., 2017a; Newman et al., 1999, Newman et al., 2000). For wild birds studied in captivity, appropriate acclimatization period, sampling methods, pre-treatment baselines, and statistical procedures are warranted.

Researchers need not confine avian petroleum ecotoxicology studies to captive birds. The realism of field experiments done *in situ* is invaluable. Homing flight studies in domestic pigeons (*Columba livia*) have proven informative and there are many examples in truly wild birds (Alonso-Alvarez et al., 2007b; Butler et al., 1988; Perez et al., 2017a, Perez et al., 2017b, Perez et al., 2017c). Future studies could take advantage of advances in tracking device technology (e.g. Perez et al., 2017c). Wild birds almost certainly work harder to move and forage on their own and are without the safety net of ad libitum access to food in captivity. Furthermore, *in situ* studies integrate important behavioral effects and ecological factors, and if desired, the effects of plumage fouling on flight performance and metabolism that simplified studies on oral toxicity necessarily exclude. Thus, field experiments provide an essential bridge between the results of conventional laboratory dosing studies and effects on free-living birds which may be exposed to petroleum at lower levels but face the challenges of survival in the wild.

While there are many studies on oiled birds that were rescued, rehabilitated, and released, many do not include the necessary controls to distinguish the effects of oiling from handling and captivity. Where this is the case, any inference of oil-related effect should be avoided or made cautiously. Data on pre-spill baselines, effects in oil exposed animals left unaided, and unoiled control groups receiving the same rehabilitation regimen would make studies in rescued birds more valuable, and good examples do exist (e.g. Golightly et al., 2002; Wolfaardt et al., 2008b).

Petroleum toxicity is systemic, affecting multiple body systems in subtle ways rather than an obvious or localized common mode of action, yet there is a chance that recent technological advances may further improve our understanding of the mechanisms at work. Advances in the study of gene expression that measures RNA within tissue and the emerging field of omics are particularly promising (reviewed in Gonzalez and Pierron, 2015). For example, gene expression analysis methods employ quantitative PCR (qPCR) to look at transcript abundance of genes of interest within a tissue, for instance the *cyp1a4* and *cyp1a5* genes that code for their respective CYP1A phase I biotransformation proteins. Gene expression with qPCR is useful for examining one or a few genes of interest, examining a suite of genes involved in a single pathway, or high-throughput gene arrays which employ a panel of genes, such as genes commonly implicated in adverse outcome pathways (Crump et al., 2016, Crump et al., 2017). RNA sequencing (RNA-Seq) is a transcriptomic tool that determines the

sequence and quantity of thousands of RNA strands or whole transcriptomes present in a sample, a method called transcriptional profiling. Transcriptional profiling (i.e. gene expression profiling) and differential gene expression can be used without a sequenced genome or known target sequences (Grabherr et al., 2011). By examining the abundance (often relative abundance) or distribution of RNA which is transcribed into proteins within the cell, qPCR and RNA-Seq offer the molecular tools to look at the effects of petroleum exposure which are the most upstream in a pathways sense. Other analogous omics tools are proteomics, which examines the abundance of proteins within tissue, and metabolomics which typically focuses on metabolites within a tissue or in circulation (Dorr et al., 2019). Collectively, qPCR, RNA-Seq, and their related omics approaches can offer snapshots of biological processes that span several levels of organization from the transcriptome to the metabolome. A caveat is that the resulting datasets can be huge and require bioinformatic methods to analyze. Applied astutely, these molecular methods may advance avian petroleum toxicology research by elucidating effects on biochemical pathways underlying the known physiological effects of toxicity.

High volume oil spills can be catastrophic to birds, yet one challenge to studying the effects of large spills is the urgent and often ad hoc nature of the response to wildlife resource damage. Based on what has been learned from past spills, including the large body of research we have synthesized in this review and older studies (Leighton, 1993), we recommend strategies for researchers and decision makers to aid the assessment of damage and recovery, while ensuring that data from the incident will further the scientific understanding of the effects of petroleum on birds (Table 1.5). Further developing the model for the effects of petroleum on birds is an unfortunate necessity, as exposure is likely to continue to occur as long as production, transportation, and consumption persist, especially near water bodies that facilitate the spread of discharged oil.

1.7. Conclusions

Birds are practical and publicly visible indicators of pollution (Elliott and Elliott, 2013; Furness and Camphuysen, 1997), and our understanding of how petroleum affects them has improved by degrees over the 27 years since last reviewed by Leighton (1993). In conclusion, we attempt to give an overview of key advancements. Feather fouling alone is harmful to thermoregulation and locomotion, and even small amounts of

oil may affect feather integrity and locomotive efficiency. Adverse toxic effects usually result from oral doses on the order of milliliters to tens of milliliters kg^{-1} bw d^{-1} over several days (Table 1.3), although the presence of concurrent stressors can increase sensitivity. The previously held notion that inhalation is an unimportant route of petroleum hydrocarbon exposure has been overturned by evidence of effects. The aromatic (PAC and BTEX) fraction of ingested petroleum causes systemic toxicity, yielding complex effects among multiple body systems. Metabolic effects of toxicity are suggested by mass loss and multiple biochemical endpoints, an important finding which merits further study by itself and how it may interact with the metabolic effects of oil-compromised plumage. As metabolism and endocrine signaling are linked, feedback effects between the two are likely. Not only would metabolic changes be expected to affect endocrine signaling, but there is also evidence that petroleum ingestion alters endocrine signaling directly, at least corticosterone signaling and stress response, but also potentially reproductive hormone and thyroid hormone signaling. In addition to the metabolic and endocrine effects of toxicity, recent work shows effects on antioxidant capacity, and there is abundant evidence that petroleum ingestion frequently results in

Table 1.5 Strategic recommendations for researchers and decision makers to prepare for and respond to a petroleum spill affecting avian wildlife resources. We make these suggestions to aid in documenting impact, assessing recovery, and ensuring that data from the incident will further the scientific understanding of the effects of petroleum on birds.

Spill phase	Goal	Strategy recommendations
1. Oil spill preparedness *Before spill* (Years prior)	Maintain evidence to demonstrate potential environmental contamination	Monitor PAC and metal contaminant concentrations in avian tissues, prey, and relevant environmental matrices
	Predict exposure risk	Document avian taxa and habitat use
	Maintain evidence to demonstrate potential effects on reproduction, populations, and demographics	Monitor avian populations, demographics, productivity, and prey at high spill risk and reference areas
2. Acute phase of spill *Visible spilled oil and oiled birds* (Days to months)	Monitor oil contamination to estimate exposure	Oil fate studies should consider avian habitat, oil deposition to benthos, and its persistence there Chemical residue analysis: •Oil grab samples •Air monitoring

Spill phase	Goal	Strategy recommendations
		•Avian prey monitoring, especially during height of oil spill contamination to generate dietary exposure estimates •Sample any oiled eggs available to determine amount on shell and PAC concentration inside
	Evaluate sublethal toxic and physical injury to bird populations	In oiled individuals and oil impacted populations: 1. Take blood samples to assess: • Hematological injury • Oxidative damage • Neurotoxicity • Cardiac damage • Corticosterone stress response • Metabolomic effects • PACs in erythrocyte fraction 2. Take liver biopsy samples to assess: • Transcriptomics and proteomics, including CYP1A and other relevant genes 3. Evaluate body condition and feather (micro)structural integrity Include reference populations and control groups
	Evaluate sublethal behavioral effects	Observe oiled wild birds to assess: • Behavioral effects • Persistence of visible plumage oiling to estimate external exposure and ingestion from preening • Breeding activity and productivity
	Maximize information collected from oil-killed birds	For *postmortem* analyses: • Include unoiled reference birds • Quantify amount of oil on plumage and percent area affected for comparison with live oiled birds • Analyze oil residue in liver tissue to confirm and quantify exposure
	Maximize information collected from rescued and rehabilitated oiled birds	Leave some oiled birds as positive control (track survival) and take some unoiled birds into captivity as control. Quantify oil on plumage and take clinical data at intake.
3. Chronic phase of spill *After visible oil disappears* (Months to decades)	Determine the long-term fate of oil impacted individuals	Tracking studies of survival, movement, habitat use, and reproduction in wild and rehabilitated birds: • Locally breeding birds • Migratory birds tracked to breeding grounds Monitor the incidence of cancerous tumors in birds

Spill phase	Goal	Strategy recommendations
		and evaluate long-term genotoxicity
	Determine cessation of avian exposure to residual contamination	Monitor hydrocarbon exposure via hepatic CYP1A and other relevant biomarkers Compare PAC and metal contaminant concentrations in avian tissues, prey, and relevant environmental matrices to pre-spill baselines
	Assess recovery of oil impacted populations	Compare avian populations, demographics, productivity, prey, and habitat use to pre-spill baselines and multiple reference areas.

oxidative damage to erythrocytes. As a result of the latter, hemolysis and erythrocyte regeneration may occur, at least in wild birds and nestlings. There is also emerging evidence of cardiotoxic effects, which together with hemolysis could reduce overall aerobic capacity. Cardiac effects may also be an important driver of embryotoxicity, which can occur from the exposure of eggs to small amounts of oil. Finally, the weight of evidence suggests immunotoxic effects, at least on the cells and organs of the lymphatic system. The toxic and physical effects of petroleum exposure can translate to emergent, ecological-level effects on behavior, reproduction, and survival. One important cause of those, though indirect, is reduced prey availability in contaminated areas.

Thoughtfully designed investigations will accelerate research progress on the myriad and often subtle ways in which petroleum and petroleum-source contaminants affect avifauna. To investigate toxicological questions in a context of environmental realism, quantitative data on petroleum exposure, estimated or empirical, are sorely needed. This includes effect thresholds for the adverse physical effects of petroleum adsorbed to the plumage, how much is ingested through preening and the diet over time, inhalation, and how much may be transferred by incubating parents to eggs. The emergence of unconventional crude petroleum warrants an evaluation of its toxicity. Robust study design and consistent reporting will improve comparability across experiments, species, regions, and petroleum types, and the continued development of powerful methods like gene expression, omics, and tracking devices may help further resolve the effects of petroleum on free-living birds. Ultimately, the better the consequences of petroleum exposure on birds are understood, the better such effects are mitigated.

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Chapter 2.

Effects of avian eggshell oiling with diluted bitumen show sublethal embryonic polycyclic aromatic compound exposure

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2.1. Chapter Abstract

Breeding birds that become oiled may contaminate the shells of their eggs, and studies of conventional crude oil suggest that even small quantities can be absorbed through the eggshell and cause embryotoxicity. Unconventional crude oils remain untested, so we evaluated whether a major Canadian oil sands product, diluted bitumen (dilbit), would be absorbed and cause toxicity when applied to eggshells of two species, domestic chicken (*Gallus gallus domesticus*) and double-crested cormorant (*Nannopterum auritum*). We artificially incubated eggs and applied lightly weathered dilbit (Cold Lake blend) to the eggshells (0.015–0.15 mg g^{-1} egg in chicken; 0.1–0.4 mg g^{-1} egg in cormorant) at various points during incubation before sampling prehatch embryos. Polycyclic aromatic compound (PAC) residue in cormorant embryos was elevated only at the highest dilbit application (0.4 mg g^{-1} egg) closest (day 16) to sampling on day 22. In contrast, cormorant liver cytochrome P450 1a4 (*cyp1a4*) mRNA expression (quantitative polymerase chain reaction assay) was elevated only in embryos treated with the earliest and lowest dilbit application (0.1 mg g^{-1} egg on day 4). These results confirm that dilbit can cross through the eggshell and be absorbed by embryos, and they imply rapid biotransformation of PACs and a nonmonotonic *cyp1a4* response. Despite evidence of exposure in cormorant, we found no detectable effects on the frequency of survival, deformity, and gross lesions, nor did we find effects on physiological endpoints indicative of growth and cardiovascular function in either chicken or cormorant. *In ovo* dilbit exposure may be less toxic than well-studied conventional

crude oils. The effects of an oil spill scenario involving dilbit to bird embryos might be subtle, and PACs may be rapidly metabolized.

2.2. Introduction

Petroleum discharges in the form of chronic small releases or large-scale oil spills can affect and potentially harm numerous birds, and the avian embryo is especially sensitive to oil contaminating the eggshell (Burger, 1993; King et al., 2021; Leighton, 1993; Wiese & Robertson, 2004). Such exposure may occur when breeding birds become oiled and in turn foul their eggs during incubation (Albers, 1980; Hartung, 1965; King & Lefever, 1979; Parnell et al., 1984). Laboratory and field studies in a variety of taxa have often shown embryotoxicity in eggs oiled with crude petroleum or refined products in the range of 0.01–0.5 mg oil g^{-1} egg (see Couillard & Leighton, 1991a; Hoffman & Albers, 1984; Macko & King, 1980; Szaro et al., 1978). Whereas substantial oiling of the eggshell surface can impair gas diffusion and negatively affect the embryo (Couillard & Leighton, 1989; Stubblefield et al., 1995), petroleum covering only a minor portion of the eggshell is capable of causing toxicity if sufficient quantities of hazardous compounds are absorbed by the embryo (Albers, 1977). Of such compounds, the most toxic to the avian embryo are polycyclic aromatic compounds (PACs; i.e., parent polycyclic aromatic hydrocarbons and their alkylated or heteroatom analogs; Albers, 1977; Ellenton, 1982; Hoffman, 1979a; Walters et al., 1987), although others may contribute to toxicity (monoaromatic compounds, aliphatic hydrocarbons, and metals; Dubansky et al., 2018; Ellenton, 1982; Hoffman, 1979b). Exposure may affect survival, development, growth, and hatching, and can also cause pathological, physiological, and metabolic effects (see Albers, 1978; Couillard & Leighton, 1990a; Hoffman, 1978; Kertész & Hlubik, 2002; Westman et al., 2013). Toxicity is modulated by the timing of exposure, because embryos are most sensitive during early to mid development (Albers, 1978; Couillard & Leighton, 1991b; Lewis & Malecki, 1984), as well as by the changes that petroleum undergoes after release into the environment, a process called weathering, which affects its physical and chemical properties (Lewis & Malecki, 1984; Macko & King, 1980; Szaro et al., 1980). The aforementioned studies and many others have thoroughly described embryotoxicity from conventional crude oil exposure *in ovo*. However, the extent to which unconventional types of crude oil, such as Canadian oil sands bitumen, pose a similar toxicity hazard to

the avian embryo remains unknown (Albers, 1979; Butler et al., 1988; Couillard & Leighton, 1990b; Finch et al., 2011; Hoffman, 1979c; Lee et al., 1986).

Unconventional crude petroleum, such as Canadian oil sand bitumen, is increasingly being produced and transported in vast quantities, but relatively little is known about its toxicity in any taxa, especially birds. Canada ranks among the nations leading in global crude oil production, and the production of unconventional bituminous crude oil in the country's oil sands region, the third largest known oil reserve in the world, has increased dramatically over the past several decades (i.e., more than double that of Canadian conventional crude oil production; King et al., 2021; Natural Resources Canada, 2019). However, in contrast to conventional crude oil, the relatively high viscosity of extracted oil sands bitumen requires reformulation into a crude product suitable for transportation, such as by pipeline or rail car. This is typically achieved by blending the bitumen with synthetic oil, natural gas condensate, or a combination thereof (Dew et al., 2015). Thus, those products' chemical composition and physical properties differ from conventional crude oils used in past scientific studies to characterize the effects of exposure to conventional petroleum in avifauna. Current initiatives to increase pipeline capacity from the oil sands region to the international port in Vancouver, British Columbia will increase the volume of bituminous crude oil transported through, and possibly discharged into, terrestrial and aquatic bird habitat (Dew et al., 2015; National Energy Board, 2016), making the lack of bituminous crude oil toxicity data a critical knowledge gap. Although the potential effects of air and water contamination from oil sands industrial activity have received substantial research attention (Green et al., 2017; King et al., 2021), toxicity data for avian exposure to bitumen-based crude oil are currently unavailable in the peer-reviewed literature.

To assess the toxicity of a representative oil sands bitumen product on a sensitive, well-described, and experimentally tractable study system, the avian embryo (Couillard & Leighton, 1991a; Hill & Hoffman, 1984; King et al., 2021), we conducted egg oiling studies with lightly weathered diluted bitumen (hereafter termed dilbit). Because any oil spill scenario will result in at least some petroleum weathering, we used dilbit that had been allowed to evaporate for 36 h. Dilbit exposures were conducted with artificially incubated eggs from two species, domestic chicken (*Gallus gallus domesticus*) and wild double-crested cormorant (*Nannopterum auritum*). The former is an established embryotoxicity model and the latter is a widespread aquatic waterbird species in areas

likely to be affected by dilbit spills. We hypothesized that different levels of dilbit exposure at various points of incubation would result in the penetration of oil material through the eggshell and absorption by the embryo, resulting in toxicity. To assess exposure, we quantified PAC residue concentrations in embryo carcasses and relative hepatic cytochrome P450 1a4 (*cyp1a4*) messenger (m)RNA gene expression indicative of PAC exposure. To assess toxicity, we measured physiological endpoints. We predicted reduced prehatch survival, and in survivors, developmental deformities, gross pathological lesions, reduced growth, and effects on cardiovascular function.

2.3. Materials and methods

Experimental work was performed at Simon Fraser University's Animal Care Facility in British Columbia, Canada (University Animal Care Committee Protocol #1268B-18). First we conducted an egg oiling experiment with chicken embryos, followed by a larger scale experiment with double-crested cormorant. Avian eggshell oiling studies have conventionally reported doses on the basis of oil volume (µl) or mass (mg) per egg, but because egg size can vary (e.g., mallard vs. chicken eggs), we instead give all doses on an oil per gram egg basis (mg g^{-1} egg) to facilitate comparison among taxa and studies, as in Finch et al. (2011) and since recommended by King et al. (2021). Dosing with viscous petroleum types such as the diluted bitumen in the present study is necessarily done on a mass rather than a volumetric basis. Accordingly, all doses in our study are reported as mg oil g^{-1} egg, as are doses or thresholds from the references we cite. For the latter, conversions were necessary. We took egg mass for a given species from the Birds of the World online database (Cornell Lab of Ornithology, 2020) and used a petroleum density of 0.9 g ml^{-1} in our calculations. In some cases, egg mass was unavailable in the Birds of the World database, so we used egg mass reported in relevant peer-reviewed articles.

2.3.1. Egg oiling experiments

Dilbit and nontoxic control oil

We used a sample of winter blend Cold Lake diluted bitumen from a producer in the Cold Lake oil sands in eastern Alberta, Canada (2018; steam-extracted oil sands bitumen [50% w/w or greater] diluted with light hydrocarbons consisting of alkanes,

cycloalkanes, and monoaromatics in the form of natural gas condensate and/or petroleum naphtha). We stored the dilbit at 4 °C and weathered it for 36 h under normal room temperature and fluorescent lighting in an open glass jar in a fume hood. Weathering reduced initial mass by 12.8%–14.0% (w/w), increased estimated density from 0.915 to 0.967 g ml^{-1} at 25 °C (H. Dettman, Natural Resources Canada, and V. Palace, International Institute for Sustainable Development–Experimental Lakes Area, personal communication, November 3, 2018), and increased viscosity during handling. Dilbit was too viscous to pipette (as in Albers, 1977), so we applied dilbit to the eggshells on a mass basis with an artist's paintbrush (Finch et al., 2011; Stubblefield et al., 1995). Given that volatile hydrocarbons are an important component of dilbit, we included part of the air sac within the area onto which oil was applied. We report the nominal amounts of dilbit applied, which were accurate (averaged by treatment group) to within ±10% of the nominal doses (Supporting Information, Table S1). As a control, we used organic safflower oil to account for the physical effects of nontoxic oils (Couillard & Leighton, 1989; Stubblefield et al., 1995).

Chicken embryo experiment

We purchased fertilized broiler chicken (*G. gallus domesticus*) eggs from a commercial hatchery in British Columbia, and the same day transported, weighed, and placed the eggs vertically in forced-air incubators (RX–2; Lyon Technologies). Incubators were maintained at a target temperature of 37.5 °C (observed $\bar{x}$ = 37.4 °C, range 37.1–38.3 °C) and a target relative humidity of 55% (observed $\bar{x}$ = 49%, range 39%–57%), and were equipped with automatic turning grids that tilted the eggs in opposing directions hourly. Chicken eggs were treated with dilbit on day 13 of incubation in amounts of: (a) 0.015 mg dilbit g^{-1} egg (n = 8), (b) 0.15 mg dilbit g^{-1} egg (n = 8), or (c) safflower oil (control; n = 8), or on incubation day 16 with (d) 0.015 mg dilbit g^{-1} egg (n = 8), (e) 0.15 mg dilbit g^{-1} egg (n = 8), or (f) safflower oil (control; n = 8; Table 2.1). Oils were applied to the egg using a paintbrush in a lengthwise rectangular area starting from the air sac margin and including a portion of the air sac (Figure 2.1A; detailed procedure given in the Supporting Information). Length and width of the oiled area was measured (±1 mm). The average egg surface area painted with oil ranged from 0.8% to 10.6%, and in controls the average area of egg covered with safflower oil exceeded or closely matched that of dilbit (not vice versa; Table 2.1).

Table 2.1 Summary of embryo exposure scenarios and oil applications for embryotoxicity studies with domestic chicken and double-crested cormorant.

Species	Treatment				Eggs (n) [a]	Sampling		Egg surface oiled (%) [b]		Measured exposure (mg oil g^{-1} egg)	
	Substance	Nominal exposure (mg oil g^{-1} egg) [c]	Day	Development (%) [d]		Day	Development (%) [d]	Mean	SD	Mean	SD
Domestic chicken	Safflower	0.15 eq.	13	62	8	18	86	10.6	3.1	0.081	0.006
	Dilbit	0.015	13	62	8	18	86	0.8	0.2	0.015	0.002
	Dilbit	0.15	13	62	8	18	86	6.4	2.1	0.156	0.009
	Safflower	0.15 eq.	16	76	8	18	86	9.9	2.2	0.082	0.004
	Dilbit	0.015	16	76	8	18	86	0.9	0.3	0.015	0.001
	Dilbit	0.15	16	76	8	18	86	10.4	2.5	0.152	0.010
Double-crested cormorant	None	0	-	-	12	22	85	-	-	-	-
	Safflower	0.2 eq.	4	15	9	22	85	4.0	0.8	0.088	0.020
	Safflower	0.4 eq.	4	15	9	22	85	11.2	0.9	0.309	0.067
	Dilbit	0.1	4	15	15	22	85	2.7	0.5	0.107	0.009
	Dilbit	0.2	4	15	15	22	85	4.5	1.0	0.204	0.018
	Dilbit	0.4	4	15	15	22	85	10.1	1.7	0.408	0.028
	Safflower	0.4 eq.	16	62	12	22	85	10.9	0.6	0.239	0.034
	Dilbit	0.2	16	62	12	22	85	5.8	0.9	0.209	0.019
	Dilbit	0.4	16	62	14	22	85	10.3	1.3	0.409	0.025

A version of this table with data from which these tabulations were derived and doses on a microliter basis are provided in the Supporting Information, Table S1.

[a] Eggs were screened prior to treatment based on presence of heart rate except for cormorant eggs treated on day 4 (n = 9 for safflower oil controls, n = 15 for dilbit treatments) and no treatment controls (n = 12). Heart rate was undetectable at early development.

[b] Proportion of the egg area affected by oil dose (square centimeters) relative to total egg surface area calculated with $Area = 4.835(Mass)^{0.662}$ from Paganelli et al. (1974) where mass is grams, and area is square centimeters.

[c] Safflower oil treatments had the oil spread over an area equivalent to the surface area affected by dilbit.

[d] Calculated from a 21-day incubation period in chicken, and a 26-day incubation period in cormorant.

Double-crested cormorant embryo experiment

Fresh (i.e., unincubated, from nests with incomplete clutches) double-crested cormorant (*N. auritum*) eggs were collected during early breeding from Pelican Lake (50.4909°N, 105.9341°W; 13 eggs on April 20, 2019, 101 eggs on May 6, 2019) and Reed Lake (50.3974°N, 107.0822°W; 6 eggs on April 20, 2019) in southern Saskatchewan, Canada. Both nesting sites are islands within rural lakes that drain

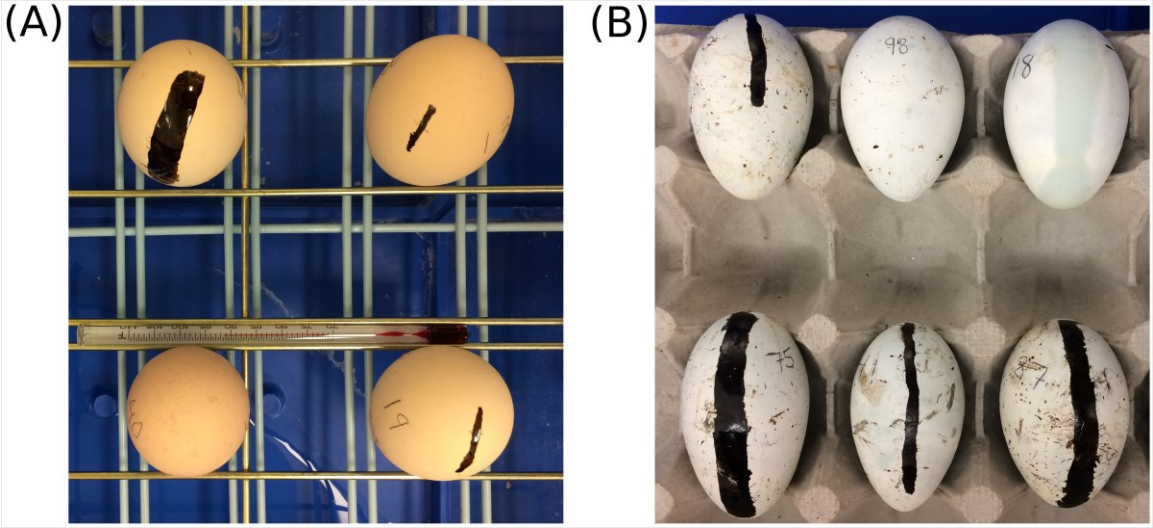

Figure 2.1 Examples of eggshell oiling treatments for eggs from left to right, top to bottom: (A) Chicken eggs held vertically in incubators with treatments of 0.15 and 0.015, safflower oil equivalent to 0.15 and 0.015 mg dilbit g^{-1} egg; (B) Double-crested cormorant eggs held horizontally at sampling with treatments of 0.1, no treatment, safflower oil equivalent to 0.4, 0.4, 0.1, and 0.4 mg dilbit g^{-1} egg.

agricultural land and have no known history of contamination, nor apparent point sources of known embryotoxic contaminants. Fresh eggs were transported by air to our facility at Simon Fraser University. Within 24–48 h of collection, the eggs were weighed and set in incubators at our facility, as just described in Chicken embryo experiment. For the cormorant eggs however, incubator egg grids were modified to hold the eggs horizontally, operated at a target temperature of 37.5 °C (observed $\bar{x}$ = 37.4 °C, range 37.0–38.3 °C) and 60% relative humidity (observed $\bar{x}$ = 60%, range 42%–72%; Supporting Information, Figure S1), and rotated 90° each day by hand, in addition to automatic turning (Powell et al., 1996). Cormorant eggs were treated with dilbit on day 4 of incubation in amounts of (a) 0.1 mg dilbit g^{-1} egg (n = 15), (b) 0.2 mg dilbit g^{-1} egg (n = 15), or (c) 0.4 mg dilbit g^{-1} egg (n = 15) or on incubation day 16 with (d)

0.2 mg dilbit g^{-1} egg (n = 12), or (e) 0.4 mg dilbit g^{-1} egg (n = 14). We also included several controls. These control groups included no treatment or safflower oil painted over an area equivalent to: (a) 0.2 mg dilbit g^{-1} egg on day 4 (n = 9), (b) 0.4 mg dilbit g^{-1} egg on day 4 (n = 9), and (c) 0.4 mg dilbit g^{-1} egg on day 16 (n = 12). No safflower oil control was used for the 0.1 mg dilbit g^{-1} egg treatment, because the percentage of egg surface area covered in oil was small (2.7%). Oil was applied over a roughly rectangular swath (Figure 2.1B) with a procedure similar to that used for chicken (detailed in the Supporting Information). Length and width of the area oiled was measured (±1 mm). The average egg surface area painted with oil ranged from 2.7% to 11.2%, in controls, and the average area of egg covered with safflower oil exceeded or closely matched that of dilbit (Table 2.1).

Heart rate determination

Heart rate was recorded (Buddy Mk2; Avitronics) at regular intervals over the course of incubation to monitor embryo survival, remove nonviable eggs from experimental treatments at mid to late development, and investigate potentially adverse effects of dilbit exposure. Heart rate was reliably quantified from incubation day 12 onward for chicken and incubation day 18 onward for cormorant. The cormorant's thicker eggshell delayed the point in development at which we could quantify or detect heart rate. For cormorant embryos, heart rate was reliably detectable at incubation day 15 but not quantifiable. It was thus possible to remove nonviable embryos from the experiment with chicken and from the cormorant experiment groups treated on day 16. Viability screening with heart rate was not done for cormorant embryos treated on day 4 because heartbeat was not yet detectable and candling proved difficult. Quantifiable heart rate measurements were recorded on incubation days 12, 15, and 17 for chicken and on days 18 and 21 for cormorant. We used these data to test for effects of dilbit on embryonic heart rate.

Embryo sampling

Embryos were sampled on day 18 for chicken and day 22 for cormorant, representing 85% to 86% of the full incubation period to hatching, which we considered 21 days in chicken and 26 days in cormorant (Cornell Lab of Ornithology, 2020; Lierz et al., 2006). This included visual inspections to identify dead embryos and gross deformities or lesions (see Franci et al., 2018). For chicken, blood was sampled by

blotting dry a major chorioallantoic vessel, usually the umbilical artery, nicking it with a hooked scalpel blade, and collecting the blood in a heparinized hematocrit tube, although this approach was not successful in the smaller cormorant embryos. Embryos were euthanized by decapitation and dissected. Hepatic tissue for *cyp1a4* gene expression analysis was excised, aliquoted, immediately flash-frozen in liquid nitrogen, and stored at −80 °C. Carcasses were stored at −20 °C with the cormorant embryos wrapped in solvent double-rinsed aluminum foil (acetone and n-hexanes).

2.3.2. Laboratory analysis

Physiological endpoints: Growth and cardiovascular function

Gross measurements on fresh embryos included body mass, tarsus length, crown–rump length (cormorant only), and fresh weight of the left liver lobe (cormorant only; liver somatic index was calculated as left liver lobe mass [mg]/total body mass [g] on a fresh weight basis). Embryo carcasses stored frozen were later re-examined to measure heart length and width, and wet and dry weight of the heart and spleen.

Blood hematocrit and hemoglobin were measured in samples from chicken embryos. Hematocrit (percentage of packed erythrocyte volume) was determined in microhematocrit tubes with digital calipers following centrifugation for 5 min at 9000 g (Microspin 24; Vulcon Technologies; Yap et al., 2017). Hemoglobin was quantified with the cyanmethemoglobin method modified for use with a microplate spectrophotometer (BioTek Powerwave 340; BioTek Instruments; Drabkin & Austin, 1932; Yap et al., 2017). We generated a standard curve with human hemoglobin (11.2 g dl^{-1}; H7506STD; Pointe Scientific) and measured the absorbance at 540 nm of whole blood in Drabkin's reagent (0.004 v/v; Cat. No. D5941; Sigma-Aldrich Canada) to quantify concentration. Mean intra- and interassay coefficients were 3.3% and 0.9%, respectively.

Biotransformation gene expression endpoint: Cormorant cyp1a4

We evaluated mRNA expression of the phase I biotransformation gene *cyp1a4* in cormorant embryo hepatic tissue with the quantitative polymerase chain reaction (qPCR) ΔΔ quantification cycle (ΔΔCq) method to compare among treatment and control groups according to the minimum information for publication of quantitative real-time PCR experiments guidelines (Bustin et al., 2009; Livak & Schmittgen, 2001). Total RNA was

extracted from frozen liver tissue for reverse transcription to complementary (c)DNA for qPCR as follows. Approximately 15 mg of left liver lobe aliquots was subsampled with sterile tools to extract RNA using a column-based kit (RNeasy® Plus Mini Kit; Cat. No. 74134; Qiagen). The sample was disrupted with 3 mm RNAse-free stainless-steel beads in lysis buffer with a mixer mill (MM 300; Retsch) for 3 min at 30 Hz, followed by genomic DNA elimination, RNA isolation, and cleanup per kit instructions. The RNA was eluted in 30 µl of nuclease-free water; total RNA yield and purity were determined spectrophotometrically using a Take3™ micro-volume plate (BioTek Instruments) with a microplate reader (EPOCH2; BioTek Instruments; 260/280 nm absorbance ratio range 2.08–2.22, $\bar{x} = 2.13$). The RNA quality was confirmed (RNA quality indicator range 8.4–10.0, $\bar{x} = 9.6$ on subset of samples, $n = 92$) with the Experion™ Automated Electrophoresis System (Bio-Rad Laboratories) RNA StdSens Kit (Cat. No. 700-7111). The RNA (1000 ng) was reverse transcribed to cDNA with the iScript™ gDNA Clear cDNA Synthesis Kit (Cat. No. 1725035; Bio-Rad Laboratories) in 20-µl reactions.

The qPCR assayed mRNA expression of *cyp1a4* relative to two reference genes, eukaryotic translation elongation factor 1a1 (*eef1a1*) and ribosomal protein l4 (*rpl4*; Supporting Information, Table S2) with the primers (Integrated DNA Technologies) described by Crump et al. (2016). We conducted the qPCR assays on a Bio-Rad CFX384 system (Bio-Rad Laboratories) with the manufacturer's 384-well plates (Cat. No. HSP3805) in duplicate 12.5-µl reactions containing forward and reverse primers (*rpl4* and *cyp1a4* at 400 nM; *eef1a1*, at 200 nM), 6.25 µl SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (Cat. No. 1725018; Bio-Rad Laboratories), 0.625 µl dimethyl sulfoxide, and nuclease-free water. Thermal conditions for qPCR were 98 °C for 3 min, followed by 40 cycles of 98 °C for 15 s and 60 °C for 1 min. For quality assurance, each plate included controls to screen for genomic DNA carryover and false-positive fluorescence signal, an interplate calibrator sample, and standard curves. Standard curves exhibited acceptable Cq efficiency (mean ± 95% CI: *rpl4* 90.8 ± 6.7, *eef1a1* 94.5 ± 1.5, and *cyp1a4* 100.8 ± 9.4) and R^2 (more than 0.98) over a cDNA input range (assuming 100% reverse transcription) from 25 to 0.006 ng; samples were assayed at dilutions equivalent to 0.390 ng cDNA on the standard curve. Dissociation curves and gel electrophoresis with amplified endpoint PCR products (abm® Taq DNA Polymerase kit; Cat. No. G009) confirmed that our primers amplified a single product the size (bp) of the target sequence. The Cq thresholds were autocalculated by Bio-Rad

CFX Maestro 2.0 software (Ver 5.0.021.0616). Intra- and interassay coefficients of variation calculated on *cyp1a4* relative quantity were 8.8% (geometric mean, n = 102) and 4.9% (arithmetic mean, n = 4), respectively.

PAC residue analysis

Cormorant embryo carcasses and the bulk dilbit were analyzed for 51 PAC analytes, including 16 parent polycyclic aromatic hydrocarbons, dibenzothiophenes, and their alkylated analogs by gas chromatography coupled with a triple quadrupole mass spectrometer used in multiple reaction monitoring mode (GC–MS/MS; Contract No. 3000686444; Centre for Oil and Gas Research and Development [ISO-17025 certified]; Winnipeg, MB, Canada). Weathered dilbit samples were pooled in solvent double-rinsed glass jars (acetone and n-hexanes). The carcasses of embryos (alive at euthanization, n = 6), less their yolk sac and the liver tissue samples removed, were pooled by treatment; the safflower oil–treated control groups (Table 2.1) were analyzed in a single pool of two embryos from each group (n = 6). Carcass pools were homogenized and stored frozen in certified amber glass jars (I-Chem™; Cat. No. 340-0250; Thermo Scientific). Stainless-steel tools were washed and double rinsed with solvents between samples to prevent any cross-contamination. Embryo homogenate extractions, cleanup, and GC–MS/MS analysis were performed as described in Idowu et al. (2018) and Xia et al. (2021). Dilbit was dissolved in hexane and cleaned up using silica/alumina adsorption chromatography (Idowu et al., 2018). Samples were injected directly into the GC–MS/MS system. The target analytes and their quantitation limits are listed in the Supporting Information, Table S3.

Data analysis

Data analysis was conducted in RStudio (Ver 1.2.5019). Egg surface area was calculated from initial egg mass with Paganelli et al.'s (1974) allometric equation. Thus, the proportion of the egg oiled was calculated from the length and width of the oiled area relative to the total egg surface area.

At sampling, some embryos were dead. Others were alive but had some type of developmental deformity or gross pathological lesion. Such observations were few across all control and treatment groups. We considered the aforementioned outcomes

adverse and analyzed their frequency across groups compared with embryos alive at sampling without suspected abnormalities using Fisher's exact test.

To compare physiological endpoints (response variable) among groups, we constructed linear mixed effect (LME) models (lme4 package in R) as follows. Experimental treatment was included as the fixed effect (predictor), and the incubator in which the egg was developed was included as a random effect to account for any unknown differences among incubator units (however unlikely; Supporting Information, Figure S1). For heart rate, which was measured repeatedly, we included day as an interaction with treatment (day × treatment) and individual as a random effect, in addition to incubator. For endpoints that we speculated might covary with egg mass or embryo body mass in our data set, we performed model selection with the Akaike information criterion estimator for small sample sizes (AICc) to decide whether either should be included as a covariate. The resulting LME models (Table 2.2) were used to test whether group means were unequal by analysis of variance (ANOVA; lmerTest package, Satterthwaite's method for degrees of freedom). Where we found a significant ANOVA result for the fixed effect ($\alpha = 0.05$), we performed pairwise tests of estimated marginal means between groups ($\alpha = 0.05$; emmeans package, Satterthwaite's method for degrees of freedom). With the LME models, we first tested for differences by ANOVA among control treatments (Table 2.1, various safflower oil treatments or no treatment), and they were seldom different. For 21 of 23 tests, there was no significant difference among control group means. Control groups were therefore combined into a single control, and the analysis was repeated to test for a difference among dilbit treatments and the combined control group by ANOVA. For those 2 exceptions of 23 where significant differences among controls were found (i.e., cormorant heart rate at day 18 and cormorant tarsus length), downstream analyses excluded the control groups with different means from the combined controls group, included them despite their difference in means, or kept all control groups separate. However, the test results concerning dilbit treatment remained unaffected. Because the way in which the multiple control groups were handled did not affect test outcome, we report the results for the analysis with control groups combined for simplicity.

The qPCR gene expression analysis used the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001). For each sample, the Cq data from the two reference genes were averaged (Eef1a1, Rpl4) and used to calculate the relative quantity of our gene of

Table 2.2 **Physiological endpoints related to growth and cardiovascular function were assessed using linear mixed effect models (LME). Where warranted, a covariate was included in the model. Egg incubator was included as a random effect, and for repeated measurements on heart rate, individual was also included as a random effect.**

				Chicken [a]				Cormorant [b]				
Physiological role	LME response	LME predictor	LME covariate	ANOVA result			Sig. post hoc contrast with control	LME covariate	ANOVA result			Sig. post hoc contrast with control
				df	F	p			df	F	p	
Growth	Body mass	Treatment	Egg mass	4, 40.3	0.132	0.970		None	5, 91.8	0.174	0.972	
	Crown-rump length	Treatment	-	-	-	-		None	5, 92.1	1.797	0.121	
	Body mass:crown-rump length	Treatment	-	-	-	-		None	5, 92.5	0.444	0.816	
	Tarsus length	Treatment	Body mass	4, 42.0	0.208	0.932		Body mass	5, 90.0	1.207	0.312	
	Liver somatic index	Treatment	-	-	-	-		None	5, 93.9	1.143	0.343	
Cardio-vascular function	Heart rate	Day	None	2, 86.0	3.367	0.039*	d 12 < d 15, d 12 < d 17	None	1, 92.1	0.895	0.347	
	Heart rate	Treatment	None	4, 127.4	0.66	0.621		None	5, 178.1	1.452	0.208	
	Heart rate	Treatment*Day	None	8, 86.0	0.527	0.833		None	5, 92.7	0.912	0.477	
	Hematocrit	Treatment	None	4, 38.6	2.762	0.041*	N	-	-	-	-	
	Hemoglobin	Treatment	None	4, 31.4	3.974	0.010*	N	-	-	-	-	
	Heart wet mass	Treatment	Body mass	4, 38.2	0.359	0.836		Body mass	5, 52.0	1.995	0.095	
	Heart dry mass	Treatment	None	4, 41.0	0.357	0.838		Body mass	5, 52.0	4.978	<0.001*	N

Heart moisture	Treatment	Body mass	4, 38.2	0.178	0.948	None	5, 53.0	0.801	0.554
Heart length	Treatment	Egg mass	4, 37.2	1.292	0.291	Body mass	5, 51.3	1.099	0.373
Heart width	Treatment	None	4, 40.0	0.205	0.934	Body mass	5, 52.0	1.662	0.16
Spleen wet mass	Treatment	None	4, 39.2	1.231	0.313	-	-	-	-
Spleen dry mass	Treatment	None	4, 37.1	1.881	0.134	-	-	-	-

[a] n ≥ 8 except for hematocrit and hemoglobin where n ≥ 5. To better conform to LME assumptions, data for heart dry mass was transformed by natural logarithm and spleen dry mass data outliers were removed (Dixon's Test).

[b] n ≥ 11 except for heart measurements where n ≥ 6. To better conform to LME assumptions, outliers were removed (Dixon's Test) from heart rate data, data for body mas was transformed by natural logarithm, and data for heart moisture was transformed by arcsine square root.

* $p < 0.05$

LME = Linear mixed effect model; ANOVA = Analysis of variance (Satterthwaite's method for degrees of freedom)

interest (*cyp1a4*). We then calculated normalized expression as the relative quantity of each sample per the mean of the control group. Statistical analysis was performed as described for physiological endpoints. Briefly, we first tested for differences between control treatments. Because there was no significant difference between control groups, they were combined, and normalized expression was recalculated. The statistical model to test for differences in normalized gene expression was an LME with treatment as a fixed effect and the incubator in which the egg was developed as a random effect.

To compare embryo homogenate PAC concentrations among experimental groups, profiles were plotted for analytes at or above the limit of quantitation (LOQ), which was 3.33 times the method limit of detection (Idowu et al., 2018). The PAC profile of the dilbit was likewise plotted. Concentrations measured in each treatment group relative to the controls (average of the no-treatment and the safflower oil control groups), and the percentage of quantified residue per calculated dose applied to the eggshell were tabulated (ng/ng × 100; Supporting Information, Table S3). The PAC profiles and tabular data were used for a qualitative, descriptive comparison of PAC residue among embryo carcass pools and the dilbit.

2.4. Results

2.4.1. Survival and abnormalities

The frequency of embryos that were dead at sampling or had some visible gross deformity or lesion (Figure 2.2), both considered negative outcomes with respect to development and survival, was low and not different among experimental groups for both cormorant ($p = 0.438$, $n = 8–15$) and chicken ($p = 0.684$, $n = 8$).

2.4.2. Physiology

There were no detectable significant effects of dilbit treatment at any dose or time point on embryo growth or organ growth ($p = 0.121–0.972$), and the few effects on cardiovascular endpoints detected were not due to differences between dilbit treatment and the control group but rather differences among treatment groups (Table 2.2). The latter included hematocrit ($F_{4,38.6} = 2.762$, $p = 0.041$) and hemoglobin in chicken

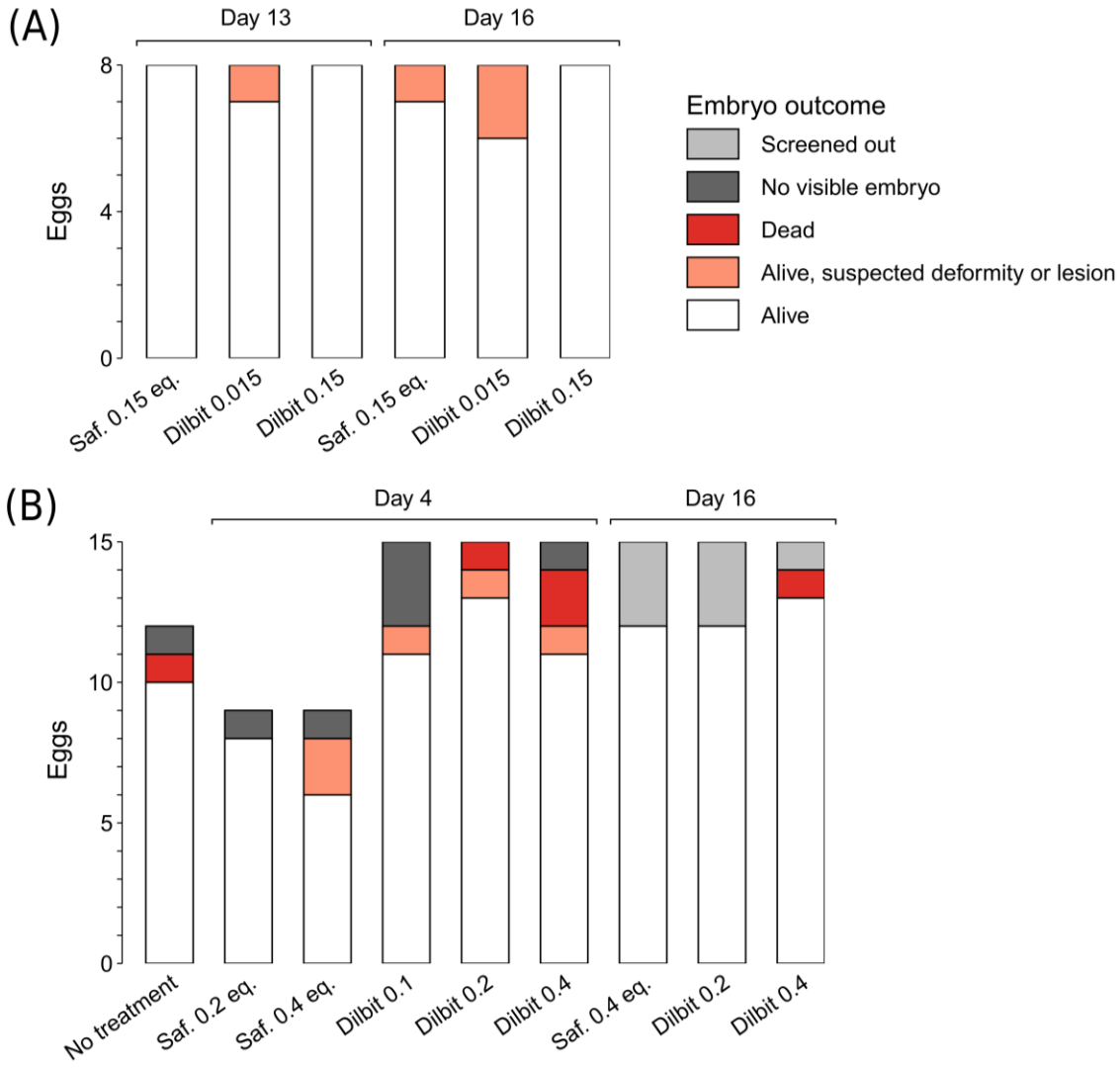

Figure 2.2 **Frequency of embryo outcome (see key) at sampling for experimental treatments: (A) Chicken eggs dosed on day 13 or 16 with safflower oil (Saf.) over an area equivalent to 0.15 mg dilbit g^{-1} egg or with dilbit amounts of 0.015 or 0.15 mg dilbit g^{-1} egg and sampled on day 18; (B) Double-crested cormorant eggs left untreated, dosed on day 4 with safflower oil over an area equivalent to 0.2 or 0.4 mg dilbit g^{-1} egg, or with dilbit amounts of 0.1, 0.2, or 0.4 mg dilbit g^{-1} egg, or dosed on day 16 with safflower oil over an area equivalent to 0.4 mg dilbit g^{-1} egg or dilbit amounts of 0.2 or 0.4 mg dilbit g^{-1} egg and sampled at day 22. The frequency of embryos with a negative outcome at sampling, either dead or some deformity or lesion, was different among groups (Fisher's exact test) for neither chicken ($p = 0.684$, $n = 8$) nor cormorant ($p = 0.438$, $n = 8$–15).**

($F_{4,31.4} = 3.974$, $p = 0.010$), as well as heart dry mass in cormorant ($F_{5,52} = 4.978$, $p < 0.001$). For embryonic heart rate, neither egg oiling treatment nor the treatment × day interaction were significant ($p = 0.208–0.833$). However, in chicken a more complete time series of heart rate over time was recorded than could be measured in cormorant, and we did detect the increase in chicken embryonic heart rate expected over the course of development (day effect, $F_{2,86.0} = 3.367$, $p = 0.039$). Day effect was not significant in cormorant ($F_{1,92.1} = 0.895$, $p = 0.347$).

2.4.3. *cyp1a4* mRNA gene expression in cormorant

For cormorant embryo hepatic *cyp1a4* mRNA gene expression data generated by qPCR (Figure 2.3), there was a significant effect of dilbit treatment on normalized *cyp1a4* expression at sampling ($F_{5,88.0} = 2.701$, $p = 0.026$). Between groups, there was higher (1.6 times) normalized *cyp1a4* mRNA expression in embryos that received the lowest dilbit dose, 0.1 mg g^{-1} egg on day 4, compared with controls ($p = 0.007$). Other dilbit treatments were not different from controls ($p = 0.774–0.968$).

2.4.4. PAC residue

In the cormorant embryos exposed on day 16 to 0.4 mg dilbit g^{-1} egg, 31 of the 51 PAC analytes were quantified in carcass homogenate (i.e., the LOQ or higher) and were qualitatively elevated (pool of $n = 6$ alive at euthanization; Figure 2.4). Those relative concentrations were 1.8–5.6 times the average concentrations in control embryos untreated or treated only with safflower oil (Figure 2.4A,B, and G; Supporting Information, Table S3). The most concentrated analytes on a wet weight basis were C3 chrysenes (2.55 ng g^{-1}), naphthalenes (nonalkylated, 1-methyl-, 2-methyl-, and C1–C3 naphthalenes; 1.02–2.07 ng g^{-1}), phenanthrene (1.40 ng g^{-1}), and C2 benzo[a]pyrenes (1.12 ng g^{-1}). However, the most abundant classes and congeners quantified in the tissue differed from those in dilbit (Figure 2.4G and H; Supporting Information, Table S3). For example, the C2 chrysene group was the most abundant PAC analyte in dilbit, yet the amount detected in embryo tissue as a percentage of C2 chrysenes present in the dilbit dose was the lowest observed (0.02%) among all 31 quantified analytes. In that example and in general for the treatment group with elevated PACs, the quantity of each

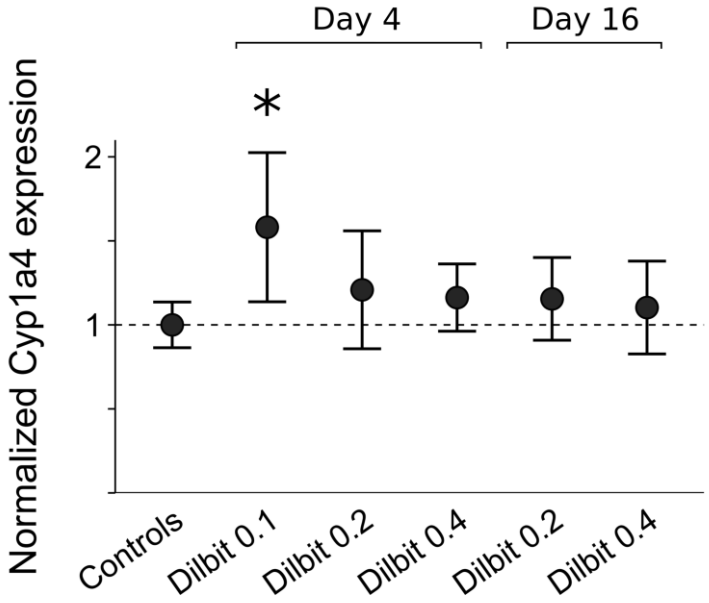

Figure 2.3 **Cormorant embryo hepatic *cyp1a4* mRNA normalized gene expression assayed by quantitative polymerase chain reaction. Dilbit treatments were applied to the eggshell on different days of incubation, and embryos were sampled at day 22. Gene expression is normalized to the control treatments (dashed line). The asterisk (*) indicates a significant difference from the controls (linear mixed effect model; *n* = 12–13 for treatments, 38 for controls). Circles show the mean by treatment and error bars bound the 95% confidence interval.**

PAC calculated to be present in the embryo as a percentage of the dilbit applied to the eggshell was low, on average 0.41% (range 0.02%–2.95%) of the applied dose and varied among analytes (although calculations do not correct for the low background concentrations typically present, as indicated by controls). In other dilbit treatment groups (Figure 2.4C–F), there was little indication that the profile or concentrations of quantified PACs differed from controls (relative concentrations 0.2–1.8 times controls). Although the 0.4 mg g^{-1} egg dilbit dose applied on incubation day 4 (Figure 2.4E) would plausibly have resulted in a level of exposure *in ovo* similar to the 0.4 mg g^{-1} egg dilbit dose applied on day 16 (Figure 2.4G), albeit to a smaller, less developed embryo, PAC profiles revealed elevated tissue concentrations only when dilbit was applied later on in incubation.

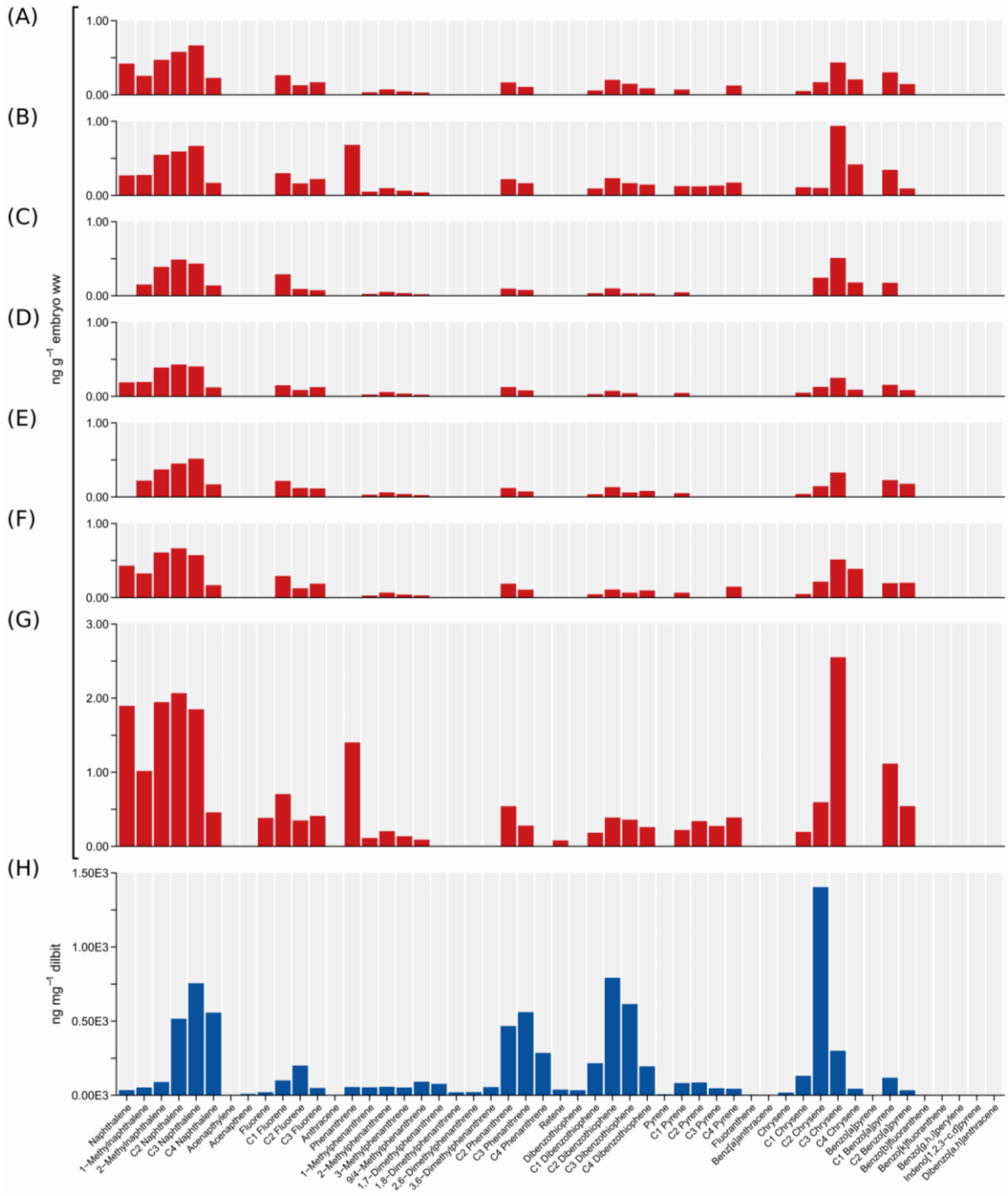

Figure 2.4 **Polycyclic aromatic compound (PAC) concentrations (limit of quantitation or higher) in embryo carcass homogenate (ng g^{-1} wet wt) from double-crested cormorant eggs at sampling, incubation day 22. Each homogenate was a composite of six pooled embryos. Control eggs were (A) left untreated, or (B) treated on day 4 with safflower oil over an area equivalent to 0.2 (n = 2) or 0.4 (n = 2) mg dilbit g^{-1} egg, or on day 16 with safflower oil over an area**

equivalent to 0.4 mg dilbit g^{-1} egg (n = 2). For dilbit treatments, eggs were oiled on day 4 with (C) 0.1, (D) 0.2, or (E) 0.4 mg dilbit g^{-1} egg, or later in development on day 16 with (F) 0.2 or (G) 0.4 mg dilbit g^{-1} egg. For comparison with embryo tissue residue, (H) PAC concentrations in the weathered Cold Lake blend dilbit (ng mg^{-1}) are given. Note that dependent scale magnitude differs among plots. Concentration values are listed in the Supporting Information, Table S3.

2.5. Discussion

In dilbit-exposed cormorant embryos, we found elevated PAC residues only in eggs that were treated with the highest dilbit dose latest in incubation (0.4 mg g^{-1} egg on day 16). Conversely, elevated *cyp1a4* gene expression was only observed in eggs treated early in incubation with the lowest dilbit dose (0.1 mg g^{-1} egg on day 4). These respective chemical and transcriptional indicators of exposure, together with incidental observations of oil spots on the interior of the eggshell membrane, confirmed that some dilbit material penetrated the eggshell and resulted in embryonic exposure despite the Cold Lake blend's high viscosity relative to conventional crude oil (4280 cSt in another 36-h weathered sample; V. Palace & H. Dettman, personal communication, November 3, 2018). Dilbit remained clearly visible on the eggshell from application until sampling (Figure 2.1). Despite clear evidence of embryonic exposure in cormorant, we detected no adverse effects on survival, development, growth, or cardiovascular function in chicken or cormorant with any dose or timing of exposure tested.

We evaluated dilbit toxicity over a range of applications known to be both realistic and unlikely to be confounded by effects on gas exchange. Information on the degree to which avian eggs could realistically be fouled by oiling remains scarce, despite numerous published egg oiling studies. Available data show that adult birds with 2 to 9 g of oil on the plumage per kg body mass, generally the ventral part of the bird, may continue to breed, potentially smearing oil over a considerable portion of the egg (Butler et al., 1988; Hartung, 1965; King & Lefever, 1979; Lewis & Malecki, 1984; Parnell et al., 1984). One study calculated that herring gulls (*Larus argentatus*) with 9 g crude oil kg^{-1} body mass on their brood patches were estimated to transfer to their eggs 0.38 (range 0.02–0.61) mg oil g^{-1} egg (Lewis, 1982). If a sufficient area of the egg is smeared with oil, impaired gas exchange through the eggshell will cause hypoxia in addition to any toxicity (Couillard & Leighton, 1989; Hartung, 1965; Stubblefield et al., 1995). Using

nontoxic oils or inert shell sealants can control for this. Accordingly, up to 12.6% egg surface area or 0.874 mg g^{-1} egg of propylene glycol caused no adverse effects in mallards (Albers, 1977), although doses of 16.7% surface area petrolatum and 0.277 mg g^{-1} egg mineral oil did cause adverse effects in mallard and chicken, respectively (Couillard & Leighton, 1989; Stubblefield et al., 1995). We therefore limited our range of dilbit applications (Table 2.1) to amounts that would affect a minor proportion of egg surface area and thereby avoid serious effects on gas exchange that could confound our evaluation of dilbit toxicity. The numerous safflower oil control groups used in the cormorant study were no different from untreated eggs in terms of survival, deformity, or lesion incidence, as well as nearly all other physiological endpoints (21 of 23) and *cyp1a4* gene expression. We are therefore confident that our assessment of dilbit toxicity was not confounded by hypoxia effects and our dilbit applications were within the range of realistic exposures.

2.5.1. Survival and abnormalities

In contrast to other types of conventional crude oil, our highest dilbit applications (0.4 mg g^{-1} egg) resulted in no reduction in prehatch survival. Exposures in that range (0.5 mg g^{-1} egg or less) reduced hatching or prehatch survival for 10 of the 11 conventional crude oils evaluated in various studies (Couillard & Leighton, 1991a; Finch et al., 2011; Hoffman & Albers, 1984; Lee et al., 1986; Macko & King, 1980; Stubblefield et al., 1995). For instance, the 25% lethal dose values reported for five unweathered crude oils in one toxicity screening ranged from 0.04 to 0.26 mg g^{-1} egg for chicken embryos exposed from incubation days 9–13 (Couillard & Leighton, 1991a). However, drawing clear conclusions about the comparative toxicity of crude oils remains difficult because many previously published studies have limited their focus to oils that are known to be toxic, their use in an unweathered state (a worst-case scenario testing approach), their application in doses that often leave the amount on a per gram egg basis and the affected surface area unclear, and exposure during early incubation. For example, timing of oiling relative to embryonic development has been shown to be important to survival, with greater mortality occurring in embryos that are younger at exposure (Albers, 1978; Lewis & Malecki, 1984; McGill & Richmond, 1979). Prehatch mortality from petroleum toxicity is substantially reduced after the outgrowth of the chorioallantoic membrane, an extra-embryonic vascular membrane within the shell

membrane (Couillard & Leighton, 1991b; Walters et al., 1987). That could explain the high prehatch survival rates we observed in our chicken (days 13 and 16) and cormorant (day 16) embryos that were exposed to dilbit after chorioallantoic membrane outgrowth would have occurred. Walters et al.'s (1987) chicken study evaluated days 12–19 Prudhoe Bay crude oil exposure, similar to our day 13 exposure in chicken and our day 16 exposure in cormorant, and reported a median lethal dose (LD50) of 0.485 mg g^{-1} egg, so it is clear that dilbit is not more toxic than Prudhoe Bay crude oil, which has well-described embryotoxic properties. For embryonic Prudhoe Bay crude oil exposure during early incubation, before chorioallantoic membrane formation, published prehatch survival LD50 values available for chicken (0.019 mg g^{-1} egg; days 7–15 exposure) and mallard (0.320 mg g^{-1} egg; days 3–18 exposure; Hoffman & Albers, 1984; Walters et al., 1987) are below the highest dilbit amounts (0.4 mg g^{-1} egg) we tested in day 4 cormorant and found no significant mortality. This suggests that if cormorants in our study are comparably sensitive at least to mallards (Farmahin et al., 2013; Head et al., 2015), then dilbit is less toxic than Prudhoe Bay crude oil during this early developmental period. A caveat is that our study and those lethality thresholds cited consider only prehatch mortality, whereas embryo mortality from toxicity can occur near hatching (Finch et al., 2011; Franci et al., 2018). Besides the factor of when during incubation egg oiling occurs, the amount of time elapsed from when oil has been discharged into the environment until eggshell contamination occurs can decrease embryotoxicity, as indicated by increased hatching success as the oil weathers and its composition changes (Lewis & Malecki, 1984; Szaro et al., 1980). Indeed, the thresholds for survival effects of weathered crude oils can be greater than the highest amount of dilbit we tested (0.4 mg g^{-1} egg) or even amounts beyond exposure estimates available for crude oil (0.61 mg g^{-1} egg or less; Finch et al., 2011 [LD50 0.50 mg g^{-1} egg]; Goodchild et al., 2020 [no-observed-adverse-effect level (NOAEL) 1.99 mg g^{-1} egg; lowest-observed-adverse-effect level 3.98 mg g^{-1} egg]; Lewis, 1982; Stubblefield et al., 1995 [NOAEL 2.43 mg g^{-1} egg]). Together, these data suggest that our weathered Cold Lake blend dilbit was not more lethal to prehatch embryos than Prudhoe Bay crude oil and likely less lethal than other conventional crude oils, as would be expected based on egg oiling studies with weathered crude oil. Although our dilbit study focused on physiological effects and exposure indicators at dilbit application amounts at which effects on gas exchange were expected to be minimal, future LDx-type studies using the methods of Hoffman and Albers (1984) or Couillard and Leighton (1991a) would be well suited to

comparing unconventional crude oil toxicity, such as dilbit and related oil sands products, as well as the effects of weathering, with the toxicity of well-studied reference oils like Prudhoe Bay crude oil in model species.

Developmental deformities and gross pathological lesions observed at sampling were infrequent and not different according to dilbit treatment in either chicken or cormorant embryos in our study. In other egg oiling studies in model species with at least six conventional crude oils, PAC-containing mixtures, and oils containing concentrated toxic metals, developmental deformities and pathological lesions were well documented among the surviving embryos (see Couillard & Leighton, 1990b; Hoffman, 1979a, 1979c). These studies most frequently reported edema, liver necrosis, and developmental malformations of organs (e.g., gastroschisis), bone (e.g., ossification), and keratin (e.g., bill). These same studies typically exposed embryos at early to mid-development and observed the lesions and abnormalities described among survivors at doses at which significant prehatch mortality was also reported. Reports of decreased hatching success without increased frequency of abnormalities among hatchlings, even with teratogenic oils, suggest that such serious developmental deformities are likely to be lethal (Albers & Szaro, 1978; Finch et al., 2011). Although we did not perform a detailed histopathological analysis, our finding of a lack of effect of dilbit treatment on developmental abnormalities or gross pathological observations is consistent with low embryotoxicity.

2.5.2. Physiology

We found no effects on the growth of dilbit-exposed embryos evident from measurements on body size or organ size. A number of studies have reported reduced prehatch growth as indicated by body weight, crown–rump length, and bill or beak length in prehatch embryos exposed to some crude oils or PAC-containing oil mixtures (Couillard & Leighton, 1990a; Hoffman, 1978, 1979a, 1979b, 1979c; Hoffman & Albers, 1984). Alternatively, an increase in prehatch body mass or the ratio of body mass to crown–rump length following exposure may result from fluid accumulation that characterizes edema (Couillard & Leighton, 1990a, 1990b, 1991a, 1991b). Our measurements on body mass, crown–rump length, their ratio, and tarsus length indicated neither stunted growth nor fluid accumulation associated with edema. Chicken embryotoxicity studies with Prudhoe Bay crude oil and PAC mixtures have also shown

that increased relative liver size can result (Couillard & Leighton, 1990a, 1990b; Westman et al., 2013, 2014). However, we found no effect on liver somatic index in our dilbit-exposed cormorant embryos.

We investigated cardiovascular function because of hemotoxic and cardiotoxic effects associated with crude oil ingestion in free-living birds and contaminant dosing studies with embryos, yet we did not find meaningful effects of embryonic dilbit exposure on those endpoints. Our heart rate measurements were sensitive enough to detect the normal age-dependent increase in chicken embryo heart rate over the developmental period (Lierz et al., 2006); however, there were no differences among the treatments. Decreased heart rate in zebra finch embryos exposed to substantial amounts of weathered Mississippi Canyon 252 crude oil (more than 0.796 $mg\ g^{-1}$ egg) applied to the eggshell surface was recently reported (Goodchild et al., 2020). For that finding, the extent to which oil toxicity per se played a role as opposed to hypoxia is unclear. Our study included nontoxic oil controls as well as dilbit applications within both previously reported exposure estimates and hypoxia thresholds, and we found no such effect on heart rate with our dilbit exposures. Effects on heart beat systole and the appearance of the heart musculature have been reported in crude oil–exposed adult birds, including the double-crested cormorant (Harr, Reavill, et al., 2017; Harr, Rishniw, et al., 2017; Horak et al., 2017), and cardiac edema resulting in distension of the heart has been described in chicken embryotoxicity from Prudhoe Bay crude oil exposure (Couillard & Leighton, 1989, 1990a, 1990b). However, we found no such effect in our examination of cardiac mass, moisture content, or size resulting from dilbit exposure. Similarly, previous reports showed that increased spleen weight in Prudhoe Bay crude oil-exposed chicken embryos was associated with increased hematopoietic tissue within the spleen (Couillard & Leighton, 1990a, 1990b). The spleen mass of chicken embryos that we exposed to dilbit remained unaffected. Because erythrocyte damage and hemolysis can occur in crude oil–exposed nestlings and wild adult birds (Fallon et al., 2017; King et al., 2021; Leighton et al., 1983), and effects on hemoglobin or hematocrit have been described in avian embryos exposed to PAC and metal contaminants that may be present in petroleum (Hoffman et al., 1982; Kertész & Hlubik, 2002), we investigated hematocrit and hemoglobin. Although hemoglobin and hematocrit varied among chicken treatment groups, none differed from controls. It remains unclear whether the hemolytic injury and anemia that can occur with crude oil ingestion in free-living birds may also

play a role in petroleum embryotoxicity. In addition, it has been speculated that cardiovascular development is an important mechanism of early life stage mortality from exposure to PAC-like compounds (Farhat & Kennedy, 2019; King et al., 2021), so further work on *in ovo* cardiovascular function impairment with embryotoxicity is warranted. Nonetheless, the endpoints we measured that are indicative of cardiovascular function and linked to petroleum toxicity were not affected by our dilbit applications to the eggshell in the chicken and cormorant models.

2.5.3. *cyp1a4* mRNA gene expression in cormorant

We assayed *cyp1a4* mRNA because it is a well-known biomarker of exposure to PACs and related compounds, and is an indicator of embryotoxicity (Manning et al., 2013; Mundy et al., 2019; Wallace et al., 2020; Yang et al., 2010), yet we found only limited up-regulation of *cyp1a4* in the liver tissue of exposed cormorant embryos. The *cyp1a4* gene is one of two avian gene isoforms that encode the transcription of cytochrome P450 (CYP) subfamily 1A enzymes (Gilday et al., 1996; Mahajan & Rifkind, 1999; Walker et al., 2000). These enzymes play a prominent role in Phase I xenobiotic transformation, which generally increases substrate aqueous solubility, and they are concentrated in hepatic tissue (Mahajan & Rifkind, 1999; Wallace et al., 2020). Molecular assays targeting mRNA that codes for those CYP1A proteins, protein concentrations, and their enzymatic activity in avian embryo tissue have shown that exposure to individual PACs, mixtures thereof, or conventional petroleum itself up-regulates the aforementioned endpoints (Brandenburg & Head, 2018; Brunström et al., 1991; Head et al., 2015; Lee et al., 1986; Mundy et al., 2019; Walters et al., 1987). However, in our study, elevated normalized *cyp1a4* gene expression at sampling on day 22 only occurred in cormorant embryos exposed in early development (day 4) to the lowest dose of dilbit applied (0.1 mg g^{-1} egg). Enzyme induction of the CYP system has been shown 24 h after oiling chicken eggs with Prudhoe Bay crude oil and its fractions in amounts similar to ours, including 0.069–0.208 mg g^{-1} egg (Lee et al., 1986; Walters et al., 1987). Similarly, *in vitro* double-crested cormorant hepatocyte exposures to oil sands industrial activity-derived PAC mixtures for 24 h increased *cyp1a4* gene expression by two- to seven-fold in a dose–response relationship that increased with ∑PAC concentration (Crump et al., 2017; Mundy et al., 2019). In contrast, our cormorant embryos receiving greater amounts of dilbit (0.2–0.4 mg g^{-1} egg), including those in

which elevated PAC residue was detected (day 16, 0.4 mg g^{-1} egg), did not exhibit elevated *cyp1a4*. Our Cold Lake diluted bitumen contained alkyl–PAC congeners such as C1 benzo[a]pyrenes and C2 chrysenes whose parent compounds are known inducers of CYP activity by binding with the aryl hydrocarbon receptor (AhR; Brunström et al., 1991; Head et al., 2015), although the relative potency of alkylated congeners remains understudied. It is known, however, that the degree and perhaps duration of *cyp1a4* up-regulation by AhR agonists varies with species and toxicant potency. For instance, prolonged *cyp1a4* up-regulation from early embryonic injections (day 0) with potent chlorinated hydrocarbon AhR agonists has been demonstrated in chicken (54-fold increase) and ring-necked pheasant (6-fold increase) hatchlings, although the effect is less consistent for Japanese quail, a less sensitive species considered more similar to the double-crested cormorant in terms of AhR ligand-binding domain amino acid sequence (Farmahin et al., 2013; Yang et al., 2010). In comparison, our results show that *cyp1a4* expression is not up-regulated at incubation day 22 in cormorant embryos exposed to most of our environmentally realistic dilbit treatments. The PACs in crude oils are less potent AhR agonists than chlorinated hydrocarbon compounds (e.g., tetrachlorodibenzo-p-dioxin; Head et al., 2015), and elevated *cyp1a4* induction by the PAC benzo[k]fluoranthene has been shown to be transient (Brandenburg & Head, 2018). Accordingly, the observed *cyp1a4* elevation only in embryos exposed to 0.1 mg dilbit g^{-1} egg on day 4 in our study may best be explained by some unidentified time-dependent *cyp1a4* expression kinetics or feedback from other metabolic pathways. A wealth of *in vitro* hepatocyte CYP enzyme activity studies (ethoxyresorufin-O-deethylase) point to dose–responses modulated by toxicant, concentration, and exposure duration but are limited to exposures lasting several hours to days (Bastien et al., 1997; Egloff et al., 2011; Farmahin et al., 2016; Hervé et al., 2010; Manning et al., 2013). Longer term *in vivo* studies are few, but they have shown some nonmonotonic *cyp1a4* expression as a function of dose or time (Farhat et al., 2014; Yang et al., 2010), spurring speculation that *cyp1a4* suppression may occur in response to oxidative stress (Egloff et al., 2011; Farhat et al., 2014), as has been demonstrated in mammalian hepatocytes (Barker et al., 1994). The fact that *cyp1a4* expression was not up-regulated in the cormorant embryo dilbit treatment in which elevated PAC residue was detected clearly highlights the gene's limitations as a dose-dependent biomarker of *in ovo* dilbit exposure after 6 or more days, although it may be a useful indicator of lower levels of embryonic exposure (e.g., 0.1 mg dilbit g^{-1} egg) when tissue hydrocarbon residue may not be elevated.

2.5.4. PAC residue

Our data are the first to characterize PAC concentrations directly in the embryo following oil exposure. Crude oils generally contain several percent of PACs (Albers, 2003), and dosing studies have shown that this hydrocarbon fraction is principally responsible for embryotoxicity (Albers, 1977; Ellenton, 1982; Hoffman, 1979a; Hoffman & Gay, 1981; Walters et al., 1987), but the few reports that have examined PAC concentrations in oil-exposed eggs have described only their presence within whole egg homogenate (Goodchild et al., 2020; Hoffman & Gay, 1981). We found elevated PAC concentrations only in carcasses of cormorant embryos exposed to the highest application most immediate to sampling (0.4 mg g^{-1} egg at day 16), which is consistent with efficient biotransformation and elimination of PAC analytes during development by embryos exposed either earlier in incubation (day 4) or to lesser amounts (0.2 mg g^{-1} egg) of dilbit so that only concentrations indistinguishable from background remained at sampling. Considerable embryonic biotransformation capacity is supported by egg injection studies showing near-complete metabolism of PACs over the course of development (Crump et al., 2021; Näf et al., 1992). Metabolism by intra- and extra-embryonic tissues also helps explain the dissimilarity between the tissue residue PAC profile and that of the dilbit, in addition to physicochemical properties governing dilbit's passage through the eggshell pores and subsequent dissolution, diffusion, and sorption processes, which would result in embryonic uptake via the network of extra-embryonic blood vessels or yolk (Annas et al., 1999; Heinrich-Hirsch et al., 1990). Once such exposure has occurred, *in vivo* PAC kinetics vary by congener innately, and each is likely further affected as but one part of a complex mixture (Näf et al., 1992; Wallace et al., 2020).

Together, the PAC residue concentrations in embryos and in the dilbit indicates that exposure to PACs known to be highly toxic was limited, which is consistent with the absence of detectable adverse biological effects we found. Embryotoxicity of PACs has been well described through experiments directly injecting those compounds (Albers, 2006). Highly embryotoxic parent PAC congeners in our dilbit applications were present in amounts well below established effect thresholds (Albers, 2006; Brunström et al., 1991; Head et al., 2015). However, among such congeners, parent chrysene and benzo[a]pyrene are known to be embryotoxic in oils (Brunström et al., 1991; Hoffman, 1979c; Hoffman & Gay, 1981), and their C2–C3 alkylated congeners were relatively

abundant in both dilbit and the embryo tissue. Overall, our dilbit sample was generally similar to the composition of a Cold Lake blend sample described previously (National Academies of Sciences, Engineering, and Medicine [NASEM], 2016), but ours had over eight-fold higher ∑C0–C3 chrysenes. Crude oils are rich in PACs with alkyl groups, which can have different toxicity compared with the parent compound (NASEM, 2016; Wallace et al., 2020); however, beyond studies in fish, alkyl–PAC toxicity remains under-researched, let alone in birds exposed to complex mixtures. At least for their better studied parent compounds, other PAC classes that we found were concentrated in dilbit but have not been shown to be very individually toxic included naphthalenes, phenanthrene, and dibenzothiophenes (Brausch et al., 2010; Brunström et al., 1991). Of course, embryotoxicity from whole oils can be greater than the toxicity of their constituent compounds or fractions (Ellenton, 1982; Hoffman, 1979c). Still, our dilbit was only 0.8% $\sum_{51}$PACs w/w, whereas oils may range from 0.2 up to 7% PAC (Albers, 2003). Except for chrysenes and benzo[a]pyrenes, the low concentrations of PAC congeners recognized for their embryotoxicity and the overall low percentage of $\sum_{51}$PACs in our Cold Lake blend sample suggest low potential for toxicity, which is altogether consistent with the absence of detectable adverse biological effects in our study.

2.6. Conclusion

To our knowledge, our study is the first to examine avian eggshell contamination with a diluted bitumen, an increasingly common oil sands product. To date, avian embryotoxicity from crude petroleum has been demonstrated, but only with approximately 11 conventional crude oils. Furthermore, our study included factors that are critical for predicting the effects of real-world exposure scenarios, including light weathering of the dilbit, a primary focus on a wild species, multiple exposure time points across the period of egg incubation, a battery of physiological endpoints, and chemical (PAC concentrations) and molecular (*cyp1a4* mRNA) exposure indicators. We found that the amount and timing of weathered Cold Lake blend dilbit exposure that could be expected in an oil spill scenario were sublethal to the prehatch embryo and did not affect critical physiological endpoints related to development. This is despite the fact that dilbit penetrated the eggshell and membranes, as shown by trace PAC analysis of the carcass and hepatic *cyp1a4* mRNA expression. Although we detected no specific adverse prehatch effects, evaluations of other dilbit and oil sands products and more

detailed study of other sublethal or delayed effects, such as on metabolism (Dorr et al., 2019; Westman et al., 2013), are warranted given our evidence of embryonic PAC exposure. Furthermore, both the fact that oil PACs were rapidly metabolized *in ovo* and the fact that *cyp1a4* gene expression, indicative of PAC exposure, was limited indicates that post oil spill biomonitoring should include multiple forensic markers of exposure when possible. Because of the sensitivity of the avian embryo to petroleum contamination, the present report constitutes an important first step toward evaluating the potential risk posed to avian wildlife with habitats through which dilbit and related bitumen products are transported in large quantities.

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Chapter 3.

Polycyclic aromatic compound and trace metal element residues in *Mytilus* mussels at marine wildlife hotspots on the Pacific coast of Canada

This chapter was submitted to Environmental Pollution July 15, 2022 as reproduced here with minor edits for clarity. Supplemental Tables (A.1−A.3) and Figures (A.1−A.4) appear in this thesis in Appendix A. A subsequent version of this chapter revised with reviewer feedback was published November 17, 2022 as Environmental Pollution 316:120624 at DOI:10.1016/j.envpol.2022.120624 © 2022 The Authors. The Supplementary Data File is available at the above DOI.

3.1. Chapter abstract

The Pacific coast of Canada has a rich marine fauna and a growing human population with increasing potential for pollution releases, but there is currently little overlap between marine wildlife hotspots and ongoing biomonitoring efforts, especially for less bioaccumulative contaminants (e.g., polycyclic aromatic compounds [PAC], trace metals [metals]). We surveyed PACs and metals at marine bird breeding colonies in coastal British Columbia in 2018 by analyzing chemical residues in the soft tissue of bivalve *Mytilus* sp. mussels collected from stations (n = 3) at seven sites. The concentration of sum PACs ($\sum_{43}$PAC) and individual high molecular weight (HMW) PACs were highest at the Second Narrows bird breeding colony in Vancouver Harbour, a highly urbanized and industrialized port within the Salish Sea. For other priority conservation areas, two Salish Sea and three Pacific Ocean coast breeding colonies, PACs were generally lower. However, $\sum_{43}$PAC, $\sum_{HMW}$PAC, and several HMW congeners at the remote site of Triangle Island, a Marine National Wildlife Area, were not significantly different from Second Narrows. These site differences were driven by HMW PACs and the dominant sources are likely pyrogenic rather than petrogenic, as suggested by PAC profiles, proportion of parent PACs, diagnostic ratios, and source-indicator congeners. For metals, site differences were found for seven out of eight priority metals, but principal component analysis indicated that site differences are likely related to environmental and biological variables including salinity, condition index, water

temperature, and shell length. Our survey across a broad coastal region shows that PAC and metal biomonitoring programs with mussels should include wildlife hotspots where the exposure of protected vertebrate species to pollutants with low bioaccumulation potential would be less obvious, and shows that collection of data on key co-variates (e.g. lipid content, salinity) will be critical to tracking long-term trends and detecting pollution release events.

3.2. Introduction

Marine wildlife hotspots are foci of conservation and resource management efforts. In British Columbia, Canada as elsewhere, wildlife research and monitoring programs have prioritized persistent and bioaccumulative (e.g. persistent organic pollutants, mercury) contaminants over other less bioaccumulative contaminants to which wildlife may nonetheless be exposed (Bianchini et al., 2022; Elliott and Elliott, 2016; Gómez-Ramírez et al., 2014; Miller et al., 2014, 2015; Noble and Elliott, 1986). Two classes of less bioaccumulative contaminants are polycyclic aromatic compounds (PAC) and trace metal elements (hereafter, simply called metals), both of which are released from a wide range of human inputs superimposed on some natural background occurrence (Bjerregaard et al., 2022; Neff, 1979). Dominant anthropogenic PAC releases to the environment are from combustion (i.e. pyrogenic), petroleum (i.e. petrogenic), or some combination of those and other minor sources. Likewise, metal-containing materials may be released by a wide range of human activities, and the presence of metals such as vanadium, nickel, and cadmium in oils and fuels means that pyrogenic emissions or petroleum spills release metals as well as PACs (Chiffoleau et al., 2004; Jervis et al., 1982; Zhao et al., 2021). The ubiquity of PAC and metal pollution sources means they can be relevant to the health of wildlife and their habitats and prey far from urban or industrialized areas (Harris et al., 2011; Miles et al., 2007; Sileo and Fefer, 1987). Sampling wildlife tissues, including non-lethal matrices (e.g. blood, fur, feathers, feces, or eggs), for PACs and metals is possible, but labor intensive (Bence and Burns, 1995; Burger et al., 2008; Harris et al., 2011; Pérez et al., 2008), and active metabolism and regulation of such compounds *in vivo* complicates estimation of exposure and inferences about sources (Bjerregaard et al., 2022; King et al., 2022; Meador et al., 1995; Ruberg et al., 2022).

Contaminants surveillance in coastal ecosystems has often focused on bivalve mussels as a tractable sentinel, especially for PACs and metal contamination that is otherwise challenging to study in vertebrates. Mussels are good sentinels for such environmental contaminants because they are widely distributed, sessile filter feeders with limited metabolic biotransformation rates, provide a time-integrated contaminant signal, and are a food source to marine wildlife (Beyer et al., 2017). Biomonitoring programs and projects with mussels have produced informative data on coastal PACs and metal contamination globally as well as in our on the Pacific coast of Canada (Farrington et al., 2016; Willie et al., 2017), but seldom with explicit sampling at protected marine wildlife areas under provincial, federal, or Indigenous nations management. The need for such surveillance data is acute in regions like British Columbia where the human population is increasing and a concomitant intensification of maritime activity is reflected by initiatives to expand the number of petroleum tanker and container ship terminals (BC Government, 2021; Global Container Terminals, 2020; Hafner et al., 2005; NEB, 2016; Van Metre et al., 2000).

Data on the occurrence of less bioaccumulative contaminants from a suitable indicator organism like mussels at wildlife hotspots could efficiently characterize the extent and potential sources of contamination that might affect wildlife and related marine resources there. To address that data gap, we collected *Mytilus* sp. mussels on Canada's Pacific coast at several remote, protected wildlife hotspots and one unprotected urban wildlife hotspot to analyse the soft tissues for PAC and metals residue. Our immediate goals were to 1) generate a snapshot of spatial differences that may exist among sites and 2) identify potential sources of contamination. We hypothesized that PAC and metal concentrations would be lower in mussels collected from remote, protected wildlife hotspots compared to a highly industrialized and urbanized site called Second Narrows in Vancouver Harbour. More generally, we aimed to inform similar contaminants monitoring studies or Mussel Watch-type programs (Beyer et al., 2017), and, for our study area, assess whether more concerted monitoring at remote conservation areas is warranted while establishing an initial baseline to serve as future reference in the event of pollution releases, such as an at-sea oil spill or increased regional combustion emissions.

3.3. Materials and methods

We collected Pacific blue mussel species complex (*Mytilus* sp.) individuals from 3 May to 25 July 2018 with protocols adapted from the US Mussel Watch program at seven coastal marine bird breeding colonies (Figure 3.1, Table 3.1): Second Narrows, Mandarte Island, Mitlenatch Island, Cleland Island, Lucy Island, Pine Island, and Triangle Island (Fisheries and Oceans Canada collection permit XMCFR 19 2018) (Apeti et al., 2012; Coan et al., 2000; Lanksbury and West, 2012; Lauenstein and Cantillo, 1998). Except for the Second Narrows site in Vancouver Harbour, all bird breeding

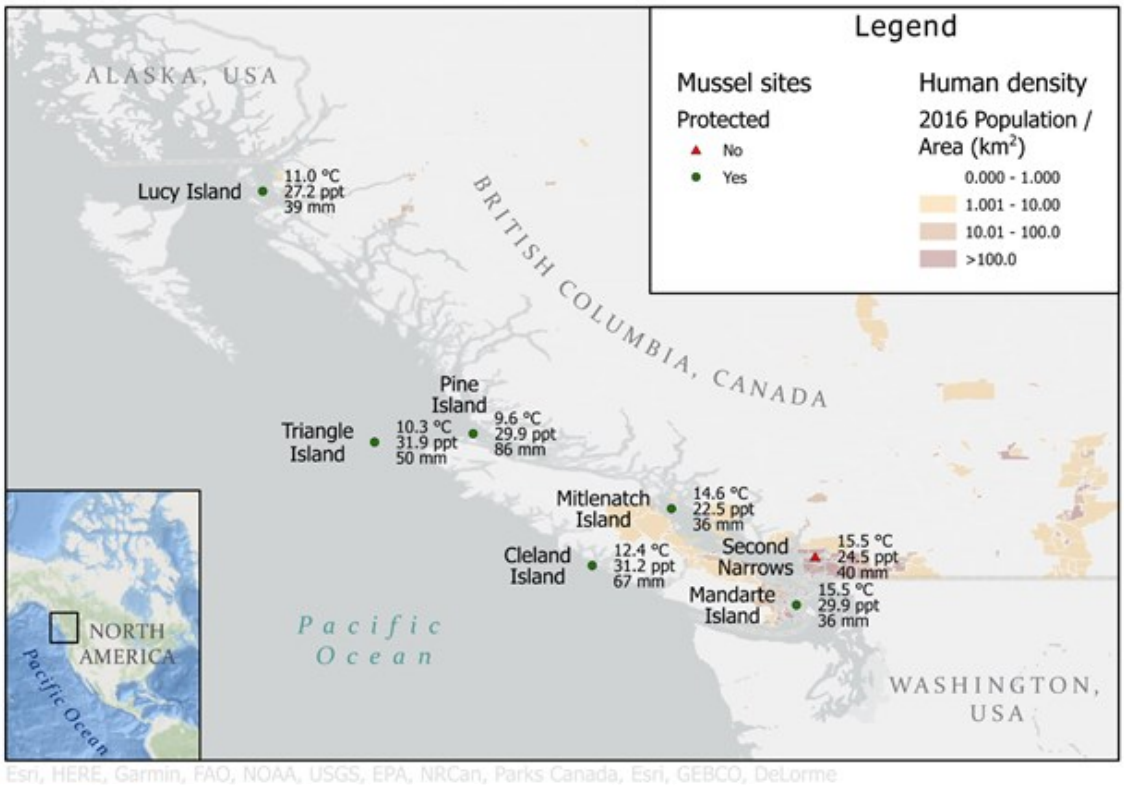

Figure 3.1 ***Mytilus*** **sp. mussel sampling sites at bird breeding colonies on the Pacific coast of Canada (2018), each with three replicate collecting stations. Sites at protected conservation areas are indicated by green circles (○) and the Second Narrows site in Vancouver Harbour without designated conservation status is a red triangle (△). Each site is labeled with average water temperature (°C), salinity (‰ NaCl), and shell length (mm). The shaded transparency layer shows human density (population/km^2).**

colonies were First Nations, federal, or provincial protected conservation areas. At each bird breeding colony site, intertidal mussels were collected by hand from three spatially

discrete stations 100 to 1,200 m apart where dense mussel beds were found. Usually these were distinct intertidal mussel beds, however, at Lucy Island and Mandarte Island multiple beds were unavailable, so mussels were sampled at three discrete points along single along-shore transects 19 and 44 m in length, respectively. In this way, each site had three replicate collecting stations (n = 3) regularly exposed to open water on a uniform type of substrate from which mussels were taken (median collection station area 10 m^2; range 2–50 m^2). At each collecting station, researchers recorded water temperature and salinity with handheld probes (Cat. No. RK-19601-21; Cole-Parmer) and targeted mussels that were within 70 to 90% of the maximum shell length apparent at a given station to reduce within-station variability (Lobel et al., 1991a). Specimens were collected by cutting their byssal threads with a knife and placing one to several individuals in zip top polypropylene bags. Although ice was unavailable to some field crews, the collected specimens were double-bagged and kept in coolers, packed in ice as soon as possible, and received by the laboratory within 3 days for storage at −20°C. For each collecting station, mussel shell external dimensions, soft tissue percent moisture, and dry weight was measured in a random subset of individuals (n = 5) not analysed for PAC residue (Supplementary Data File). Condition index was calculated according to Lobel et al., (1991a).

Mussel tissue was pooled for each collecting station by randomly selecting individual mussels, allowing them to thaw briefly, opening the shells, and shucking the soft tissue directly into certified amber glass jars (I-Chem™; Cat. No. 340-0250; Thermo Scientific). While shucking the mussels analysed for PACs, laboratory blanks consisting of empty jars and n-hexanes-filled jars were placed in a nearby fume hood. The resulting PAC tissue pools consisted of 10 individuals with shell length >5 cm from Cleland and Pine or 20 individuals with shell length <5 cm from other sites (Lanksbury and West, 2012), less a 3 mm strip of mantle tissue from a subset of 3 mussels for every 10 for other analyses not reported here. Tissue pools, which ranged in mass from 13.4 to 243.6 g, and laboratory blanks were shipped on dry ice to the Centre for Oil and Gas Research and Development (COGRAD [ISO-17025 certified]; Winnipeg, MB, Canada) for homogenization and PAC analysis. Likewise, the remaining mussels were shucked and pooled for metals analysis: 4 to 5 individuals with shell length >5 cm from Cleland and Pine or 6 to 10 individuals with shell length <5 cm from other sites (Lanksbury and West,

2012). Pools ranged in mass from 4.8 to 97.1 g and were shipped to the National Wildlife Research Centre (NWRC) for homogenization and analysis.

We measured 43 PAC analytes in tissue pools, including 16 parent polycyclic aromatic hydrocarbon compounds, dibenzothiophenes, and their alkylated analogs by gas chromatography coupled with a triple quadrupole mass spectrometer used in multiple reaction monitoring mode (GC–MS/MS; COGRAD Contract No. 3000686444). Soft tissue pool extractions, cleanup, and GC–MS/MS analysis were performed as described in Idowu et al. (2018) and Xia et al. (2021). Lipid content was determined gravimetrically and reported on a percent wet sample weight basis. The full dataset of target analytes, method limits of detection (MLOD), and concentrations in mussel tissue pools are listed in the Supplementary Data File. We give all PAC concentrations on a blank-corrected, wet sample weight basis in ng analyte per g homogenized soft tissue pool.

For metals analysis, tissue pools were homogenized and freeze-dried. Aliquots were analyzed with a direct mercury analyzer (DMA-80 evo; Milestone Srl) for total mercury as described by Hebert and Popp (2018) or microwave digested in acid (2 mL 70% HNO_3 per dg sample dry weight; MARS 6, CEM), diluted to a volume of 10 mL with ultra-pure water, and analyzed at 10X dilution by ICP-MS (NexION 300D; PerkinElmer) as in Thomas et al. (2021) for total concentration of 25 trace elements (NWRC Laboratory Services Project No. JE19-2020). Of those, we report on eight metals that are most relevant to marine pollution monitoring in mussels, especially with respect to petroleum spills and fossil fuel combustion, are considered a priority from a regulatory perspective, are potentially toxic to mussels and wildlife, and are least likely to be confounded by the potential presence of sediment in the intestinal tract (Beyer and Meador, 2011; Bou-Olayan et al., 1995; Celo et al., 2015; Chiffoleau et al., 2004; Davies and Vethaak, 2012; Fowler et al., 1993; Kimbrough et al., 2008; Lobel et al., 1991b; USEPA, 2021): cadmium, copper, lead, mercury, nickel, selenium, vanadium, and zinc. The full data set with all 26 metals, MLODs, and their concentration in mussel tissue pools are given in the Supplementary Data File. All metal concentration data is given on a dry sample weight basis as µg analyte per g homogenate.

Data analysis was conducted in RStudio version 2021.09.0 (R Ver 4.1.1; $\alpha = 0.05$). All metal concentrations were above MLODs; PAC concentration data included

below MLOD observations. Accordingly, statistical analysis for PACs focused on analytes detected in all samples at all sites or sum concentrations such that below MLOD observations were considered negligible and handled as zeros. For certain calculations we used methods for censored data (<MLOD, i.e. an unknown value from zero to MLOD) or substitution methods as detailed below (Helsel, 2012). All chemical concentration data was natural log transformed as Ln(x) or Ln(x + 1) to better conform to statistical model assumptions of normal residuals and homogeneity of variance.

First, we compared mussel soft tissue percent lipid and percent moisture among sites by Analysis of Variance (ANOVA, n = 3), which indicated no difference among sites in percent lipid ($F_{6,13}$ = 0.57, p = 0.750, one high outlier identified with Dixon's Test [outliers package in R] was removed from this test and subsequent among site comparisons) on percent moisture ($F_{6,14}$ = 1.18, p = 0.371). Exploratory linear regression models showed evidence that sum PACs or individual congeners at higher concentrations were correlated with percent lipid. Therefore, to answer our primary research question of whether PAC concentration differed by site and control for the potential variation in lipid content (Elliott and Norstrom, 1998; Hebert and Keenleyside, 1995), we first performed an analysis of covariance (ANCOVA: Ln[*PAC analyte conc.*] ~ *Site* + *Percent lipid*) to determine whether percent lipid was a significant covariate. If it was, we tested for differences in marginal means among sites with the ANCOVA model (emmeans package in R with Satterthwaite's method for degrees of freedom and Tukey's adjusted p values for multiple pairwise contrasts). If percent lipid was not significant as a covariate, we used an ANOVA to test for differences with *Site* as the sole predictor in the model, again using emmeans for post hoc pairwise contrasts. Limited sample size (n = 3) precluded any meaningful evaluation of whether correlation between percent lipid and PAC concentration was site-dependent (i.e. a *Site* × *Percent lipid* interaction in the ANCOVA model), and ANCOVA/ANOVA tests were limited to sum concentrations or the twelve individual PAC analytes detected at all station and sites (i.e. n = 3).

To calculate mean concentrations of individual congeners for each site where there were 2 or 3 detections (i.e. ≥MLOD observations) without introducing bias through substitution (e.g. 0, ½ MLOD, and so forth), we used regression on ordered statistics (ROS), a method for censored data (NADA package for R) (Helsel, 2012). ROS mean concentrations were calculated for 25 to 41 PAC analytes at our different sites

(Supplementary Data File). These values were used to plot PAC profiles by site and were also summed as an additional total PAC metric.

To make inferences about potential pyrogenic and petrogenic PAC sources, we considered several lines of qualitative (e.g. homologous series distributions in PAC profiles) and quantitative (e.g. parent compound versus alkyl homologs, tracer compounds like dibenzothiophenes) evidence. The latter included three well-described diagnostic ratios that can help discriminate between pyrogenic and petrogenic sources: 1) ∑[Select 3–6 ring parent PACs] / ∑[Select C_0–C_n homologous series] with slight modifications described in the footnotes of Table A.1, 2) [fluorene] / [fluorene + pyrene], and 3) [anthracene + phenanthrene] / [anthracene + phenanthrene + C1 phenanthrene] (Meniconi and Barbanti, 2007; Wang et al., 1999b; Yunker et al., 2002). Subsequently we refer to those ratios as Wang et al.'s (1999b) index, Fl / [Fl + Py], and [Anth + Phen] / [Anth + Phen + C1 Phen], respectively. For calculating diagnostic ratios, some observations were below MLODs, so we substituted ½ the MLOD so as not to bias ratios by substituting zero or the MLOD.

For metal concentrations, we compared among sites with ANOVA models (ANOVA: Ln[*Metal analyte conc.*] ~ *Site*) as described for PACs, followed by principal components analysis (PCA, factoextra package in R) to further examine major axes of variation in the metals data. The PCA coordinates for individuals (i.e. collecting stations) projected onto the first and second principal components were correlated (Hmisc package in R) with relevant chemical, biological, and environmental response variables: sum PAC concentrations, lipid content, shell length, soft tissue dry mass, condition index, salinity, water temperature, and collection date. Correlations (n = 21) used Spearman's ρ (because of limited improvement to the normality of response variable data by transformations) and the Benjamini-Hochberg false discovery rate correction was applied to the 16 resulting p values. For the 44 m sampling transect at Mandarte Island, replicate salinity and water temperature measurements were not available, but because all three collecting stations were in close proximity and those measurements usually varied little within sites (as suggested by F ratios, salinity $F_{6,12}$ = 72.178, water temperature $F_{6,12}$ = 84.671), the single measurement at station one was also used for nearby stations two and three in the correlation so as not to reduce sample size unnecessarily.

3.4. Results and discussion

We hypothesized that PAC and metal residue in mussel tissue would be higher at the urban, industrialized Second Narrows site in Vancouver Harbour compared to six other remote conservation areas (Figure 3.1). We found that significant differences among sites depended on the congeners examined and that the sum of the 43 PAC analytes ($\sum_{43}$PAC), sum high molecular weight (HMW) PACs ($\sum_{HMW}$PAC), and several HMW congeners were not significantly lower than Second Narrows at some remote

Table 3.1 Study sites on the Pacific coast of Canada (2018), with a brief description of their locations and data from analysis of the mussel soft tissue pools (n = 3 unless otherwise indicated by footnote): percent lipid, total number of polycyclic aromatic compound (PAC) analyte detections (out of 43 analytes times three sites), sum PACs ($\sum_{43}$PAC), sum of PAC congener regression on ordered statistic means ($\sum_{ROS}$PAC), and sum of the 12 PACs detected at all stations and sites ($\sum_{12}$PAC).

Site	Location	Lipid content (%)		Total PAC congener detections	$\sum_{43}$PAC (ng/g)		$\sum_{ROS}$ PAC (ng/g)	$\sum_{12}$PAC (ng/g)	
		Mean	SD		Mean	SD		Mean	SD
Second Narrows	Urban fjord within Salish Sea	0.22	0.10	118	20.10	13.38	20.07	12.24	8.04
Mandarte	Salish Sea	0.20	0.02	81	3.80	1.58	3.61	2.91	1.42
Mitlenatch	Salish Sea	0.28 [a]	0.06 [a]	98	5.73	1.12	5.69	4.48	1.30
Cleland	Coast	0.19	0.13	73	2.59	0.31	2.59	2.21	0.22
Lucy	Coast (Sound)	0.22	0.04	90	4.87	2.10	4.68	3.47	1.21
Pine	Coast	0.25	0.02	72	4.19	1.59	4.12	3.83	1.50
Triangle	Coast (Offshore)	0.18	0.06	98	10.39	4.65	10.33	6.23	1.62

[a] Single outlier removed = 0.92, n = 2

SD = Standard deviation

areas such as Triangle Island, a Marine National Wildlife Area, whereas low molecular weight (LMW) PACs generally did not differ (e.g. $\sum_{LMW}$PAC, naphthalenes) among sites (Table 3.1, Figure 3.2). The combined evidence from PAC congener distribution,

diagnostic ratios, and tracer compounds all indicates that PACs likely originate from combustion-dominated sources. Seven of the eight metals tested were significantly different among sites, but Second Narrows was never the highest and PCA indicated that residues were likely related to biological and environmental variation among sites (Figure 3.3). We present and discuss those findings in detail below.

3.4.1. PACs in mussel tissue

Of the PACs detected in mussel tissue pools, total concentrations varied significantly by site. The sites with the most PAC analytes detected (i.e. >MLOD) were Second Narrows, followed by Triangle Island and Mitlenatch Island (Table 3.1). Each of the 43 PAC analytes were detected in at least one sample in our study, so concentrations of the 43 PAC analytes detected were summed (i.e. $\sum_{43}$PAC) (Table 3.1; Figure A.1). $\sum_{43}$PAC varied significantly among sites ($F_{6,12} = 6.295$, $p = 0.003$) and percent lipid was a significant covariate ($F_{1,12} = 4.983$, $p = 0.045$) (Figure 3.2 A): marginal means for $\sum_{43}$PAC were significantly greater at Second Narrows than Lucy, Pine, Mandarte, and Cleland, but not different at Second Narrows compared with Triangle or Mitlenatch. Mean $\sum_{43}$PAC at Cleland was also significantly lower than Triangle and Mitlenatch. Congeners detected infrequently, at only one of the three sampling stations for a given site, were present at low concentrations and their influence on $\sum_{43}$PAC concentrations was minor, as indicated by the similarity between $\sum_{43}$PAC (n = 3) and sum ROS mean PAC concentrations ($\sum_{ROS}$PAC, n = 2 to 3) (Table 3.1). Twelve PAC analytes were detected at all stations and sites, and their summed concentration ($\sum_{12}$PAC) showed a pattern analogous to that found for $\sum_{43}$PAC (Table 3.1; Figure A.1). For $\sum_{12}$PAC, percent lipid was not a significant covariate but there was significant among-site variation ($F_{6,14} = 3.288$; $p = 0.031$) (Figure 3.2 B). However, post-hoc contrasts on marginal means showed fewer between-site differences for $\sum_{12}$PAC than for $\sum_{43}$PAC: the only significant pairwise contrast was that Cleland was lower than Second Narrows for $\sum_{12}$PAC. Overall, these results showed that total PAC concentrations differed by site, and also suggested that certain congeners and their covariation with lipid content may contribute to these differences.

To assess patterns in individual PAC concentrations, we analysed among-site variation for each of the 12 PACs detected at all sites (i.e. n = 3, Figure A.1, A.2) with, or without, percent lipid as a covariate, and we qualitatively compared PAC profiles for

each site generated from ROS mean concentrations (i.e. n = 2 to 3, Figure A.3). None of the naphthalene series analytes with two aromatic rings were significantly different among sites: naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and C4 naphthalenes, ($F_{6,14}$ = 0.463 to 0.809, p = 0.580 to 0.824; Figure A.2 A–D). In contrast, differences among sites were significant for all three to four ring compounds except

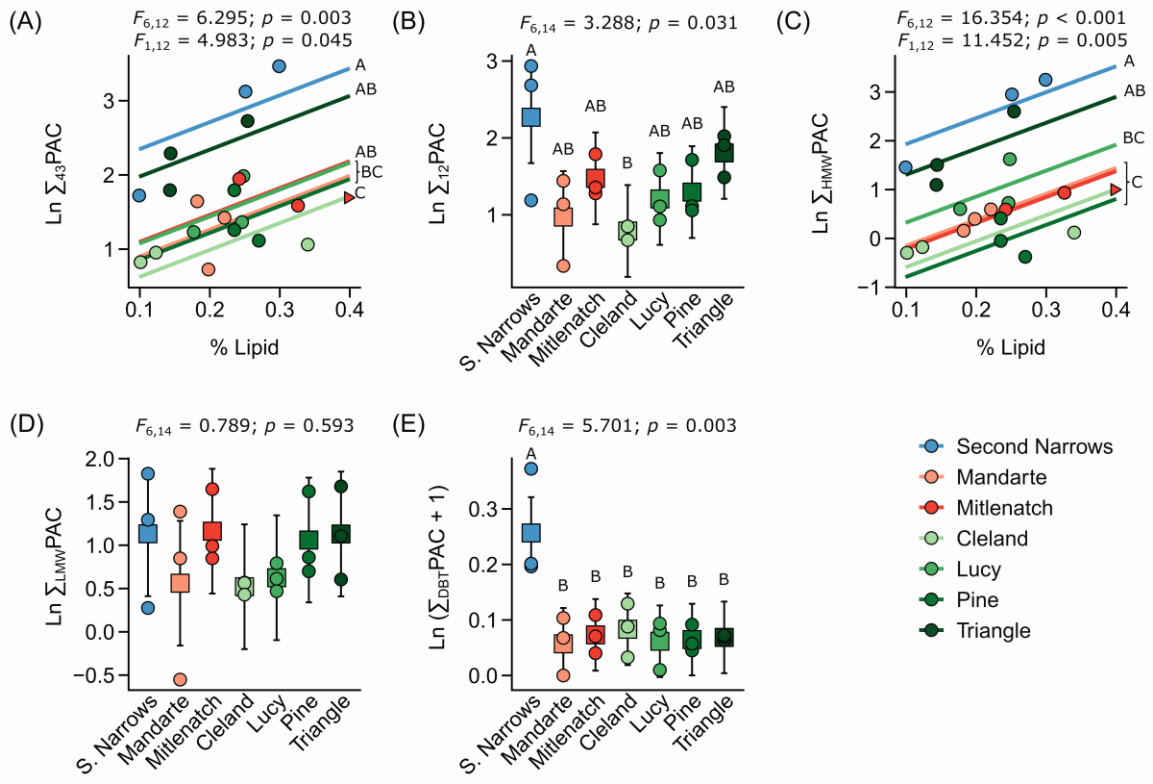

Figure 3.2 **Among site (n = 3) comparisons of natural log-transformed sum polycyclic aromatic compound (PAC) concentrations (ng/g wet weight) in mussel soft tissue pools from the Pacific coast of Canada (2018) by ANOVA (B, D, & E; means are shown as squares [□] with 95% confidence intervals), or where significant, ANCOVA with percent lipid (A and C; regressions plotted as lines). *F* test statistic and p values are given above each plot (α = 0.05), and significant pairwise contrasts are labeled with differing letters. A percent lipid outlier from a Mitlenatch Island sample was removed and its position on the vertical axis is plotted as the right-facing triangle (▷). Abbreviations in the axes are as follows: Second Narrows (S. Narrows; B, D, & E), sum of the 43 PAC analytes ($\sum_{43}$PAC; A), sum of the 12 PACs detected at all stations and sites ($\sum_{12}$PAC; B), sum high molecular weight PACs ($\sum_{HMW}$PAC; C), sum low molecular weight PACs ($\sum_{LMW}$PAC; D), and sum dibenzothiophenes ($\sum_{DBT}$PAC; E).**

2-methylphenanthrene ($F_{6,14}$ = 2.827 to 5.133, p = 0.051): fluorene, 3-methylphenanthrene, C2 phenanthrenes, and C1 chrysenes differed significantly by site ($F_{6,14}$ = 3.580 to 5.133, p = 0.006 to 0.023) (Figure A.2 E, H, I, & L), and phenanthrene, C3 phenanthrenes, and fluoranthene covaried significantly with lipid content and differed significantly by site ($F_{1,12}$ = 7.074 to 12.937, p = 0.004 to 0.021; $F_{6,12}$ = 9.350 to 21.210, p < 0.001) (Figure A.2 F, J, & K). Of those PACs with a significant difference among sites, significant pairwise contrasts varied by congener, but concentrations were always highest at Second Narrows, and Triangle was never significantly lower than Second Narrows (Figure A.2). PAC profiles of ROS mean concentrations by site (Figure A.3 & Supplementary Data File) additionally suggested elevated benz[a]anthracene and chrysene at Second Narrows and Triangle Island, which are four ring PACs. Overall, naphthalene is a major component of $\sum_{43}$PAC and $\sum_{12}$PAC concentrations but exhibits large variation within and among sites and does not correlate with tissue lipid content (Figures A.1–A.3). In contrast, two other major congeners, phenanthrene and fluoranthene, which both correlate with lipid, and perhaps the influence of other three to four ring compounds appear to account for $\sum_{43}$PAC's significant correlation with lipid and pairwise site differences (Figures 3.2, A.1–A.3).

Because heavier PACs with more rings appeared to drive among-site differences in total PAC concentrations, we examined how heavier PACs might affect our comparison of total PAC concentrations among sites. Accordingly, we split all PACs into two categories: one category consisting of LMW, lower Kow parent PACs with two to three rings and their alkyl homologues (naphthalene through fluorene in Figure A.3; MW 128.2–166.2, log Kow 3.3–4.2) and a second category with HMW, higher Kow three to six ring PACs and their alkyl homologues (anthracene through dibenzo[a,h]anthracene in Figure A.3; MW 178.2–278.3, log Kow 4.5–6.7). For each LMW and HMW PAC category, concentrations were summed to generate $\sum_{LMW}$PAC and $\sum_{HMW}$ PAC for each site. Accordingly, $\sum_{HMW}$PAC with the 32 heavier PACs was significant for site ($F_{6,12}$ = 16.354, p < 0.001) as well as covariation with percent lipid ($F_{1,12}$ = 11.452, p = 0.005) (Figure 3.2 C). In contrast, $\sum_{LMW}$ PAC with the 11 lighter PACs was not significantly different among sites by ANOVA on $\sum_{LMW}$ PAC ($F_{6,14}$ = 0.789, p = 0.593; no effect of percent lipid; Figure 3.2 D). This shows that higher molecular mass, higher Kow PAC congeners drive the differences among sites we observed with $\sum_{43}$PAC concentrations, and why differences among sites were less significant when examined as $\sum_{12}$PAC

concentrations, which includes several naphthalenes and fluorene. Any LMW-HMW dichotomy is artificial, and one reason why is that molecular mass and log Kow can increase greatly within a homologous PAC series with increasing number of alkyl carbon atoms (e.g. R < 1-methyl-R < 1-ethyl-R). However, because our dataset was dominated by parent PACs (Figure A.3, Table A.1), splitting analytes into LMW and HMW categories served to answer the question of whether spatial differences may be molecular mass, and therefore Kow, specific.

Mussels are exposed to PACs via seawater, a medium in which these compounds' behavior is complex due to chemical, physical, and biotic factors. Among them, aqueous solubility and hydrophobic partitioning to lipid and carbon-rich organic matter are critical (Neff, 1979). It is PACs in the aqueous phase and those adsorbed to filtered particles, especially phytoplankton, which mussels assimilate (Björk and Gilek, 1999; Gossiaux et al., 1998; Maldonado et al., 1999; Wang and Chow, 2002). Among PACs, LMW compounds tend to be more soluble and therefore abundant in the dissolved phase, whereas HMW compounds are less water soluble and adsorb more readily to suspended particulate matter (Hellou et al., 2005; Law et al., 1997). The lipid content of mussel tissue can further affect PAC uptake and kinetics through lipid partitioning (Bruner et al., 1994). We found differences in mussel tissue PAC concentrations for individual HMW compounds and $\sum_{HMW}$PACs. Researchers often invoke Kow to explain mussel tissue PAC distributions found in field studies, for instance depletion of heavier PAC congeners (e.g. ≥252.3 MW, 5–6 ring) (Baumard et al., 1999b, 1999a, 1998a; Francioni et al., 2007). Likewise, the fact that we found site differences consistent with our hypothesis exclusively for HMW compounds supports the idea of congener mass and Kow influencing tissue residue. However, few individual congeners actually covaried significantly with lipid content, which suggests that the importance of lipid partitioning is congener-dependent or possibly related to concentration. In our study, lipid covariation was strongest for phenanthrene and fluoranthene which were among the highest concentrations observed. It is unclear whether the high within-site variability for the naphthalenes (parent naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and C4 naphthalene) and lack of site variation in $\sum_{LMW}$PACs reflects widespread and variable LMW PAC sources at our sites or suggests some unidentified factor decoupling LMW PAC from the HMW PACs that did differ among sites. Our data shows that PAC pollution monitoring studies with mussels should avoid simplistic

comparisons of summed PAC concentrations alone which risks missing information about spatial differences, and should include lipid content as a covariate where statistically supported (Francioni et al., 2007; Hebert and Keenleyside, 1995).

3.4.2. Toxicological implications of PAC residue

PAC residues measured in mussel tissue at our study sites in 2018 were low and are unlikely to pose an immediate toxicity risk to mussels and wildlife. Genotoxicity biomarkers such as micronuclei are among the most sensitive adverse effects measurable in *Mytilus* mussels and correlate with contamination in field studies reporting total PAC in the range of tens to hundreds of ng/g, but the PAC concentrations we observed ($\sum_{43}$PAC 2.59–20.10 ng/g) are on the low end of that range and are below effect thresholds from laboratory PAC exposures (e.g. approx. ≥108 ng/g ww) (Francioni et al., 2007; Sundt et al., 2011; Turja et al., 2020). Mussels exposed to heavy petroleum contamination would be expected to have total PAC in the range of at least hundreds of ng/g ww (Bence and Burns, 1995; Hwang et al., 2014; Soriano et al., 2006). At those high PAC concentrations, toxic effects on mussel physiology may occur, such as on antioxidant capacity, immune function, and filtration rate (Dyrynda et al., 2000; Eertman et al., 1995; Grundy et al., 1996; Turja et al., 2020). Beyond adverse effects in mussels, abundant avian wildlife such as sea ducks and shorebirds, or in some locations sea otters, at our study sites could feed on PAC contaminated mussels or similarly contaminated prey items. Such exposure is associated with biotransformation enzyme induction (ethoxyresorufin-O-deethylase, EROD) and is putatively linked to wildlife demographic effects where heavy contamination exists (Bustnes, 2013; Esler et al., 2018; Miles et al., 2007; Trust et al., 2000). A study by Willie et al. (2017) in the same inlet as our Second Narrows site reported high mussel PAC concentrations (e.g. approximately $\sum_{17}$PAC 135 ng/g) co-occurring with elevated EROD in Barrow's goldeneye (*Bucephala islandica*) at one nearby sampling location. While we observed lower PAC concentrations at Second Narrows ($\sum_{43}$PAC 20.10 ng/g) than in that study, it does indicate the possibility of wildlife exposure to localized PAC releases at the Second Narrows site or similarly PAC-affected locations like Triangle Island and suggests that more routine monitoring at protected wildlife areas and better resolution of potential sources is warranted.

3.4.3. Forensic indicators of PAC sources

To determine whether the sources of the PACs were from petrogenic sources like oil discharges, or pyrogenic sources like combustion exhaust, soot, or smoke, we considered the weight of evidence from a combination of qualitative interpretation of PAC analyte abundance and three PAC source diagnostic ratios. Regarding analyte abundance, the predominance of unalkylated parent PACs over alkylated PACs, decreasing analyte concentration with increasing alkylation within homologous series (e.g. C0 >> C1 > C2 > C3 > C4), and the presence of HMW four to six ring PACs at the Second Narrows and Triangle Island sites (Table A.1, Figure A.3) all suggest combustion-dominated sources (Hwang et al., 2014; Wang et al., 1999a; Wang and Fingas, 2003). Using PAC concentrations, we calculated three diagnostic ratios that were based on well-described principles, were supported for use in mussel tissue based on data reported in past petroleum exposure studies (Table A.1, A.2), and compatible with our dataset (Meniconi and Barbanti, 2007; Wang et al., 1999b; Yunker et al., 2002). For the pyrogenic index described by Wang et al. (1999b), our samples ranging from 0.10 to 1.07 (Table A.1) were either greater than the range reported for petroleum samples (<0.06) or within the range of combustion product samples (0.5–2.0) in that study. Ratios of Fl / [Fl + Py] ranged from 0.60 to 0.95 and ratios of [Anth + Phen] / [Anth + Phen + C1 Phen] ranged from 0.85–0.99 (Table A.1). The latter two diagnostic ratios are often much lower in *Mytilus* mussels after substantial petroleum exposure in laboratory experiments or from oil spills (Table A.2). Accordingly, the weight of evidence from the relative abundance of analyzed PACs and the high values for the three diagnostic ratios we calculated were consistent with PAC sources dominated by pyrogenic sources.

PAC diagnostic ratios for source identification are based on the concentration of certain congeners in PAC-source material such as oils or exhausts, and the accuracy of diagnostic ratios in contaminated environmental media therefore depends on the conservatism of the ratio's congeners during the transport processes to those compartments such as water or marine sediments (Boehm et al., 2018; Yunker et al., 2002). That PAC assimilation in bivalves is equal among congeners present in seawater or sediment is an oversimplified assumption due to the varied chemical behaviour of individual compounds discussed previously (Baumard et al., 1999a; Bihari et al., 2007; Murray et al., 1991; Yunker et al., 2011). Therefore, diagnostic ratios for a certain

medium such as mussel tissue must be applied cautiously where their use is unsupported. Previous PAC pollution monitoring studies indicate that diagnostic ratios with mussel tissue concentration data may be informative, for instance indicating potential uptake of oil from the *Prestige* wreck, but have applied several diagnostic ratios to make inferences about unknown sources without critical appraisal of whether the extension of those ratios and break point thresholds (e.g. <0.5) to bivalve tissue is justified (Amin et al., 2011; Baumard et al., 1998b; Francioni et al., 2007; Maioli et al., 2010; Soriano et al., 2006). Our reference diagnostic ratios from well-described *Mytilus* exposures to petroleum pollution in laboratory and field studies (Table A.2), generally supports their use and suggests that the Fl / (Fl + Py) and the (Anth + Phen) / (Anth + Phen + C1 Phen) ratios are often decreased by petroleum exposure (Hwang et al., 2014; Pérez-Cadahía et al., 2004; Sundt et al., 2011; Turja et al., 2020). However, that data underscores the necessity of drawing inferences about PAC sources from the combined evidence of multiple ratios, as we have done, the inadvisability of overreliance on thresholds reported for sediment, and the need for more information on this topic. Regarding Wang et al.'s (1999b) pyrogenic index, this ratio requires more PAC congeners than are normally available in published reports, and more studies would better support this diagnostic ratio's use in mussel tissue, though analogous pyrogenic indices have been used in published *Mytilus* mussel studies (Boehm et al., 2004). Our data in Table A.2 supporting diagnostic ratio use in *Mytilus* species will facilitate more work on the topic to improve robust source identification in biomonitoring studies with mussels.

For individual PAC congeners in our dataset that can be useful as source tracers for fuels, we found that dibenzothiophenes were elevated at Second Narrows. Concentrations of dibenzothiophenes C0 through C3 were summed ($\sum_{DBT}$PAC) and compared among sites, which was significant ($F_{6,14} = 5.701$, $p = 0.003$): $\sum_{DBT}$PAC at Second Narrows was significantly higher than all other sites (Figure 3.2 E). Dibenzothiophene and alkyl-dibenzothiophenes are tracers for fossil fuels due to their presence in fuels and relative stability in the marine environment and mussels (Berthou and Vignier, 1986; Hwang et al., 2014; Kira et al., 1983). However, $\sum_{DBT}$PAC concentrations at Second Narrows and at other sites were low overall (≤0.45 ng/g; Table A.1, Supplementary Data File). Besides direct releases of petroleum fuels, uncombusted fuel in engine exhausts is thought to also be an important source of dibenzothiophenes

to the environment (Ha et al., 2012; Takada et al., 1991), and this or some minor petroleum discharges at Second Narrows may be the source of greater dibenzothiophenes at that urban port location. Besides dibenzothiophenes, we examined retene (1-methyl-7-propan-2-ylphenanthrene), which is a C4 phenanthrene tracer for conifer combustion products, such as may be generated by the forest fires seasonally common in western Canada (Ramdahl, 1983). We calculated low ROS mean retene concentrations for Second Narrows (0.02 ng/g, n = 2) and Lucy (0.02 ng/g, n = 3) and infrequent quantitations above MLOD at the other sites (n < 2) (Supplementary Data File). That indicated that softwood combustion is unlikely to be a major source of the PACs we quantified. Thus, low or infrequently detected dibenzothiophenes and retene concentrations at our study sites were consistent with combustion-dominated PAC sources other than pine forest fires.

The dominance of pyrogenic PACs in our dataset is consistent with studies on coastal sediment in Vancouver Harbour and other industrialized harbours that most often report pyrogenic-source PACs (Meniconi and Barbanti, 2007; Yunker et al., 2015, 2002; Zeng and Vista, 1997). Petrogenic-source PAC residue in mussels tends to be reported where there are occasional or localized petroleum discharges such that petrogenic residues are superimposed on more persistent inputs from non-point combustion (Francioni et al., 2007; Hwang et al., 2014; Soriano et al., 2006). Indeed, first approximations suggest that most of the petroleum used for human activity in the urban environment at Second Narrows in Vancouver Harbour is combusted as fuel in comparatively larger quantities than that accidentally spilled, and therefore combustion constitutes the baseline signal. Other possible PAC sources are biogenic or geologic natural processes, or creosote harbour pilings at Second Narrows, which should both be evaluated in future studies with appropriate hydrocarbon analytes as tracers (Brenner et al., 2002; Neff, 1979; Yunker et al., 2015). Past sediment studies in the region indicate that natural oil seeps and carbon-rich rock minerals are unlikely to constitute a major source of bioavailable PACs (Yunker et al., 2015, 2014) and sediment cores suggest that post-industrial anthropogenic parent and alkyl-PAC inputs in the Strait of Georgia are an order of magnitude higher or more than pre-industrial baselines (Yunker and Macdonald, 2003). Besides the abundant combustion sources around Second Narrows, creosote pilings also present at port facilities could have contributed to high concentrations of parent phenanthrene, fluoranthene, and pyrene at the site (Brenner et

al., 2002). Wildfires may be relevant sources of bioavailable PACs to the marine environment (Vidal et al., 2011; Yunker et al., 2015; Yunker and Macdonald, 2003), but low retene concentrations at all sites and the high PAC at the Triangle Island sampling site furthest from land masses runs contrary to wildfires as an explanation. Accordingly, the most parsimonious explanation for elevated PAC concentrations quantified in mussel tissue at sites like Second Narrows and Triangle Island may well be pollution mainly from anthropogenic combustion sources.

3.4.4. Metals in mussel tissue

Mussel tissue concentrations varied significantly among sites for each of the eight priority metals with the exception of copper (Cu) ($F_{6,14}$ = 3.973 to 18.708, p = 6.06×10^{-6} to 0.016, Figure A.4). However, the patterns of significant between-site differences found for metals (cadmium [Cd], lead [Pb], mercury [Hg], nickel [Ni], selenium [Se], Vanadium [V], and Zinc [Zn]) were dissimilar to that found for PAC residue (Figures 3.2, A.1–A.3). Instead, metal concentrations appeared related to one another and possibly other variables besides site.

To examine the apparent covariation among certain metals and other biological or environmental variables, we performed a PCA (Figure 3.3, Table A.3). PCA with the eight trace elements captured 43.7% of variation in the first principal component (PC1) with loadings dominated by Ni, Hg, Cd, and Zn. The second principal component (PC2) accounted for 21.7% of variation in the dataset with loadings dominated by Cu, V, and Se. The third principal component capturing 13.2% of data variation had variable loadings dominated by Pb. Correlations of PC1 and PC2 with $\sum_{43}$PAC and tissue lipid content were not significant (ρ = −0.11–−0.18 p = 1.00), but among other biological and environmental variables measured at field collection or during laboratory processing, condition index (ρ = −0.67, p = 0.010) (Lobel et al., 1991a), salinity (‰ NaCl; ρ = 0.65, p = 0.010), water temperature (°C; ρ = −0.59, p = 0.027), and shell length (mm; ρ = 0.56, p = 0.033) were significantly correlated with PC1, but not PC2 (Table A.3). In contrast, soft tissue dry mass (g; ρ = 0.38, p = 0.243) and collection date (day of year; ρ = −0.45, p = 0.134) were not significantly correlated with PC1 or PC2.

Metal concentrations in mussel tissue likely vary with site-specific seawater chemistry that governs metal speciation, abundance, complexation, and bioavailability or

mussel size and condition (Bruland et al., 2014; Casas et al., 2008; Cossa, 1989). For instance, significant positive correlation of shell length and salinity and significant negative correlation of temperature with PC1 on which Ni, Hg, Cd, and Zn load heavily is consistent with cold, salty waters of the open ocean, where longer mussels are found (Figure 3.1). Regarding the negative correlation of condition index with PC1, our three Strait of Georgia locations, Second Narrows, Mandarte, and Mitlenatch, are located where there are regionally high satellite chlorophyll measurements, so higher primary

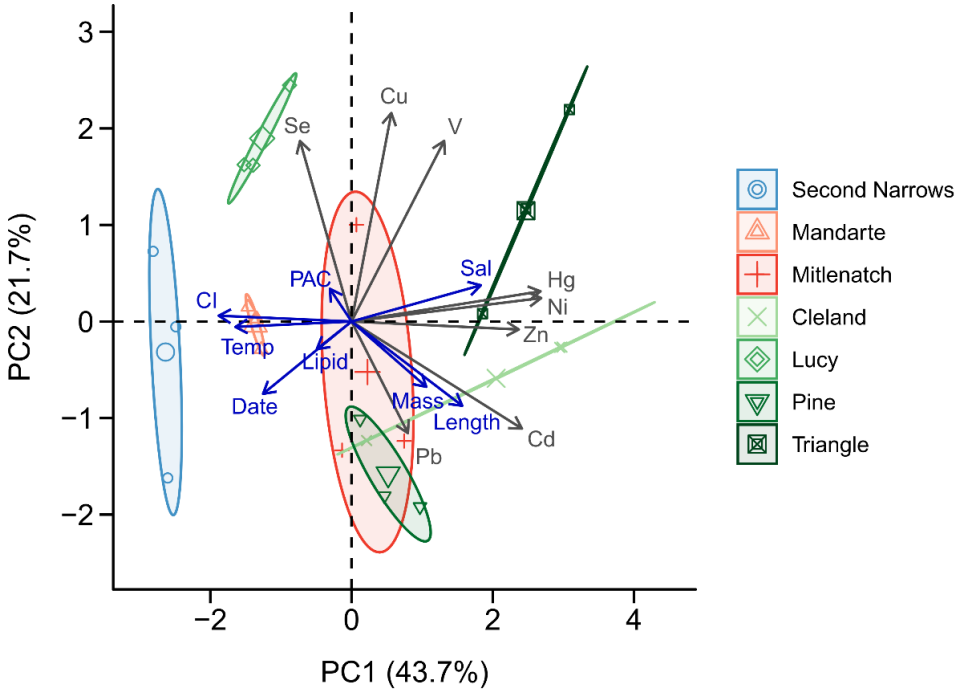

Figure 3.3 **Principal component analysis on natural log-transformed metal concentration in mussel soft tissue pools from sites (shaded ellipses) on the Pacific coast of Canada (2018). Metals are depicted as vectors (grey → labeled with their element symbol) of the first and second principal components (PC1 and PC2), which capture 43.7% and 21.7%, respectively, of variation in the data. Blue vectors (→) show correlation of total polycyclic aromatic compounds ($\sum_{43}$PAC), lipid content, and other biological and environmental variables taken during collection and processing of mussels with principal components (i.e. Spearman's ρ times a scaling constant for plotting) abbreviated as: salinity (Sal), shell length (Length), soft tissue dry weight (Mass), collection date (Date), water temperature (Temp), and condition index (CI).**

productivity there may correspond to higher condition index (Jackson et al., 2015; Lobel et al., 1991a). PC2 was not associated with environmental or biological variables and is

more difficult to interpret. In contrast to other metals studied, Pb loaded primarily to PC3. High Pb observations at Pine Island are consistent with its known history of Pb and hydrocarbon contamination at a light station there that is designated a federal contaminated site (Government of Canada, 2022). Mussel Pb concentrations were 3.01 µg/g at one of our collecting stations less than 100 m downslope from the light station and 0.97 µg/g at another station, whereas Pb ranged from 0.14 to 0.66 µg/g at all other stations and sites we studied. While the aforementioned elevated Pb concentrations at certain collecting stations show that mussels can be useful sentinels for local metal pollution, the profound differences in most metals among our sites are likely due less to any pollution sources there than environmental and biological factors that vary among locations. For instance, a strong positive correlation between mussel size and Cd concentration like that in our data is well described, and some metals in seawater vary according to salinity (Bruland et al., 2014; Cossa, 1989).

Given the considerable spatial variation in metal concentrations across our study region, the baseline concentration data we collected is all the more critical to identifying temporal trends in metals that may be influenced by pollution at the wildlife hotspots we studied, for instance in an oil spill event or increased combustion sources (Celo et al., 2015; Chiffoleau et al., 2004). Furthermore, our PCA and correlations of principal components with biological and environmental variables indicates that statistical linear models (like we used for percent lipid and PACs) may facilitate more informative inter-site comparisons of pollution-source metals where predictor variables are well supported (e.g. Hg, Ni, Zn, Cd), but the independent variables of such models might well differ among metals (e.g. Hg versus V).

3.5. Conclusion

An exception to the pattern of generally low PACs at remote conservation areas was found for Triangle Island, which despite being an offshore island furthest from permanent human settlement, had $\sum_{43}$PAC, $\sum_{HMW}$PAC, and individual PAC concentrations which were never significantly lower than at the urban Second Narrows site in Vancouver Harbour. At all sites, information available from multiple lines of evidence suggests that PACs in the mussels are predominantly of pyrogenic origin. For metals residue in mussels, unlike PACs, values at Second Narrows were never higher than most conservation areas even though significant site differences were apparent for

Cd, Pb, Hg, Ni, Se, V, and Zn. PCA with metal concentrations indicated that variation in metals was related to water conditions and mussel biology, suggesting such factors may control baseline metal residue in mussels in our study to a greater extent than any metal pollution.

Variation is inherent to large study regions like ours. Adequate replication ($n \geq 3$) and well-understood supplementary variables that relate to contaminant residue in mussels can provide information to both better understand differences among sites and enable multivariate statistical methods. We found that mussel lipid content was a relevant covariate for high concentration or $\sum_{HMW}$PAC, and further resolving environmental and biological factors affecting metal residue in the marine environment may improve future studies. We suggest that the inclusion of additional easily obtained seawater parameters like suspended solids, dissolved organic matter, and chlorophyll may provide more insight into the among site differences in metals, and possibly PAC analyte results as well.

All of the sites in this study are breeding colonies for birds like cormorants, auks, and gulls. Several sites are also habitat for mammals such as otters, pinnipeds, and cetaceans. Importantly, our PAC evidence suggests that pollution is not necessarily lower at remote locations like Triangle Island with designated conservation status. More routine pollution monitoring at the wildlife hotspots in our study region and other jurisdictions for contaminants like PACs and metals can support resource conservation goals, and mussels are a useful organism for such contaminants which are less bioaccumulative and therefore more difficult to interpret in vertebrates. This study constitutes an important first step to establishing PAC and metal baselines at these wildlife hotspot locations and identifying relevant covariates. Though the PAC concentrations we report are unlikely to yield ecotoxicological effects, follow-up studies will be useful to characterize temporal variation and further resolve PAC sources affecting wildlife conservation areas.

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Chapter 4.

Hepatic mRNA expression-augmented contaminant biomonitoring with a qPCR array to assess potential chemical effects in wild-collected seabird embryos

4.1. Chapter abstract

Birds can bioaccumulate persistent contaminants, and maternal transfer to eggs may expose sensitive embryos to high concentrations. However, using tissue residue concentrations alone to infer whether contaminant effects are occurring suffers from uncertainty, and efficient, sensitive biomarkers remain limited in wildlife. We studied relationships between whole embryo contaminant concentrations (total mercury, organochlorine pesticides, perfluoroalkyl substances, polychlorinated biphenyls, and halogenated flame retardants) together with mRNA gene transcript expression in embryonic liver tissue from a Pacific Ocean seabird, the rhinoceros auklet (*Cerorhinca monocerata*). Fresh eggs were collected, incubated under controlled conditions, and from the pre-hatch embryo, hepatic tissue mRNA was extracted for qPCR array analysis to measure gene expression ($2^{-\Delta Cq}$), while the remaining embryo was analyzed for contaminant residues. Contaminant and gene expression data were analysed with a combination of multivariate approaches and linear models. Results suggest correlation between embryonic total mercury and several genes such as *sepp1*, which encodes selenoprotein P. Correlation was likewise found for the biotransformation gene *cyp1a4* and C7 and C8 perfluoroalkyl carboxylic acids (PFHpA, PFOA). This study demonstrates that eggs like those routinely collected for contaminant biomonitoring programs can be used to relate chemical residues to *in ovo* effects in free-living birds.

4.2. Introduction

Marine birds can serve as useful indicators of ecosystem processes and pollution hazards (Furness and Camphuysen, 1997; Piatt et al., 2007). This is especially true for bioaccumulative contaminants because marine birds range widely, feed at high topic positions, and breed on land where non-lethally sampled matrices like eggs can be obtained (Elliott and Elliott, 2013). Accordingly, bird eggs have been the cornerstone of

contaminants biomonitoring programs for decades to track temporal trends in priority contaminants (e.g. Stockholm Convention listed persistent organic pollutants, mercury [Hg] per the Minamata Convention) and continue to play a role in monitoring emerging chemical hazards (Bianchini et al., 2022; Elliott et al., 1992; Gómez-Ramírez et al., 2014).

Chemical hazard to wildlife is often recognized by some combination of persistent, bioaccumulative, and toxic properties. Such compounds with long *in vivo* half lives and an affinity for lipid or protein biomolecules tend to be deposited in the egg which constitutes the embryo's nutritional supply (Hitchcock et al., 2019). Thus, the developing embryo is exposed to those maternally transferred contaminants and sufficient concentrations may cause adverse effects during sensitive early life stages (Ackerman et al., 2016; Beyer and Meador, 2011; Hoffman et al., 2003), which could translate to demographic effects in characteristically K-selected marine birds (Bustnes et al., 2015; Goutte et al., 2014; Verreault et al., 2010). However, whether chemical residues measured in eggs are actually associated with adverse biological effects remains a major source of uncertainty in contaminant biomonitoring, and sensitive biomarkers remain limited or unvalidated in many wildlife species.

With the aim of improving avian egg-based marine contaminants surveillance to better evaluate *in ovo* biological effects, the objectives of this study were to survey a broad suite of persistent contaminants in the eastern North Pacific ecosystem using seabird eggs from the rhinoceros auklet (*Cerorhinca monocerata*), and in those same samples assess the extent to which molecular qPCR-based gene expression methods could quantify potential effects in the embryo. The rhinoceros auklet, hereafter simply auklet, is a common, widely distributed Alcidae seabird that forages mainly in the continental shelf zone on fish, euphausiids, and squid (Davies et al., 2009; Gaston and Dechesne, 2020; Ito et al., 2009). For decades it has been a focal species for marine wildlife and contaminants monitoring programs in Canada and the United States (Elliott et al., 1992; Government of Canada, 2015; Jarman et al., 1996; Pyle et al., 1999). In this study, we collected fresh auklet eggs with undeveloped embryos from four island breeding colonies spread over 1,600 km of coastline and incubated the eggs under controlled conditions until the embryo was nearly fully developed to samples tissues. We analyzed the tissue residue of 95 contaminants and three stable isotopes in the whole embryo carcass in tandem with hepatic mRNA expression for 23 genes using a qPCR

array. With that dataset, we characterized contaminant residue in the embryos, including trophic information from stable isotopes, and assessed correlational relationships between contaminant burdens and hepatic gene expression.

4.3. Materials and methods

4.3.1. Egg collection, incubation, and embryo sampling

At island breeding colonies in coastal British Columbia and Alaska (Lucy Island [54°17’45” N, 130°36’33” W], Middleton Island [59°25’43” N, 146°20’10” W], Pine Island [50°58’37” N, 127°43’28” W], and Triangle Island [50°51’50” N, 129°4’55” W]), we conducted daily searches of auklet burrows. This seabird typically produces a single-egg clutch that is incubated for a mean period of 45 days (Gaston and Dechesne, 2020). Freshly laid eggs collected from 1 to 14 May 2018 were stored and transported at ambient temperature in foam-lined coolers for up 6 days, and upon arrival to Simon Fraser University the eggs were placed in forced-air incubators (RX-2, Lyon Technologies, Inc.) equipped with turning grids that tilted the eggs hourly. Incubators were operated at 37.5°C and 70% relative humidity. After incubation for 34–36 days, developed embryos were euthanized by decapitation. Aliquots of tissue from the left liver lobe were dissected, flash frozen in nitrogen, and stored at −80°C. The gall bladder was removed for another study, and the remaining embryo, less the tissues sampled, was placed in acetone- and n-hexanes-rinsed aluminium foil and stored at −20°C for chemical analysis.

4.3.2. Chemical analysis of rhinoceros auklet embryos

The accredited chemical laboratories at Environment and Climate Change Canada’s National Wildlife Research Centre (Ottawa, ON) performed all tissue residue analyses for halogenated flame retardants (FRs), organochlorine pesticides (OCPs), polycyclic chlorinated biphenyls (PCBs), perfluorinated compounds (PFCs) and total mercury (THg) (organic analytes listed in Appendix B Table B.1). Samples were homogenized with a ball mill (Retsch) and aliquoted for different chemical analyses.

For analysis of OCP, PCB, and FR, an aliquot of embryo homogenate was mixed with diatomaceous earth and spiked with ^{13}C-labeled internal standards. Organic

compounds were extracted by accelerated solvent extraction (Dionex), large biogenic material was removed using gel permeation chromatography (GX-271 Liquid Handler, Gilson Inc.), and purification was accomplished with solid phase extraction (ISOLUTE® SI, Biotage). PCBs and OCs in purified sample extracts were quantified by triple quadrupole GC-MS/MS (Agilent Technologies 7890B/7000C) in multiple reaction monitoring mode using electron impact ionization. Target FRs were quantified by single quadrupole GC-MS (Agilent Technologies 7890B/5977A) in selected ion monitoring mode using negative chemical ionization. Analysis and quantification were achieved with methods based on Gauthier et al., (2008).

Likewise, aliquots of embryo homogenate for PFC analysis were spiked with mass-labelled internal standards and extracted with acetonitrile-formic acid solution by homogenization. One aliquot of the extraction solvent was cleaned up through solid phase extraction using a weak anionic exchange (i.e. acetonitrile fraction) (Oasis® WAX, Waters™), whereas a second aliquot (i.e. formic acid fraction) was filtered prior to analysis. The separate sample extracts were analysed with a UPLC-MS/MS system (Xevo TQ-S, Waters™) using negative ion mode electrospray ionization and the manufacturer's PFC analysis kit. Analysis and quantification were achieved with methods based on Letcher et al., (2015).

We report organic contaminant (OC, PCB, FR, and PFC) concentration data at or above the laboratory method limit of reporting (MLOR, Table B.1) on a wet weight basis (ng/g wet wt.). Concentration data were recovery corrected with spiked-in mass-labelled compounds and blank corrected with extraction method blanks. Internal spike-in standards, duplicate samples per extraction batch, and reference materials were within laboratory control limits for analytes we report on.

Aliquots of embryo homogenate for THg analysis were freeze-dried. Moisture content was determined gravimetrically, and the samples were placed in nickel boats and analysed in an automated mercury analyser (DMA-80, Milestone, S.r.L.). Analytical blanks consisting of empty boats, duplicate analyses, and reference material was within laboratory control limits. All concentrations in our embryo homogenate samples were above the MLOR and are reported on a dry weight (ng/g dry wt.) basis unless stated otherwise (see 4.3.4 Data analysis).

For stable isotope analysis, an aliquot of embryo homogenate was freeze-dried and placed in tin capsules. The Ján Veizer Stable Isotope Laboratory (Ottawa, ON) performed all stable isotope analyses. Carbon (^{13}C) and nitrogen (^{15}N) isotope concentrations were determined with an elemental analyser (Vario EL Cube, Elementar Analysensysteme, G.m.b.H) interfaced with an isotope ratio mass spectrometer (IRMS) (Finnigan DELTAplus™ Advantage, Thermo Fisher Scientific). Likewise, sulphur isotope (^{34}S) concentrations were determined with a separate element analyser system (Vario ISOTOPE Cube, Elementar Analysensysteme, G.m.b.H) interfaced with an IRMS (Finnigan DELTAplus XP, Thermo Fisher Scientific). Isotope data was normalized to internal standards that were calibrated to international standards (e.g. IAEA, NBS, USGS), and data was reported as δ notation (‰) relative to air for nitrogen, V-PDB for carbon, and V-CDT for sulphur. Duplicate sample and reference material analyses were within laboratory control limits.

4.3.3. qPCR gene array assay on hepatic mRNA

Approximately 16 mg of liver tissue was subsampled from frozen aliquots with sterile tools to extract total RNA using a column-based kit (RNeasy® Plus Mini Kit; Cat. No. 74134; Qiagen). The sample was disrupted in lysis buffer with 3 mm RNAse-free stainless-steel beads in a mixer mill (MM 300; Retsch) for 3 min at 30 Hz, followed by genomic DNA elimination, RNA isolation, and cleanup per kit instructions. The RNA was eluted in 30 µl of nuclease-free water. Total RNA yield and purity were determined spectrophotometrically using a micro-volume plate (Take3™; BioTek Instruments) with a microplate reader (EPOCH2; BioTek Instruments; 260/280 nm absorbance ratio 2.18–2.21, yield 105–1,800 ng), and quality was determined on an Experion™ Automated Electrophoresis System (Bio-Rad Laboratories) RNA StdSens Kit (Cat. No. 7007104) (RQI 7.7–9.7, $\bar{X}$ = 9.3). One thousand ng RNA was reverse transcribed to cDNA in 22 µl reactions with kit-supplied reagents (SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme VILO RT; Cat. No. 11766050; Invitrogen™).

Genes selected for the auklet qPCR array (Table 4.1) included two well-established reference genes and 23 genes of interest with described biological functions and contaminant-modulated hepatic tissue expression in avian toxicity studies (as assayed by qPCR and related ToxChip gene arrays, e.g. Crump et al., 2016; Porter et al., 2014; Xia et al., 2020; Zahaby et al., 2021). To design primers, we used Clustal

Omega's Multiple Sequence Alignment tool to identify highly conserved coding sequences from multiple avian species available in the National Centre for Biotechnology Information's (NCBI) Nucleotide database, then generated primer sequences (Appendix B Table B.2) with Primer3Plus and screened for specificity to avian isoforms with NCBI's Primer-BLAST.

Table 4.1 A list of 23 genes of interest and two reference genes (symbol and name) assayed with our rhinoceros auklet qPCR array. These genes have been routinely investigated by ecotoxicity studies (e.g. Crump et al., 2015, 2016). They are listed here grouped by relevant effects pathways with a brief description of important protein functions.

Role	Gene symbol	**Gene name** (synonymous gene symbols) & relevant protein function(s)
Calcium homeostasis	*rgn*	**Regucalcin** Calcium binding protein responsible for calcium homeostasis across membranes
Heme synthesis	*alas1*	**Aminolevulinate, delta, synthase 1** Mitochondrial heme synthesis isozyme
Immune function	*batf3*	**Basic leucine zipper transcription factor, ATF-like 3** Required for the generation of type I dendritic cells and activates transcription of multiple interleukins in T helper cells
Interactions with electrophiles	*gpx3*	**Glutathione peroxidase 3** Extracellular selenoprotein which reduces hydrogen peroxides and hydroperoxides using selenocysteine and glutathione
	gstm3	**Glutathione s-transferase M3** Cytosolic conjugation of electrophilic xenobiotics and reactive oxygen species with glutathione
	mt4	**Metallothionein 4** Metal binding protein, characteristically binds divalent cations
	sepp1	**Selenoprotein P** Selenium transportation and peroxidase activity
Lipid, bile acid, & cholesterol metabolism	*acsl5*	**Acyl-CoA synthetase long-chain family member 5** Mitochondrial and endoplasmic reticulum located fatty acid metaboliser; converts fatty acids to acyl-CoAs used for lipid synthesis and β-oxidation
	apob	**Apolipoprotein B** Lipid sequestration into very low density lipoproteins
	cyp7b1	**Cytochrome P450, family 7, subfamily B, polypeptide 1** Neurosteroid and sex hormone associated endoplasmic reticulum protein involved in cholesterol catabolism and bile acid synthesis
	fgf19	**Fibroblast growth factor 19** Multifunctional enterokine with effects on glucose, glycogen, lipid, and bile acid metabolism, hepatocyte proliferation and organogenesis
	hmgcr	**3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase** Endoplasmic reticulum glycoprotein that synthesizes cholesterol
	lbfabp	**Liver Basic Fatty Acid Binding Protein** (*L-babp*) Cytosolic bile-acid chaperone protein
	pdk4	**Pyruvate dehydrogenase kinase, isozyme 4** Mitochondria cytosolic inhibitor of pyruvate conversion to acetyl-CoA, increasing dependence on non-glucose energy sources

Phenylalanine catabolism	*pah*	**Phenylalanine hydroxylase** Catabolizes toxic phenylalanine into tyrosine
Steroid metabolism	*ncoa3*	**Nuclear receptor coactivator 3** (*src3*) Nuclear hormone (e.g. androgen, estrogen, and glucocorticoid hormones) receptor coactivator
Thyroid hormone circulation	*ttr*	**Transthyretin** Hepatically produced plasma carrier proteins for thyroxine
Tumor regulation	*mdm2*	**MDM2 Proto-Oncogene** Ubiquitinase involved in cell cycle regulation
	p53	**Tumor Protein P53** Transcriptional regulator located in nuclei that activates cellular repair or senescence and apoptosis
	p53r2	**p53 inducible ribonucleotide reductase** Recruits and supplies dNTPs in early cell cycle phases
Xenobiotic metabolism	*cyp1a4*	**Cytochrome P450 1A4** Phase I xenobiotic and endogenous ligand metabolism induced by the aryl hydrocarbon receptor
	cyp3a37	**Cytochrome P450 A37** Phase I metabolism of xenobiotics, steroids, bile acids, and fatty acids
	ugt1a1	**UDP glucuronosyltransferase 1A1** (*ugt1e1*) Phase II hormone, endogenous substance, and xenobiotic metabolism
Reference genes	*eef1a1*	**Eukaryotic translation elongation factor 1 alpha 1** Subunit of the elongation factor complex which transports tRNA to the ribosome
	rpl4	**60S ribosomal protein L4** Subunit of the ribosome

Each 20 µl qPCR reaction contained custom forward and reverse primers for the target cDNA sequence at 200 to 400 nM (Table B.2) as well as 50% commercially available qPCR supermix (SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix; Cat. No. 172-5017; Bio-Rad Laboratories), 4% DMSO, and cDNA template. We conducted the qPCR assays in technical duplicates on a Bio-Rad CFX384 system (Bio-Rad Laboratories) with the manufacturer's 384-well plates (Cat. No. HSP3805). Operating conditions were 98°C for 3 minutes, 40 cycles of 98°C for 15 seconds and 60°C for one minute, followed by stepwise fluorescence measurements at 0.5°C, 5 second intervals from 65 to 95°C to generate product dissociation curves. Cq thresholds were autocalculated by Bio-Rad CFX Maestro 2.0 software (version 5.0.021.0616). We first generated standard curves with six to seven serial dilutions of pooled cDNA in the range of 2.75 to 3.78×10^{-3} ng cDNA / µL (E = 94.1–110.7%; R^2 = 0.964–0.998) for the genes assayed, each with no template controls and no reverse transcriptase controls, followed by plates with assays on each gene (i.e. gene array) with 0.102 ng cDNA / µL from every individual (n = 32), and replicate no template controls, no reverse transcriptase controls, and a positive control (cDNA pool) with *eef1a1* primers (standard curve performance given in Appendix B Table B.3).

Gene array qPCR data used to calculate gene expression for downstream analysis passed post-assay evaluations (Table B.3), detailed here. qPCR machine fluorescence readings for each well were manually inspected for evidence of amplification, a dissociation curve consistent with the expected product, and Cqs within the linear range of the assay's standard curve. In a few cases (7 of 736 non-reference gene assays), the former two criteria were met but the Cq was higher than the standard curve range, so a "template below linear assay range" qualifier was added to the observation (see 4.3.4 Data analysis). Otherwise, failed reactions were deleted. Assay technical duplicate repeatability was generally high, with mean intra-assay Cq standard deviations ≤0.25. Reference gene *eef1a1* and *rpl4* Cqs were consistent among samples, as assessed visually by plotting, and not significantly different among sites (analysis of variance [ANOVA], $F_{3,29} \leq 1.539$, $p \geq 0.225$). Therefore, reference gene Cqs were averaged and used to calculate (i.e. normalize) expression for genes of interest with the $2^{-\Delta Cq}$ method, hereafter simply termed gene expression (Schmittgen and Livak, 2008). Between plate, inter-assay coefficient of variation on gene expression ($2^{-\Delta Cq}$) was ≤9.8% for 20 genes, although Ncoa3 (14.8%), Pdk4 (27.0%), and Alas1 (31.3%) were more variable (n = 2), while inter-assay standard deviation on positive control Cqs was 0.45 (n = 8), which together indicated low among-plate assay variability. Negative controls, together with melt curves, indicated that assay Cqs were not gDNA or other non-target artifacts. Gel electrophoresis on endpoint PCR reactions confirmed that our primers amplified a single product the size of the target sequence (61 to 201 bp with the following reagents and conditions: 2% agarose in TAE with Invitrogen™ SYBR™ Safe DNA Gel Stain [Cat. No. S33102] and Thermo Scientific™ DNA Gel Loading Dye [Cat. No. FERR0611] combined 1:5 with PCR products [abm® Taq DNA Polymerase kit; Cat. No. G009: 100 ng cDNA, 3 mM $MgSO_4$, 2.5 U Taq, and forward and reverse primers at 200–400 nM for 35 PCR cycles at an annealing temperature of 60°C]). Bands were excised from the gel, the DNA extracted (GeneJET Gel Extraction kit; Cat. No. K0691; Thermo Scientific), Sanger sequenced with our custom primers by the University of British Columbia Sequencing & Bioinformatics Consortium, and the results searched with NCBI's Nucleotide BLAST tool to determine whether our amplified sequence matched the coding sequence for the target gene in avian taxa.

4.3.4. Data analysis

Embryo carcass contaminant residue data and liver tissue gene expression data ($2^{-\Delta Cq}$) were analysed with R version 4.1.1 (R Core Team, 2021) run in R Studio version 2021.09.0. Statistical analyses used dry weight basis THg concentrations. Where we report wet weight basis THg for comparison with organic contaminants in this study or other reports, conversion was made with percent moisture determined from freeze dried embryo homogenate. Sum contaminants, e.g. ∑PCB, were calculated on ≥MLOR values (i.e. <MLOR observations handled as zeros). Contaminant concentrations were $\log_{10}$ transformed and gene expression data were $\log_2$ transformed to improve the normality of the data for parametric procedures. However, in many cases we used non-parametric, ranks-based correlation procedures (Spearman's ρ, Kendall's τ) that allowed us to include a limited number of observations in our analyses that were chemical quantitations below MLOR or gene expression observations below the linear assay range by handling those observations as low ties (i.e. censored values) instead of substituting or excluding them (Helsel, 2012).

We performed principal component analyses (PCA, n = 32) on centred and scaled concentrations of frequently quantified contaminants (listed in section 4.4 Results and discussion) with the *prcomp* function and visualization tools in the *factoextra* package. PCAs included 1) a set of eight priority contaminants with a small minority of observations below the MLOR handled as zero (nine for hexabromocyclododecane and two for ∑polybrominated diphenyl ethers), as well as 2) a set of 18 individual contaminants quantified in all samples (i.e. ≥MLOR, no zero substitutions). Gene expression values in a supplemental data matrix were correlated with first and second principal component (PC1 and PC2) coordinates for individuals using Spearman's ρ (*rcorr* function in *Hmisc* package) which handled seven censored gene expression observations as low ties. Correlation coefficients (ρ) were multiplied by a scaling constant for visualization with the *fviz_add* function.

We performed three separate cluster analyses to look at covariation among individuals and among variables of three different categories: stable isotopes, contaminants, and genes. Distance matrices for clustering were calculated with the *get_dist* function in the *factoextra* package. Agglomerative hierarchical clustering was performed with the averaging method and visualized as heatmaps with the *pheatmap*

function. Distance matrices for each cluster analysis were calculated on data that was centered and scaled with the following modifications. For contaminants, to maximize the number of analytes included while minimizing bias from using substitution for censored (≤MLOR) observations, we used Kaplan-Meier estimated mean and standard deviation to centre and scale the data (*censtats* function in *NADA* package). Gene expression data was unsuited to the Kaplan-Meier method, however, but censored values were fewer and exerted minor influence on data scaling, so those few censored observations (1 to 4 observations for four genes, 7 total) were excluded (i.e. handled as NA) to estimate mean and standard deviation for data centering and scaling. Just as data centering and scaling procedures were optimized for each variable category, distance matrix calculations were likewise tailored to best suit inferences that could be made from each data category. For cluster analysis on stable isotopes (n = 32), the data approximated a normal distribution, so the isotope distance matrix was calculated with the Pearson correlation distance method to examine covariation among stable isotope elements (heatmap columns) and, to examine differences of magnitude among individuals, that distance matrix was calculated with Euclidean distance (heatmap rows). For cluster analysis on contaminants (n = 32), a distance matrix for chemicals was calculated with Spearman's ρ with censored values handled as tied low values to examine covariation among contaminants (rows), and a distance matrix was calculated with Euclidean distance to examine differences of magnitude among individuals (columns). For cluster analysis on gene expression data, one sample was omitted because of incomplete gene expression data where the *fgf19* qPCR reaction failed (individual LI-12, remaining n = 31). A distance matrix was calculated with Spearman's ρ with censored values handled as tied low values to examine covariation among genes (rows), and to examine covariation in gene expression profiles among samples, another distance matrix was calculated also with Spearman's ρ (columns). A clustered heatmap was produced for each analysis, and supplemental variables were plotted to aid in interpretation, although those supplemental variables did not feed into the clustering algorithm. Supplemental variables included site for the stable isotope and contaminant analyses, as well as contaminant concentrations for the gene expression analysis. To statistically test the association apparent between the supplemental variable THg and individuals that formed major clusters based on gene expression, we used linear models (i.e. ANOVA, $\log_{10}$[THg concentration] ~ branch) with the *Anova* function (Type II) and

estimated marginal means with the *emmeans* function (Satterthwaithe method for degrees of freedom, *emmeans* package).

We used bivariate correlation (*cor.test* function) to evaluate trends between the expression of each gene and each contaminant frequently quantified (≥MLOR concentrations in at least 3 samples per site, resulting in 19 to 32 observations for each contaminant). First, we generated a correlation matrix of gene expression as a function of chemical concentration with Kendall's τ such that censored observations were handled as low ties. Due to the many tests, we adjusted p-values with the Benjamini-Hochberg false discovery rate procedure (Q = 10%; *p.fdr* function in the *FDRestimation* package). Significant gene-contaminant associations identified in the correlation matrix were further examined with Pearson correlation tests without the censored observations (i.e. parametric correlation; n = 27 to 32; $\alpha = 0.05$) and visualized as linear regression models with the *geom_smooth* function.

Among site comparisons were made on contaminant concentration or gene expression data as described above using linear models (ANOVA), with seabird breeding colony as the predictor variable. Censored observations were excluded as they were few and their omission was inconsequential to the analysis (0–2 of 32 individuals per test).

4.4. Results and discussion

THg was the highest concentration contaminant in auklet embryo carcasses, followed by generally low persistent organic pollutants. Based on data reported in the literature, we speculate that the range of THg concentrations we observed are plausibly high enough that a biological response might exist. In support of that, multivariate approaches indicated that the expression of several genes in liver tissue was correlated (PCA, correlation matrix, linear regressions) or otherwise associated with elevated THg (clustered heatmap): *fgf19*, *sepp1*, *lbfabp*, and *cyp3a37*. Furthermore, PFC contaminants displayed carbon (C) chain length-dependent covariation in embryo carcass concentrations, and two specific C7 and C8 perfluoroalkyl carboxylic acid (PFCA) congeners correlated with a well described xenobiotic biotransformation gene, *cyp1a4*. That association between C7–C8 PFCA and *cyp1a4* was likewise evident in a

spatial comparison of differences among egg collection sites, although there was no difference among sites in THg.

4.4.1. Contaminants residue in embryo carcass

The highest concentration contaminant in the embryo carcasses was THg, whereas most organic contaminants were lower. Concentrations summed for each analytical group were ranked by their medians (n = 32) as follows: THg > OCP > PCB > PFC > FR (Figure 4.1). Within those categories, notably high (e.g. median >10.0 ng/g wet wt.) concentration analytes were THg (range 30.5–366.2 ng/g wet wt.), p,p'-DDE (16.1–199.0 ng/g wet wt.), PFUdA (12.0–57.2 ng/g wet wt.), and PFOS (6.9–23.6 ng/g wet wt.) (Table B.4).

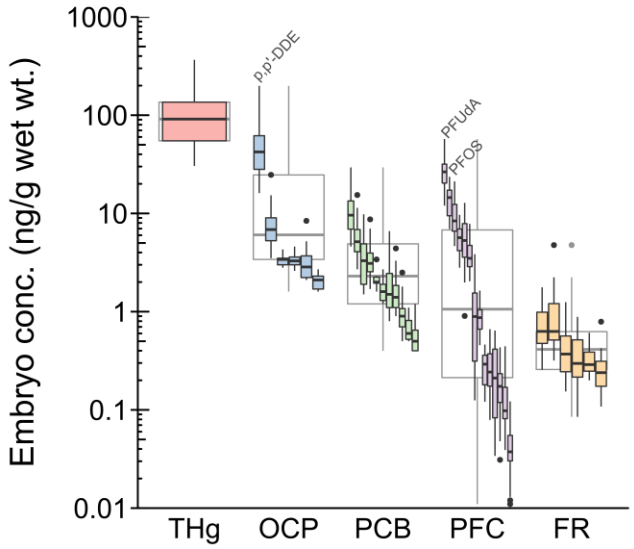


Figure 4.1 **Rhinoceros auklet embryo carcass residue (ng/g wet wt.) for chemicals with ≥5 MLOR quantitations (i.e. n = 5 to 32) for each of five contaminant analytical classes: total mercury (THg), organochlorine pesticides (OCP), polychlorinated biphenyls (PCB), perfluorinated compounds (PFC), and flame retardants (FR). The sum concentration for each analyte class is indicated with grey-outlined boxplots; individual chemicals appear as coloured boxplots. Analytes other than THg with median concentrations above 10 ng/g are labled. Abbreviated analyte names are given in full in Table B.1; data given in Table B.4.**

With the exception of THg, these concentrations were typically orders of magnitude below effect thresholds reported in the literature for embryotoxicity, and in the case of p,p'-DDE, eggshell thinning as well (Table B.5). Piscivorous seabirds like the auklet and other marine biota, often exhibit high mercury tissue residues (Ackerman et al., 2016). In bird eggs and embryonic tissues, nearly all the THg measured is methylmercury (MeHg) (Ackerman et al., 2013; Cappon and Smith, 1981; Perkins et al., 2017; Rutkiewicz and Basu, 2013). If Ackerman et al.'s (2013) factor (96%) is applied to our auklet embryo carcass THg concentrations, then the approximate range of estimated MeHg in our samples (29.2–351.6 ng/g wet wt.) overlaps with LC50 estimations from egg injection studies with MeHg chloride (≥280 ng/g wet wt.) in species from the order Charadriiformes to which the rhinoceros auklet belongs (Heinz et al., 2009), which suggests that the concentrations we found might plausibly affect the biology of the auklet embryo (see section 4.4.2).

We used PCA (n = 32) to examine variation in auklet embryo contaminant residues in the dataset, first with a PCA on a small set of priority contaminants (Figure 4.2 A) representative of each analyte class: the sum of structurally similar congeners (∑PCBs, two PFC subclasses, ∑perfluoroalkyl carboxylic acids [∑PFCA] and ∑perfluoroalkyl sulfonates [∑PFAS], one FR subclass ∑polybrominated diphenyl ethers [∑PBDE]) and individual, structurally unique compounds, detected in most samples (p,p'-DDE, hexachlorobenzene [HCB], and hexabromocyclododecane [HBCD]). A second PCA was performed with individual chemicals detected in every sample (Figure 4.2 B). For these PCA analyses in Figure 4.2 A and B, the vector lengths representing loadings (i.e. rotation) are relatively high (i.e. longer) along PC1 and/or PC2 for organic contaminants (all except PFNA, PFDS), but low (i.e. shorter) for THg. Thus, these first two PCA axes capture organic contaminant variation to a larger extent than THg. Additionally, Figure 4.2 vectors pointing in a similar direction indicate co-variation among contaminants, with some groupings apparent. Among those, it is noteworthy that 1) THg does not group with organic contaminants, and that 2) PFCA's in Figure 4.2 B which have carbon chain lengths greater than 10 atoms load heavily to PC2 (e.g. PFTrDA and nearby vectors), a grouping dissimilar to the shorter chain length PFCA and PFAS compounds with 10 or fewer carbon atoms (analyte names and abbreviations are given in Table B.1). Individuals plotted in Figure 4.2 with 95% confidence ellipses for each egg collection location shows some trends in chemical concentrations among sites, for

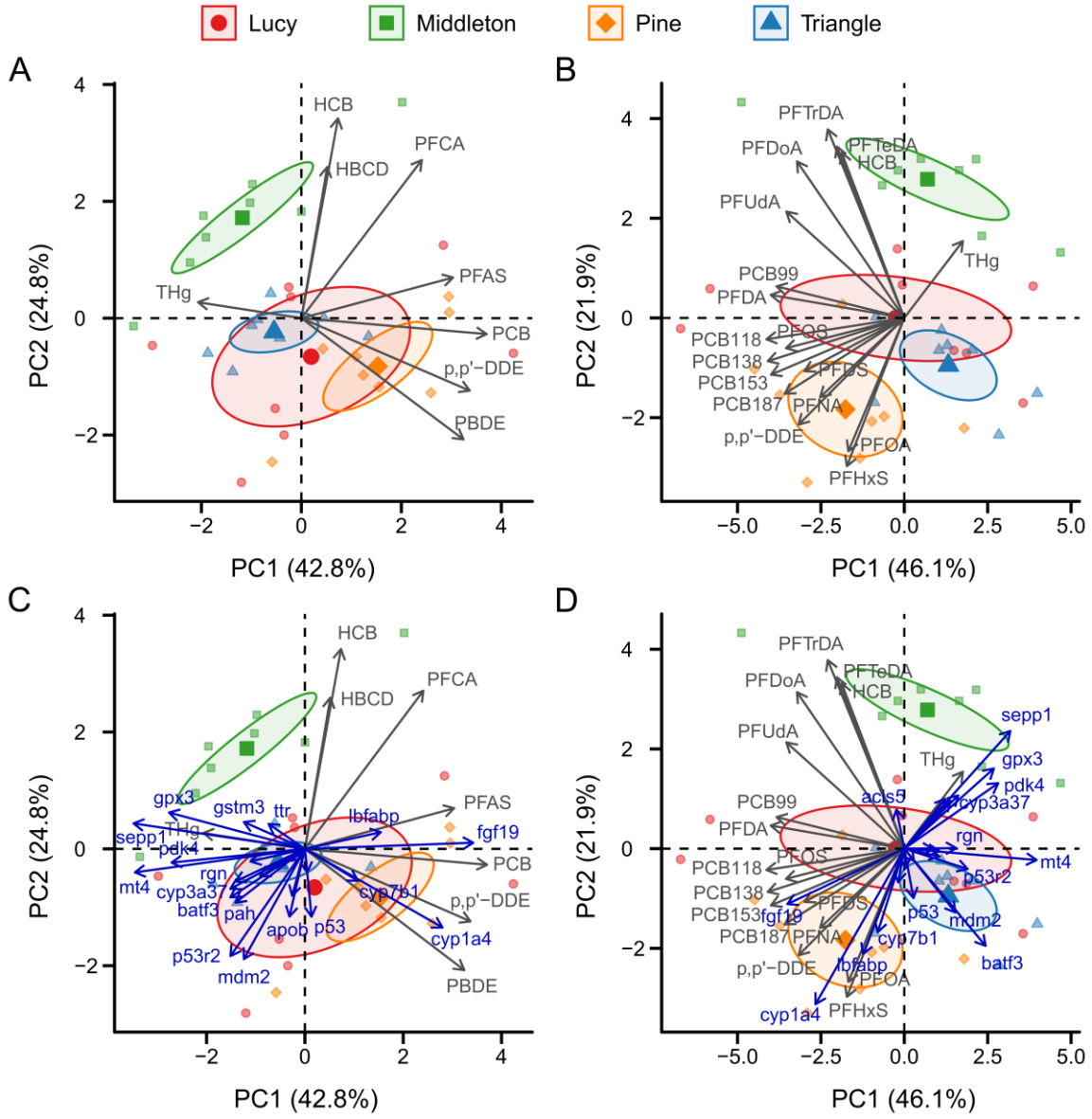


Figure 4.2 **PCA on contaminant concentraions in embryo carcass (grey vectors and labels) with colored 95% conficene ellipses and individuals for each site (see legend at top). Note that PCB, PBDE, PFCA, and PFAS in panel A and C represent sum concentrations. Gene expression values for individuals were correlated with first and second principal component (PC1 and PC2) coordinates for individuals using Spearman's ρ. That correlation coefficient, multiplied by a scaling constant, is plotted for each gene (blue vectors and labels), although labels for some genes with low coefficients are omitted for calrity. Chemical names are given in Table B.1 and gene names are given in Table 4.1.**

instance lower concentrations of chemicals like ∑PBDE, p,p'-DDE, ∑PCB, and PFHxS at Middleton Island and higher concentrations at Pine Island. These minor, analyte-specific spatial differences are consistent with whole egg homogenate in this species collected from British Columbia breeding colonies in 2014 to 2017 (Elliott et al., 2021).

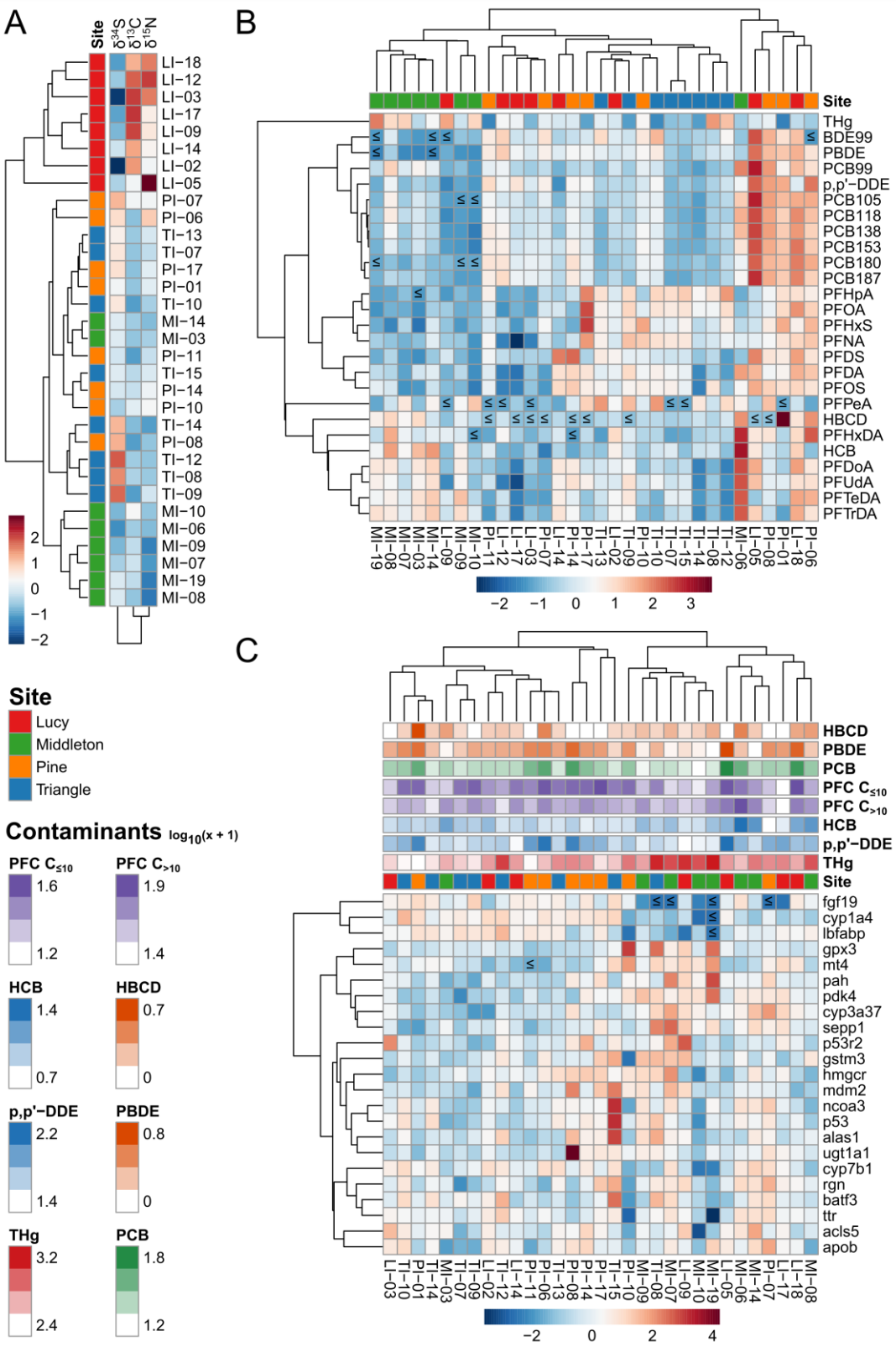
To further investigate the potential cause and extent of co-variation among the tissue residue concentrations of certain chemicals, as was suggested by our PCAs and is consistent with bioaccumulation theory, we used cluster analyses. Cluster analysis on stable isotope data (Figure 4.3 A) resulted in a separate and distinct cluster with Lucy Island embryos having higher $\delta^{13}C$, $\delta^{15}N$, and lower $\delta^{34}S$. Six of eight Middleton samples had low $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$, and the remainder of individuals did not cluster in a way that clearly related to site. That suggests a difference in maternal prey stable isotope composition among certain sites, perhaps with Lucy Island birds in Chatham Sound consuming higher trophic level coastal prey during deposition of egg material (higher $\delta^{15}N$, $\delta^{13}C$), and at the far offshore Middleton Island, birds taking lower trophic level pelagic prey (Hobson, 1999, 1995). Like stable isotopes, THg concentrations we measured likely reflect maternal mercury exposure in pre-laying auklets near the breeding grounds, whereas the organic contaminants we measured are assimilated over potentially longer time periods, and may be influenced by non-local diet outside of the breeding season (Elliott et al., 2021; Hitchcock et al., 2019; Monteiro and Furness, 1995). Subsequent cluster analysis with contaminant concentration data produced groupings largely different from the aforementioned stable isotope grouping. This is unsurprising, as previous studies in this species show that $\delta^{13}C$ and $\delta^{15}N$ are minor and inconsistent predictors of most organic contaminants and THg in eggs or adult blood, where significant at all (Elliott et al., 2021; Hipfner et al., 2011). Yet, the clustering based on contaminants was consistent with the results of PCA on contaminants discussed earlier (compare Figures 4.2 A, B ellipses with Figure 4.3 B column-wise clustering on individuals). Clustering by contaminant concentration (row-wise clustering in Figure 4.3 B) grouped THg and organic contaminants separately. Within the organic contaminants branch, ∑PBDE and PBDE congener BDE99, PCBs, p,p'-DDE, and PFCs with six to ten carbon (C) atoms clustered in one branch, whereas the C5 PFC perfluoropentanoic acid (PFPeA) and five larger C11 to C16 carbon atom PFCs clustered separately together with HCB and HBCD. That split of PFCs in into separate carbon chain length-related clusters suggests that PFCA and PFAS compounds with C6 to C10 tend to be present in

auklet embryo tissue at concentrations somehow decoupled from the concentrations of other size compounds. Clustering of individuals in Figure 4.3 B shows that a subset of six individuals from Middleton (n = 1), Lucy (n = 2), and Pine (n = 3) had relatively higher organic contaminant burdens, but that, as seen in the PCA (Figure 4.2), high organic contaminant residue did not necessarily co-occur with high THg concentration.

4.4.2. Gene expression in embryo liver tissue

The multivariate approaches used for the analysis of chemical data above were likewise used to assess covariation among genes and covariation between gene expression and carcass contaminant concentrations. We computed the correlation coefficients (Spearman's ρ, n = 32) for gene expression with the contaminant PCA PC1 and PC2 coordinates for individuals (Figure 4.2, A, B), and found that many genes, including those with the highest correlation coefficients (i.e. longer vectors), grouped with THg concentration (Figure 4.2 C, D). That suggested covariation between THg and the expression of those genes. Likewise, a clustered heatmap showing covariation of gene expression data (Figure 4.3 C; n = 31) resulted in a dendrogram that, viewed together with contaminants (those identified from analysis in Figure 4.3 B), grouped embryos with higher THg concentrations together based on their gene expression profiles. That is evident in the initial dendrogram split with 14 individuals in the right cluster (Figure 4.3 C from PI-10 through MI-08, left to right) which had significantly higher THg than the 17 individuals in the left branch ($F_{1,29}$ = 17.898, p = 0.0002; marginal mean THg 793 versus 346 ng/g dry wt.). Within that right branch, a second split occurs between seven individuals (PI-10 through MI-19 from left to right in Figure 4.3 C) with yet again

Figure 4.3 Following page–Clustered heatmaps with individual emryos and (A) stable isotopes in the carcass, (B) contaminants in the carcass, and (C) hepatic gene expression. Site (A–C) and contaminants (C) are shown to aid in interpretation and do not affect the clustering. Heatmap shading is comparable among individuals, as the isotope, contaminant, and gene variables in each plot are centered and scaled. Censored values handled as low ties are labeled (≤). PBDE, PCB, and PFC are the sum of congeners in those groups. One sample is omitted from the analysis in panel C due to missing gene expression data. Chemical names are given in Table B.1 and gene names are given in Table 4.1.


A
Site
δ34S
δ13C
δ15N
LI−18
LI−12
LI−03
LI−17
LI−09
LI−14
LI−02
LI−05
PI−07
PI−06
TI−13
TI−07
PI−17
PI−01
TI−10
MI−14
MI−03
PI−11
TI−15
PI−14
PI−10
TI−14
PI−08
TI−12
TI−08
TI−09
MI−10
MI−06
MI−09
MI−07
MI−19
MI−08
2
1
0
−1
−2
B
Site
THg
BDE99
PBDE
PCB99
p,p'−DDE
PCB105
PCB118
PCB138
PCB153
PCB180
PCB187
PFHpA
PFOA
PFHxS
PFNA
PFDS
PFDA
PFOS
PFPeA
HBCD
PFHxDA
HCB
PFDoA
PFUdA
PFTeDA
PFTrDA
−2 −1 0 1 2 3
C
HBCD
PBDE
PCB
PFC C≤10
PFC C>10
HCB
p,p'−DDE
THg
Site
fgf19
cyp1a4
lbfabp
gpx3
mt4
pah
pdk4
cyp3a37
sepp1
p53r2
gstm3
hmgcr
mdm2
ncoa3
p53
alas1
ugt1a1
cyp7b1
rgn
batf3
ttr
acls5
apob
−2 0 2 4
Site
Lucy
Middleton
Pine
Triangle
Contaminants log10(x + 1)
PFC C≤10
1.6
1.2
PFC C>10
1.9
1.4
HCB
1.4
0.7
HBCD
0.7
0
p,p'−DDE
2.2
1.4
PBDE
0.8
0
THg
3.2
2.4
PCB
1.8
1.2

significantly higher THg than the other seven individuals in that branch as well as distinctive heatmap shading ($F_{1,29}$ = 7.667, p = 0.017; marginal mean THg 1095 versus 561 ng/g dry wt.). The dendrogram clustering of individuals and heatmap shading in Fig 4.3C suggests that among contaminants, gene expression may relate to THg, and that several of the genes we assayed may have concordant trends with THg concentration such that their expression could together be considered a transcriptomic profile for THg affected individuals. For instance, the dendrogram on rows in Figure 4.3 C indicates two groups of genes (top branch: *fgf19*, *lbfabp*, and *cyp1a4*; upper bottom branch: *gpx3*, *mt4*, *pah*, *pdk4*, *cyp3a37*, and *sepp1*) with intense heatmap shading (i.e. low/high expression values) in same the column-wise dendrogram branch that we identified above with the highest THg embryos.

To examine the correlation of gene expression with contaminants suggested by both the PCAs and the clustered heatmap (Figure 4.2, 4.3 C), we generated a correlation matrix (Table 4.2) evaluating the positive or negative association between each gene's expression and contaminant concentration in the auklet embryos (Kendall's τ, n = 31 to 32). The expression of four genes, *cyp3a37*, *fgf19*, *lbfabp*, and *sepp1*, were significantly correlated with THg concentration. Another gene, *cyp1a4*, was significantly correlated with C7 and C8 chain length PFCA concentrations: PFHpA and PFOA. Additional linear correlations (Pearson's *r*) with those contaminant-gene combinations were significant (Figure 4.4) (DF = 25 to 30, $p \leq 9.61\times10^{-3}$, $|r|$ = 0.48 to 0.70).

4.4.3. Spatial comparison by egg collection location

Contaminant biomonitoring often features spatial comparisons, such as contrasting sites known or suspected to be affected by some type of pollution with less or unaffected reference (i.e. control) sites. Even though all of the seabird breeding colonies where we collected eggs are remote coastal areas with limited human activity, our multivariate analyses indicated among site differences in embryo concentrations of certain contaminants (Figure 4.2 A, B, 4.3 B). For instance, lower p,p'-DDE, PCBs, PBDEs, and $C_{\leq 10}$ PFCs at Middleton Island in the Gulf of Alaska than Pine Island in the Queen Charlotte Strait.

Given the relationships we found between contaminant concentration and gene expression for two PFCAs and THg with embryos regardless of site, we investigated

Table 4.2 **Correlation coefficients (Kendall's τ, n = 32) of embryo hepatic gene expression (rows) as a function of contaminant concentration in the carcass (columns). Positive (red) or negative (blue) associations that are significant (false discovery rate Q = 10%) are indicated with asterisks and outlined (*). Chemical names are given in Table B.1 and gene names are given in Table 4.1.**

Gene	∑All	THg	OCP			PCB							
			β-HCH	HCB	p,p'-DDE	∑PCB	PCB99	PCB105	PCB118	PCB138	PCB153	PCB180	PCB187
acls5	-0.05	-0.07	0.15	0.07	0.07	0.04	0.05	0.05	0.06	0.02	0.03	0.06	0.09
alas1	0.06	0.14	0.03	-0.03	0.01	-0.06	-0.04	-0.07	-0.09	-0.06	-0.10	-0.07	-0.08
apob	0.09	0.16	0.03	-0.07	0.06	0.03	0.00	0.04	0.04	0.00	0.03	0.02	0.08
batf3	-0.19	-0.10	-0.07	-0.13	-0.06	-0.15	-0.16	-0.18	-0.20	-0.16	-0.18	-0.12	-0.16
cyp1a4	-0.15	-0.41	-0.09	-0.17	0.25	0.18	0.02	0.17	0.12	0.16	0.16	0.29	0.17
cyp3a37	0.33	0.45*	0.00	-0.01	0.00	0.03	0.10	0.06	0.06	0.05	0.03	-0.03	0.07
cyp7b1	-0.13	-0.21	0.01	-0.16	0.15	0.07	0.02	0.08	0.05	0.03	0.04	0.09	0.06
fgf19	-0.18	-0.50*	0.02	0.01	0.18	0.23	0.06	0.20	0.19	0.22	0.24	0.32	0.21
gpx3	0.05	0.32	-0.08	-0.02	-0.28	-0.26	-0.13	-0.25	-0.21	-0.22	-0.24	-0.29	-0.26
gstm3	0.10	0.18	-0.04	-0.12	-0.14	-0.18	-0.14	-0.18	-0.18	-0.17	-0.18	-0.17	-0.17
hmgcr	0.02	0.09	-0.01	-0.07	0.01	-0.07	-0.07	-0.05	-0.08	-0.10	-0.10	-0.11	-0.06
lbfabp	-0.21	-0.46*	0.09	0.06	0.14	0.05	-0.02	0.07	0.03	0.05	0.04	0.08	0.02
mdm2	0.07	0.17	-0.07	-0.20	0.02	-0.04	0.00	-0.06	-0.08	-0.06	-0.07	-0.05	-0.02
mt4	-0.06	0.28	-0.20	-0.19	-0.29	-0.32	-0.23	-0.29	-0.30	-0.26	-0.30	-0.28	-0.26
ncoa3	-0.04	-0.02	0.12	-0.03	-0.01	-0.12	-0.05	-0.10	-0.11	-0.14	-0.15	-0.14	-0.11
p53	-0.12	-0.08	0.03	-0.09	0.07	-0.01	-0.07	-0.02	-0.02	-0.01	-0.03	0.03	0.00
p53r2	0.04	0.23	-0.26	-0.26	-0.07	-0.04	-0.13	-0.10	-0.07	-0.08	-0.05	0.01	0.03
pah	0.19	0.34	0.02	-0.02	-0.02	-0.03	0.05	-0.03	-0.02	-0.04	-0.03	-0.07	0.01
pdk4	0.16	0.41	-0.07	-0.12	-0.12	-0.20	-0.12	-0.18	-0.18	-0.17	-0.22	-0.24	-0.18
rgn	-0.01	0.12	0.02	-0.09	-0.05	-0.10	-0.10	-0.08	-0.11	-0.10	-0.11	-0.12	-0.10
sepp1	0.17	0.46*	-0.01	-0.01	-0.19	-0.24	-0.08	-0.22	-0.22	-0.21	-0.25	-0.29	-0.20
ttr	-0.02	0.00	0.04	-0.05	-0.04	-0.02	-0.02	-0.03	-0.05	-0.03	-0.05	-0.02	-0.01
ugt1a1	0.06	0.14	0.03	-0.06	0.10	0.03	0.02	0.05	0.04	0.03	0.02	0.00	0.04

Table 4.2 (Continued)

Gene	PFCA											
	∑PFCA	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUdA	PFDoA	PFTrDA	PFTeDA	PFHxDA
acls5	0.03	0.15	0.23	-0.12	-0.15	-0.15	0.02	0.07	0.05	0.03	-0.05	0.05
alas1	0.00	0.20	0.15	0.16	0.07	0.03	-0.09	-0.03	-0.07	0.00	0.01	0.12
apob	0.04	0.17	0.16	0.03	-0.04	-0.06	0.03	0.04	0.06	0.03	-0.12	-0.03
batf3	-0.26	0.01	-0.06	0.25	0.16	0.01	-0.25	-0.28	-0.33	-0.29	-0.21	-0.08
cyp1a4	0.11	-0.02	0.07	0.47*	0.42*	0.37	0.24	0.10	-0.02	-0.15	-0.06	-0.09
cyp3a37	-0.09	0.06	0.10	-0.28	-0.25	-0.27	-0.21	-0.10	-0.02	0.11	-0.06	0.12
cyp7b1	0.06	0.14	0.11	0.25	0.22	0.19	0.10	0.06	0.01	-0.09	-0.03	-0.03
fgf19	0.21	-0.02	-0.05	0.26	0.31	0.34	0.40	0.23	0.12	-0.02	0.11	-0.04
gpx3	-0.10	-0.06	-0.06	-0.13	-0.20	-0.15	-0.16	-0.15	-0.08	0.02	-0.04	0.02
gstm3	0.00	0.05	0.20	0.05	0.03	-0.05	-0.07	-0.02	0.00	0.09	0.10	0.18
hmgcr	0.07	0.20	0.13	0.20	0.10	0.15	0.08	0.05	0.00	-0.02	0.03	-0.08
lbfabp	0.03	0.06	0.01	0.33	0.34	0.22	0.10	0.03	-0.04	-0.17	-0.07	-0.05
mdm2	-0.21	0.09	0.15	-0.02	-0.08	-0.01	-0.21	-0.24	-0.22	-0.13	-0.11	0.03
mt4	-0.29	-0.08	-0.18	-0.16	-0.15	-0.14	-0.33	-0.33	-0.31	-0.18	-0.15	-0.17
ncoa3	0.01	0.16	0.12	0.07	-0.02	-0.02	-0.08	0.00	-0.02	-0.01	-0.05	-0.03
p53	-0.02	0.13	0.14	0.14	0.07	0.04	0.00	-0.04	-0.10	-0.07	-0.10	-0.06
p53r2	-0.18	-0.11	0.07	-0.11	-0.14	-0.20	-0.15	-0.18	-0.15	-0.06	-0.08	-0.04
pah	-0.04	0.16	0.20	-0.36	-0.32	-0.20	-0.13	-0.06	0.02	0.09	-0.05	0.09
pdk4	-0.10	-0.03	0.09	-0.27	-0.27	-0.23	-0.21	-0.11	-0.09	0.07	-0.04	-0.07
rgn	-0.05	0.12	0.20	0.04	0.00	-0.07	-0.14	-0.09	-0.11	-0.04	-0.10	-0.02
sepp1	-0.06	0.02	0.09	-0.28	-0.29	-0.32	-0.27	-0.09	-0.02	0.14	0.04	0.08
ttr	-0.03	0.01	0.09	0.09	0.10	-0.02	-0.11	-0.05	-0.04	0.00	-0.01	0.14
ugt1a1	0.02	0.12	0.19	0.07	0.00	0.01	-0.02	-0.02	-0.02	0.00	-0.01	0.08

Table 4.2 (Continued)

Gene	PFAS					FR	
	∑PFAS	PFHxS	PFOS	PFDS	∑PBDE	BDE99	HBCD
acls5	-0.05	-0.17	-0.04	-0.04	-0.05	0.01	-0.10
alas1	-0.03	0.06	-0.04	-0.09	-0.06	0.03	-0.10
apob	0.10	0.02	0.10	0.04	0.03	0.09	-0.14
batf3	-0.24	0.13	-0.24	-0.21	-0.07	0.02	0.00
cyp1a4	0.28	0.35	0.28	0.35	0.31	0.25	-0.13
cyp3a37	-0.14	-0.17	-0.13	-0.18	-0.05	-0.05	0.00
cyp7b1	0.02	0.25	0.01	0.08	0.08	0.02	-0.04
fgf19	0.28	0.18	0.29	0.33	0.26	0.19	0.05
gpx3	-0.11	-0.14	-0.12	-0.15	-0.22	-0.12	-0.04
gstm3	-0.08	-0.02	-0.07	-0.08	-0.13	-0.14	0.08
hmgcr	0.17	0.20	0.18	0.05	-0.04	-0.11	-0.14
lbfabp	0.04	0.20	0.04	0.07	0.12	0.13	0.04
mdm2	-0.19	0.08	-0.20	-0.14	-0.04	-0.03	-0.10
mt4	-0.29	-0.11	-0.28	-0.27	-0.20	-0.14	0.00
ncoa3	-0.13	0.03	-0.12	-0.19	-0.14	-0.09	-0.02
p53	0.02	0.09	0.02	0.02	0.09	0.13	-0.10
p53r2	-0.01	-0.11	0.01	0.00	-0.03	-0.09	-0.14
pah	-0.11	-0.17	-0.12	-0.19	-0.09	-0.07	-0.06
pdk4	-0.26	-0.23	-0.25	-0.30	-0.22	-0.24	0.02
rgn	-0.18	-0.05	-0.17	-0.13	-0.09	-0.02	-0.06
sepp1	-0.30	-0.22	-0.29	-0.36	-0.35	-0.32	0.01
ttr	-0.17	0.10	-0.18	-0.16	-0.12	-0.08	0.08
ugt1a1	0.03	0.06	0.03	-0.05	0.01	0.03	-0.16

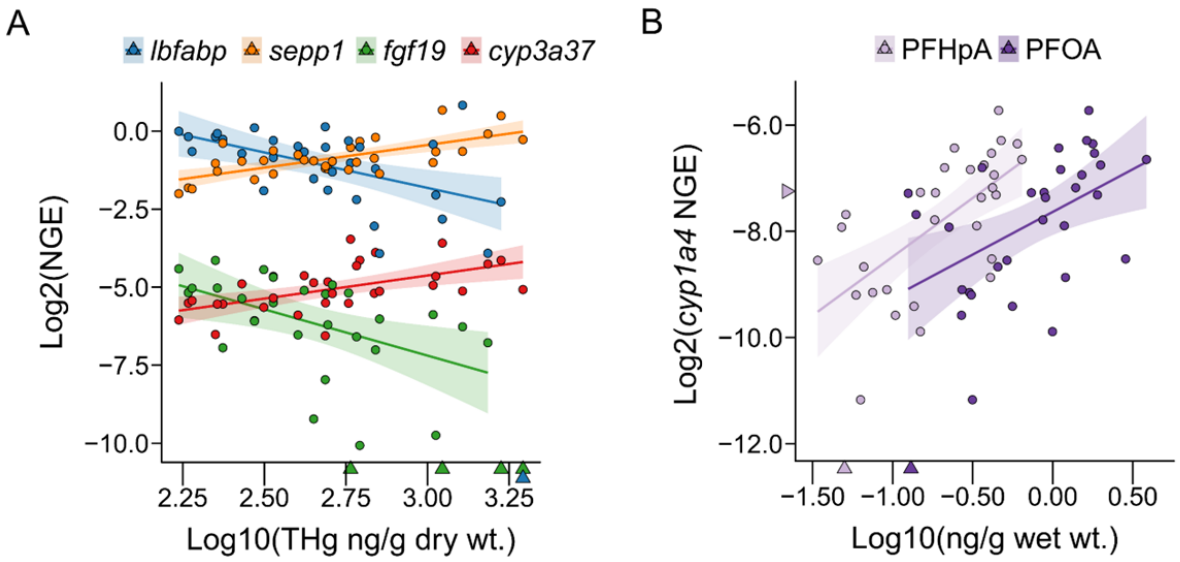


Figure 4.4 **Linear regressions where the log_2 expression of genes in liver tissue (vertical axis) was significantly correlated (Pearson's r) with log_{10} embryo carcass concentrations (horizontal axis) of (A) THg, (B) PFHpA, and PFOA. Shaded lines and 95% confidence intervals in panel A represent the genes *lbfabp* (blue, n = 31), *sepp1* (orange, n = 32), *fgf19* (green, n = 27), and *cyp3a37* (red, n = 32). Likewise, the regression of gene *cyp1a4* with PFHpA (light violet, n = 30) and PFOA (dark violet, n = 31) is shown in panel B. Triangles (Δ) indicate the axis position of observations where the matching value is censored (i.e. unkown) such that it cannot be plotted and is excluded from analysis. Chemical names are given in Table B.1 and gene names are given in Table 4.1.**

whether those contaminants varied by egg collection location, and if so, whether the spatial patterns in gene expression corresponded to contaminants. There was good concordance in among site differences in PFHpA and PFOA residue in embryos and liver tissue *cyp1a4* gene expression; THg did not vary by site (ANOVA, Table 4.3). That result indicates how gene expression effects can support contaminants biomonitoring, as has been suggested previously (Xia et al., 2020), even for analytes present at very low concentrations like PFOA (range 0.13 to 3.85 ng/g) and PFHpA (range <0.016 [MLOR] to 0.64 ng/g).

Table 4.3 Linear models for THg and PFCA contaminants (ng/g) in the auklet embryo carcass or *cyp1a4* (normalized gene expression) in liver tissue. Where the ANOVA is significant (α = 0.05), significant pairwise contrasts are indicated by differing capital letters, and 95% confidence are intervals given in parentheses.

		THg	C7 PFHpA [a]	C8 PFOA [a]	*cyp1a4*
Test statistics	n [b]	32	31	32	31
	DF	3, 28	3, 27	3, 28	3, 27
	F	1.93	17.31	11.25	6.15
	p	0.148	1.81e-06	5.13e-05	2.52e-03
Post hoc contrasts	Triangle	na	A (0.296-0.657)	A (0.940-2.441)	A (0.0044-0.0125)
	Pine	na	A (0.200-0.444)	AB (0.747-1.940)	A (0.0037-0.0105)
	Lucy	na	B (0.072-0.160)	BC (0.349-0.905)	AB (0.0026-0.0073)
	Middleton	na	B (0.050-0.118)	C (0.168-0.437)	B (0.0010-0.0030)

DF = Degrees of freedom

[a] Non-homogenous variance among test groups (Levene's test)

[b] Of 32 samples, chemical concentrations below the MLOR or gene expression observations below the qPCR linear assay range were omitted from the analysis.

4.4.4. Contextualizing contaminants measured in auklet embryos

We quantified contaminant concentrations in the whole embryo carcass, but it is unclear how comparable those observations are to the wealth of literature values from the analysis of whole egg content homogenate. Our embryos weighed only about a third of total egg weight, and some compounds may be more or less actively absorbed or biotransformed by the developing embryo (Beyer and Meador, 2011; O'Brien et al., 2009b; Rutkiewicz and Basu, 2013). Nonetheless, THg concentrations we report here (range 30.5–366.2 ng/g wet wt., n = 32) are similar to embryos from a pilot sampling year (2017, range 35.1–567.9 ng/g wet wt., n = 15) and are similar to whole egg homogenate concentrations for this species (approximately 50–500 ng/g wet wt.) (Elliott et al., 2021; Elliott and Elliott, 2016). The THg concentration range we observed is comparable to some literature values for embryo lethality mentioned previously (Heinz et al., 2009), as well as other reported adverse effects (Ackerman et al., 2016). If the equations and synthesis in Ackerman et al., (2016) for whole egg residues are applied to our embryos, they would be considered above background, but lower risk for adverse

effects (<1 µg/g wet wt. blood equivalent). Additional research would be necessary to indicate whether the THg concentrations we report are associated with adverse effects such as reduced hatching success. Although Elliott and Elliott (2016) reported no significant THg trend in rhinoceros auklet eggs over the 1970 to 2010 period, Hg biogeochemical data for our region is limited but indicates that inputs to the eastern North Pacific Ocean may be increasing (Obrist et al., 2018).

Organic contaminants (OCPs, PCBs, PFCs, and FRs) measured in embryos agreed well with concentrations in whole egg homogenate for this species in recent studies (Elliott et al., 2021; Miller et al., 2014, 2015a, 2015b). Comparison with eggs sampled from 2014 to 2017 in another study at our three sites in Pacific Canada indicates that some contaminants in whole eggs were more concentrated (e.g. p,p'-DDE ≤611.0 ng/g, PBDEs ≤5.46 ng/g, and PCBs ≤76.6 ng/g wet wt.) and more frequently above MLOR than in embryos in this study (e.g. cyclodiene pesticides) (Elliott et al., 2021). Conversely, PFCAs in our auklet embryos were generally higher, especially the C7 PFHpA and C8 PFOA congeners that were positively correlated with *cyp1a4* gene expression (PFOA [geometric mean±standard deviation ng/g wet wt.] 0.90±0.46 in embryos versus 0.28±0.37 in eggs; PFHpA 0.24 ±0.15 in embryos versus 0.03±0.06 in eggs) (Elliott et al., 2021). This is consistent with egg injection studies where PFOA was concentrated in hepatic tissue to a greater degree than other PFCs like PFUdA and PFOS (O'Brien et al., 2009a, 2009b). While temporal PFC trends in seabird eggs appear to vary by species and congener, rhinoceros auklets did not show PFC declines for dominant congeners (PFUdA, PFOS, PFTrDA) over the 1990 to 2007 period in line with North American industry production scale-backs (Miller et al., 2015b). Our PFC data indicating carbon chain length-dependent covariation among analytes in auklet embryos suggests that congener-specific analyses of PFCs in seabird eggs may provide more valuable information than sum concentrations alone, especially as congeners like C8 PFOA and C7 PFHpA present at low concentrations may concentrate in tissue and cause *in vivo* effects.

4.4.5. Interpreting the biological relevance of gene expression trends

The gene expression-contaminant concentration associations we describe are biologically plausible and are supported by toxicological studies, to varying degrees. Previous avian embryo hepatic tissue studies on organic contaminants reported gene

expression effects on four of the five genes we found significant gene expression-contaminant residue correlations for: *cyp3a37*, *cyp1a4*, *lbfabp*, and *fgf19* (Crump et al., 2017, 2016, 2015; Mundy et al., 2019; Porter et al., 2014; Xia et al., 2020). The other gene, *sepp1*, was not measured in those early life stage studies, but it is upregulated along with many other selenoproteins (though not *gpx3*) in circulating immune cells (neutrophils) studied in young chickens dosed with related metals (selenite, lead [II], or both) (Li et al., 2017).

The gene *sepp1* encodes selenoprotein P, which is secreted by the liver and is the primary plasma protein to which Hg complexes bind (Achouba et al., 2016; Liu et al., 2018; Manceau et al., 2021b). Embryonic exposure to Hg is likely primarily complexed as MeHg-cysteine initially (Manceau et al., 2021b; Perkins et al., 2017; Rutkiewicz and Basu, 2013). Elaborate systems involving *sepp1* regulate mercury in the body via deposition to feathers, demethylation, and a combination of biomineralization and biliary elimination pathways conserved across taxa, particularly in waterbirds with high-Hg diets (Dutczak et al., 1991; Eagles-Smith et al., 2009; Manceau et al., 2021a, 2021b; Scheuhammer et al., 2008). However, the mechanisms involved and their activity in developing embryos remain incompletely known.

Cyp3a37 is typically upregulated by organic contaminants or drugs (Crump et al., 2017, 2016, 2015; Mundy et al., 2019; Ourlin et al., 2000; Xia et al., 2020), so the correlation we found between *cyp3a37* expression and THg was unexpected. However, experiments in various invertebrate and rodent models link increased expression of *cyp3a* homologs to Hg and other toxic divalent metals (Cd^{2+}, Pb^{2+}) (Cao et al., 2012; Lu et al., 2011; Rand et al., 2012; Zhang et al., 2019). Instead of MeHg binding to the CXR (avian PXR homolog) nuclear receptor to upregulate its expression, MeHg may relate to *cyp3a37* expression through bile acid precursor hydroxylation pathways, and in that way may help to explain the trends we observed in *fgf19* and *lbfabp* expression (Chiang, 2009; Goodwin et al., 2003; Jia et al., 2019; Katafuchi and Makishima, 2022; Ourlin et al., 2000; Wagner et al., 2005).

Interpreting *fgf19* expression is challenging because the preponderance of data focuses on liver tissue as the *target* of intestinally produced FGF19 protein to exert negative feedback on bile acid synthesis, although a similar autocrine function within liver may exist (Song et al., 2009). The role of hepatic *lbfabp* is, similarly, unknown

beyond the ability of its protein to bind bile acids (Monaco, 2009). If gene expression patterns in our high-THg auklet embryos (Figure 4.4 A) are conserved among species and between tissue types, then the relatively decreased *fgf19* and *lbfabp* would be consistent with bile acid synthesis, while increased *cyp3a37* may indicate hydroxylation of sterols or bile acids for excretion or detoxification, as these three genes are associated with pathways involving the FXR receptor (Chiang, 2009; Jia et al., 2019; Katafuchi and Makishima, 2022). Although the functions, pathway involvement, and expression dynamics of these genes are incompletely known, enhanced bile acid synthesis would be consistent with the fact that MeHg-Cysteine undergoes biliary cycling (Clarkson et al., 2007) and drug induction of *cyp3a37*-like genes can increase MeHg excreted in bile (Klaassen, 1975). For instance, a dosing study in captive sanderling (*Calidris alba*) with polycyclic aromatic hydrocarbons found that hepatic *lbfabp* downregulation was associated with decreased circulating bile acids in serum, which may indicate hepatic clearance of bile acids (Bianchini et al., 2021; Bianchini and Morrissey, 2018). Alternatively, other roles of the FGF19 protein that remain areas of active research may be involved, which are wide ranging and include cholesterol metabolism, endocrine signaling, and participation in pathways involving glucose, lipid, and protein metabolism (Bianchini et al., 2021; Dolegowska et al., 2019; Katafuchi and Makishima, 2022).

Besides the aforementioned genes associated with THg concentration in embryos, we also found that *cyp1a4* gene expression was significantly positively correlated with two PFC compounds, C8 PFOA and C7 PFHpA, instead of PCBs which are dioxin-like compounds (DLCs) with well described effects on *cyp1a4* via aryl hydrocarbon receptor agonism (Kennedy et al., 1996; Manning et al., 2013). The *cyp1a4* gene encodes an avian cytochrome P4501A protein that catalyzes the phase I biotransformation of xenobiotics (Head and Kennedy, 2007). Although PFCAs are not considered DLCs, effects of PFCAs on hepatic gene expression have been reported previously in avian studies (Hickey et al., 2009; Nakayama et al., 2008; O'Brien et al., 2009b; Watanabe et al., 2009; Yeung et al., 2007) and some of that data supports a possible relationship between medium carbon chain length PFCAs and *cyp1a4* gene expression. Nakayama et al. (2008) reported that the concentrations of perfluorononanoic acid (PFNA, a C9 PFCA) and DLC compounds in wild collected common cormorant (*Phalacrocorax carbo*) liver tissue were significant, positive multiple

linear regression model terms predicting *cyp1a4* expression, but chemical analysis detection limits were not sensitive enough to report on C8 PFOA. Subsequent *in vitro* exposure studies with avian primary hepatocytes show mixed results on C7 PFHpA and C8 PFOA upregulation of *cyp1a4*. Notably, C8 PFOA effects on *cyp1a4* expression in one *in vitro* study did not result in a clear dose response, but elevated expression was induced at lower concentrations than its C8 sulfonate analog, perfluorooctane sulfonate (PFOS) (Watanabe et al., 2009). Another study indicated that the magnitude of *cyp1a4* induction varied by chain length, with perfluorohexanoic acid (PFHxA, a C6 PFCA) eliciting the greatest *cyp1a4* increase in chicken embryo hepatocytes (Hickey et al., 2009). We detected no significant relationship between C6 PFHxA and *cyp1a4*, even though concentrations were not far below C7 PFHpA concentrations we measured (C6 <0.034 [MLOR] to 0.44 versus C7 <0.016 [MLOR] to 0.64 ng/g). Because the aryl hydrocarbon receptor's (AhR) ligand binding cavity volume differs among species (Farmahin et al., 2013) and survival data from egg injection studies suggests different sensitivity to PFOA among species (Nordén et al., 2016), it could be that *cyp1a4* induction is AhR genotype dependent. That might explain the considerable *cyp1a4* induction by C6 PFHxA in chicken that have a smaller AhR binding cavity volume, but association between *cyp1a4* and longer C7 and C8 PFCAs in the rhinoceros auklet which is likely the genotype with a larger and less sensitive AhR ligand binding cavity (Farmahin et al., 2013; Hwang et al., 2016).

Even where the functional relevance of observed gene transcription is not yet known, previous gene array studies support the idea that certain gene expression profiles may be indicative of exposure to site-specific contamination (Crump et al., 2016, 2015; Mundy et al., 2019; Xia et al., 2020), and we note that our clustered heatmap findings indicate covariation among certain genes in auklet embryos with high THg burden (Figure 4.3 C). Ultimately, our correlational study with only a few genes each from many toxicologically relevant pathways is designed to explore gene-contaminant relationships in wild collected embryos of a free-living seabird. Experimental manipulations are necessary to further support and fully explain those relationships.

4.4.6. Synthesis and future directions

In pollution studies, applicable effect thresholds are not always readily available, as is the case for many of the chemicals we examined. In this study, THg was both the

highest contaminant concentration found and had the strongest evidence for affecting gene expression. However, the tendency to focus on numerically dominant analytes when reporting on contaminant residues can ignore the fact that different chemical classes or congeners can have substantial differences in their potency to elicit biological effects or that some compounds are subject to *in vivo* metabolism that decouples measured residues from exposure (Crump et al., 2021; King et al., 2022; Manning et al., 2013). For example in our data, the dominant PFC congeners on a concentration basis, PFUdA and PFOS, were not the same as those with the strongest evidence for effects, PFHpA and PFOA. When it comes to contaminant biomonitoring, this highlights the advantage of including measures of biological effects (i.e. biomarkers), like gene expression assayed with qPCR in this study. Further experimental work, for instance hepatocyte or egg injection experiments, can evaluate whether causal relationships exist for the gene-contaminant associations we found in our correlational analysis, and can investigate other genes involved in relevant pathways more fully, for example, mercury binding and transport, bile synthesis and energy substrate signaling, and the AhR pathway. A caveat is that gene expression like we measured in this study is a useful indicator of biological processes *in vivo* but do necessarily indicate adverse effects.

Researchers have articulated that monitoring programs should quantify sublethal effects from pollution like metals (Cooke et al., 2021), specifically in marine bird egg contaminant monitoring programs, with tools such as qPCR gene arrays (Bianchini et al., 2022) that have recently been developed for wildlife monitoring (Crump et al., 2016; Zahaby et al., 2021). Gene arrays and whole transcriptome sequencing will likely play a growing role in this research area (e.g. Bonisoli-Alquati et al., 2020) as wildlife genomes become sequenced and annotated, and robotics, RNA Seq technologies, and transcript count data analysis methods develop. Indeed, as contaminant monitoring programs with avian eggs are expected to continue into the future, the information gained from using those animals should be maximized. This study points to the utility of using gene expression methods together with contaminant residue analysis by establishing that hepatic gene expression in a free-living seabird correlates with measured contaminant concentrations in its body.

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Appendix A.

Supplemental tables and figures from Chapter 3

Table A.1 Sum concentrations and diagnostic ratios indicative of petrogenic-dominated or pyrogenic-dominated polycyclic aromatic compound (PAC) sources for mussel soft tissue pools from each collecting station at study sites on the Pacific coast of Canada in 2018. Sum concentrations include total parent (i.e., unalkylated) PACs ($\sum_{Parent}$PAC), alkylated PACs ($\sum_{Alkyl}$PAC), and dibenzothiophenes ($\sum_{DBT}$PAC, sum of parent and C1–C3). See table footnotes for details on diagnostic ratios.

Site	Station	$\sum_{Parent}$ PAC	$\sum_{Alkyl}$ PAC	$\sum_{DBT}$PAC	Pyrogenic index[a, b]	FI / (FI + Py) [b]	(Anth + Phen) / (Anth + Phen + C1 Phen) [b]
S. Narrows	1	28.72	3.25	0.22	0.94	0.72	0.96
S. Narrows	2	4.32	1.29	0.22	0.57	0.71	0.94
S. Narrows	3	19.31	3.42	0.45	0.67	0.73	0.92
Mandarte	1	3.32	0.83	0.07	0.24	0.83	0.89
Mandarte	2	4.78	0.40	0.00	0.23	0.80	0.89
Mandarte	3	1.30	0.77	0.11	0.28	0.82	0.85
Mitlenatch	1	4.31	0.58	0.04	0.28	0.74	0.93
Mitlenatch	2	6.26	0.74	0.07	0.13	0.70	0.93
Mitlenatch	3	4.60	0.71	0.12	0.29	0.69	0.92
Cleland	1	2.00	0.89	0.14	0.16	0.80	0.89
Cleland	2	2.26	0.34	0.03	0.17	0.70	0.95
Cleland	3	1.68	0.61	0.09	0.17	0.71	0.89
Lucy	1	2.71	0.71	0.09	0.27	0.62	0.92
Lucy	2	3.20	0.71	0.10	0.27	0.60	0.92
Lucy	3	6.98	0.29	0.01	0.67	0.70	1.00
Pine	1	5.53	0.48	0.10	0.10	0.77	0.98
Pine	2	3.09	0.43	0.05	0.21	0.78	0.95
Pine	3	2.74	0.31	0.06	0.13	0.80	0.95
Triangle	1	13.89	1.39	0.07	1.07	0.95	0.93
Triangle	2	5.17	0.85	0.07	0.35	0.88	0.93
Triangle	3	9.17	0.70	0.08	0.48	0.89	0.96

[a] Described by Wang et al., (1999b, see section 3.7 References) as ∑(Select 3–6 ring parent PACs) / ∑(Select C0–Cn homologous series) and calculated here as: ∑(acenaphthylene, acenaphthene, anthracene, pyrene, fluoranthene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene, dibenz[a,h]anthracene) / ∑(C0–C4 naphthalenes, C0–C4 phenanthrenes, C0–C3 dibenzothiophenes, C0–C2 fluorenes, C0, C1, C3 chrysenes). Biphenyl, Benzo[e]pyrene, Perylene, C3 Fluorene, and C2 Chrysene were not analyzed in this study and therefore omitted. Here, we estimated C1 napthalenes (Nap) as ∑(1-MeNap, 2-MeNap).

[b] Where quantitation <method limit of detection (MLOD), a value of ½ MLOD was substituted so as not to bias ratios.

Fl = fluoranthene, Py = pyrene, Anth = anthracene, Phen = phenanthrene, DBT = dibenzothiophenes

S. Narrows = Second Narrows

Table A.2 Summary of two diagnostic ratios calculated from polycyclic aromatic compound data reported in peer-reviewed journal publications for petroleum exposed *Mytilus* mussels. Exposures were either controlled laboratory tests or field studies with known petroleum releases.

Mytilus species	Petroleum type	Time (days)	Fl / (Fl + Py)						(Anth + Phen) / (Anth + Phen + ∑MePhen)						Reference
			Laboratory				Field		Laboratory				Field		
			Control	Low	Med	High	Ref	Impact	Control	Low	Med	High	Ref	Impact	
M. trossulus	Arctic Crude Oil	4	0.302	0.322	0.363	0.411			0.410	0.418	0.323	0.183			Turja et al., 2020
M. trossulus	Arctic Crude Oil	14	0.328	0.373	0.414	0.468			0.461	0.446	0.262	0.149			Turja et al., 2020
M. edulis	Oil rig produced water	29	0.601	0.308	0.288	0.267									Sundt et al., 2011
M. edulis	Oil rig produced water	48					0.729	0.658 [a]							Sundt et al., 2011
M. gallopro-vincialis	*Prestige* wreck oil	12	NA	0.373	0.322	0.259									Perez-Cadahia et al., 2004
M. spp.	IFO 380 bunker fuel	Before & after					0.566	0.190							Hwang et al., 2014
M. edulis chilensis	Port facility fuel spillage	NA					0.500	0.199 [b]					0.850	0.644 [b]	Amin et al., 2011

[a] average of six sites

[b] average of two sampling years

Fl = fluoranthene, Py = pyrene, Anth = anthracene, Phen = phenanthrene, ∑MePhen = sum methylphenanthrenes, NA = Not analyzed, Ref = Reference site

No exposure data available for the diagnostic ratio described by Wang et al., 1999b

Table A.2 References

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Table A.3 **Principal component analysis (PCA) on metal concentrations (natural log transformed) in mussel soft tissue pools collected from the Pacific coast of Canada in 2018: PCA loadings and their percent contributions for principal components 1 and 2 (PC1, PC2) (top) and correlation of sum polycyclic aromatic compounds ($\sum_{43}$PAC), lipid content, and other biological and environmental variables taken during collection and processing of mussels with PC1 and PC2 by Spearman's ρ with Benjamini-Hochberg false discovery rate-adjusted p values (bottom).**

		PC1			PC2	
		Loading	%		Loading	%
PCA on metals	Cd	0.45	20.1		-0.29	8.6
	Cu	0.10	1.1		0.57	32.4
	Pb	0.15	2.2		-0.30	9.3
	Hg	0.50	24.7		0.08	0.7
	Ni	0.50	24.9		0.06	0.4
	Se	-0.14	1.8		0.49	24.3
	V	0.24	5.9		0.49	24.3
	Zn	0.44	19.3		-0.02	0.0
		ρ	adj. p		ρ	adj. p
Spearman correlation with supplementary variables	$\sum_{43}$PAC	-0.11	1.000		0.12	1.000
	Lipid content	-0.18	1.000		-0.10	1.000
	Shell length	0.56	0.033	*	-0.31	1.000
	Soft tissue dry mass	0.38	0.243		-0.24	1.000
	Condition index	-0.67	0.010	*	0.02	1.000
	Salinity	0.65	0.010	*	0.13	1.000
	Water temperature	-0.59	0.027	*	-0.02	1.000
	Collection date	-0.45	0.134		-0.27	1.000

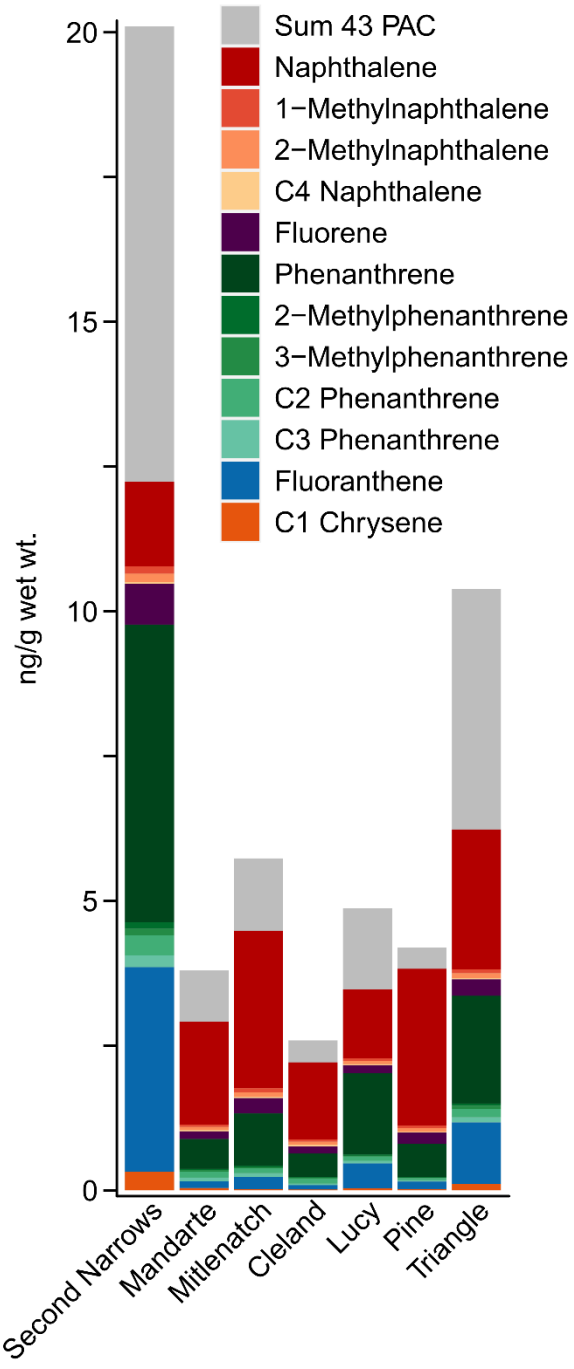

Figure A.1 **Mean polycyclic aromatic compound (PAC) concentrations (ng/g wet weight) by site (n = 3) in mussel soft tissue pools collected from the Pacific coast of Canada in 2018: sum PACs ($\sum_{43}$PAC, grey) and each of the PACs detected at all stations and sites ($\sum_{12}$PACs) as stacked colored bars.**

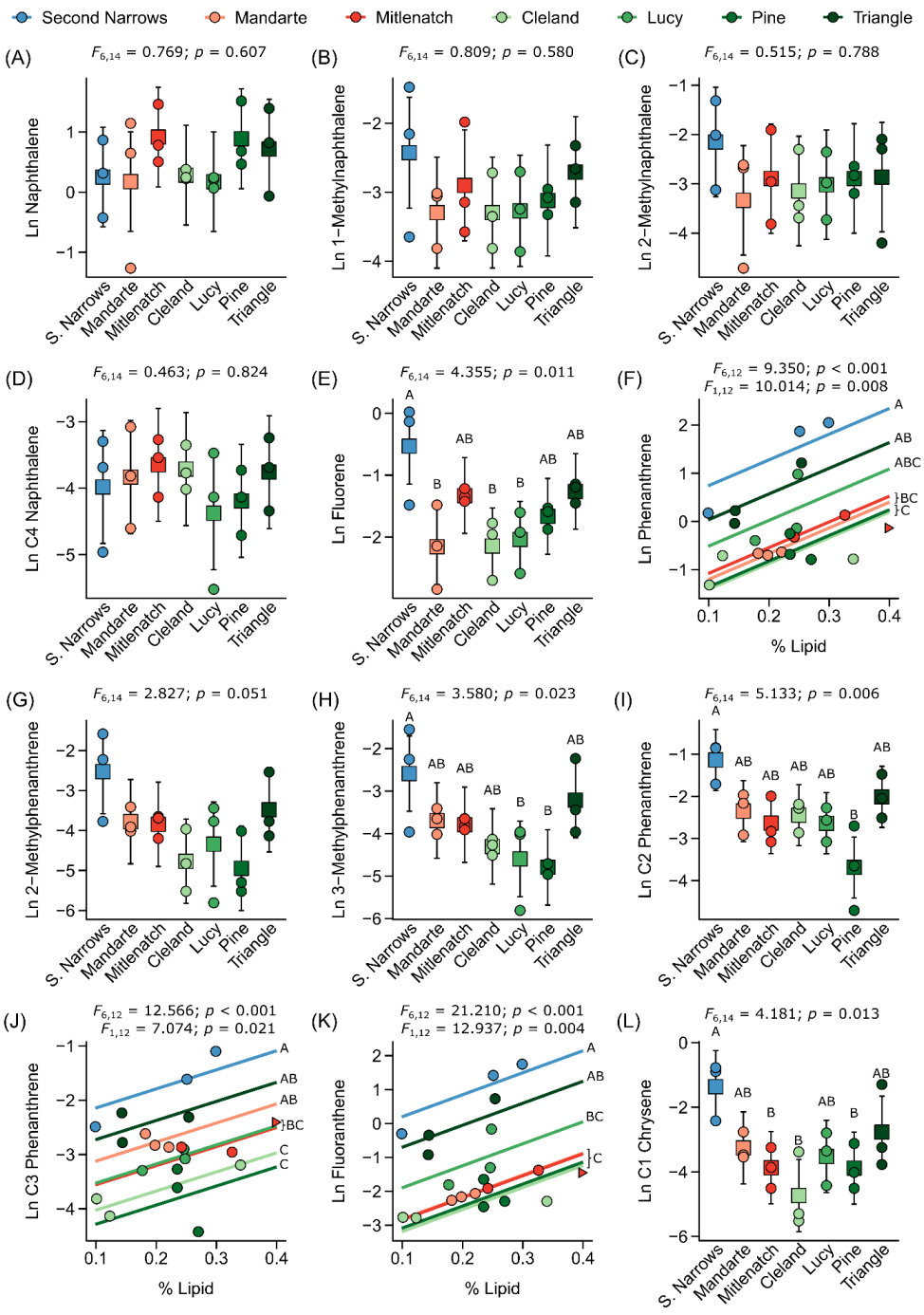

Figure A.2 **Comparison of natural log-transformed polycyclic aromatic compound (PAC) concentrations (ng/g wet weight) among sites for congeners detected in mussel soft tissue pools from all three sampling stations per site (i.e. n = 3) on the Pacific coast of Canada (2018) by ANOVA (A–E, G–I, & L; means shown as squares [□] with 95% confidence intervals), or where significant, ANCOVA with percent lipid (F, J, & K; regressions plotted as lines). F test statistic**

and p values are given above each plot (α = 0.05), and significant pairwise contrasts are labeled with differing letters. A percent lipid outlier from a Mitlenatch Island sample (0.92) was removed and its position on the vertical axis is plotted as the right-facing triangle (▷). The Second Narrows site is abbreviated in axes as S. Narrows.

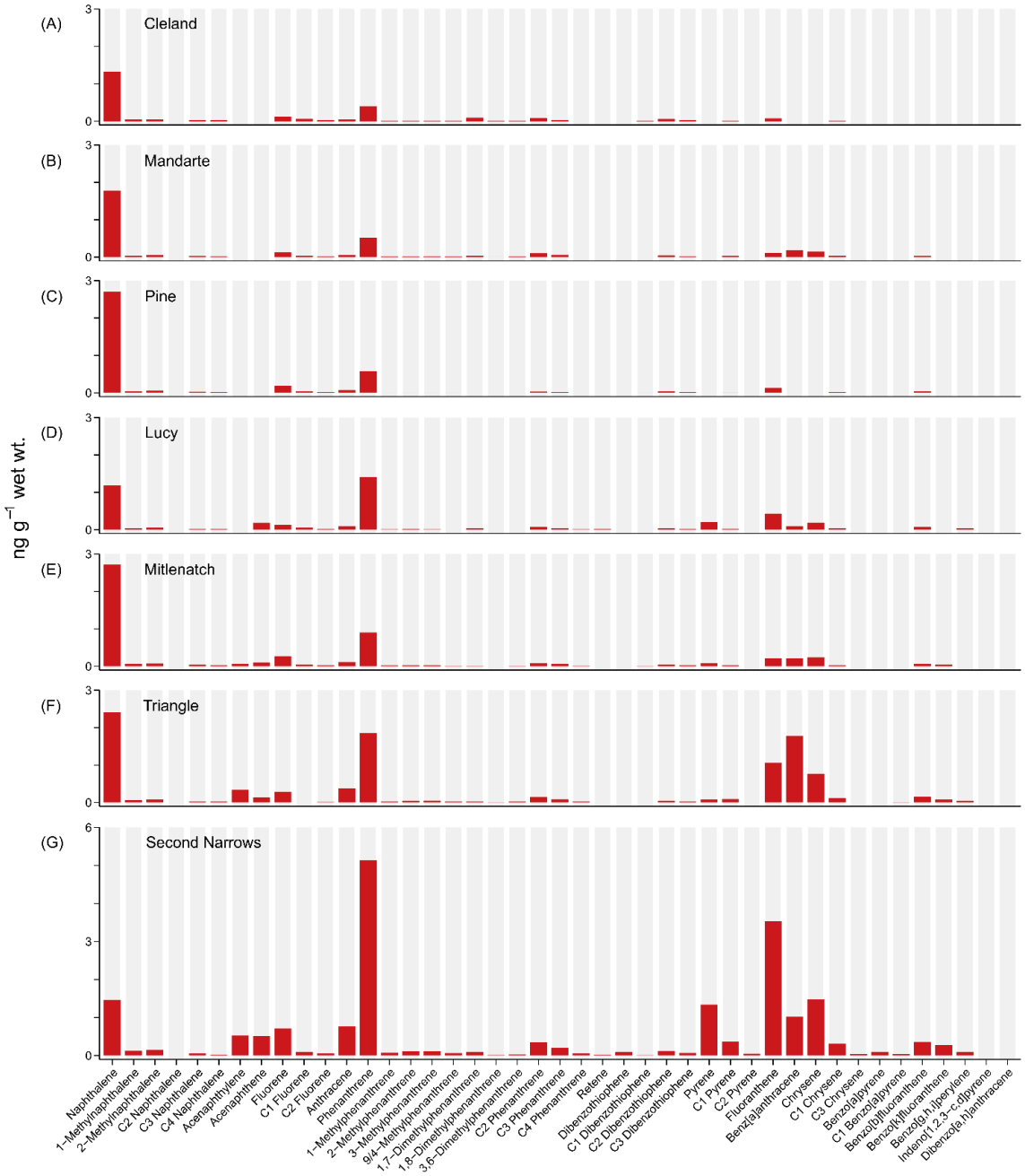

Figure A.3 **Profiles of mean polycyclic aromatic compound (PAC) concentrations (ng/g wet weight) in mussel soft tissue pools by site (A–G) on the Pacific coast of Canada (2018) calculated by regression on ordered statistics (ROS) for congeners detected in mussel soft tissue pools from 2 to 3 of the 3 stations per site; analytes detected at 1 or 0 stations per site are not plotted. Plot panels are ordered by ascending $\sum_{ROS}$PAC concentration by site from A (Cleland) to G (Triangle). In panel G, note the doubled vertical axis scale. Analytes listed in the horizontal axis generally increase in molecular weight, log Kow, and ring number from left to right.**

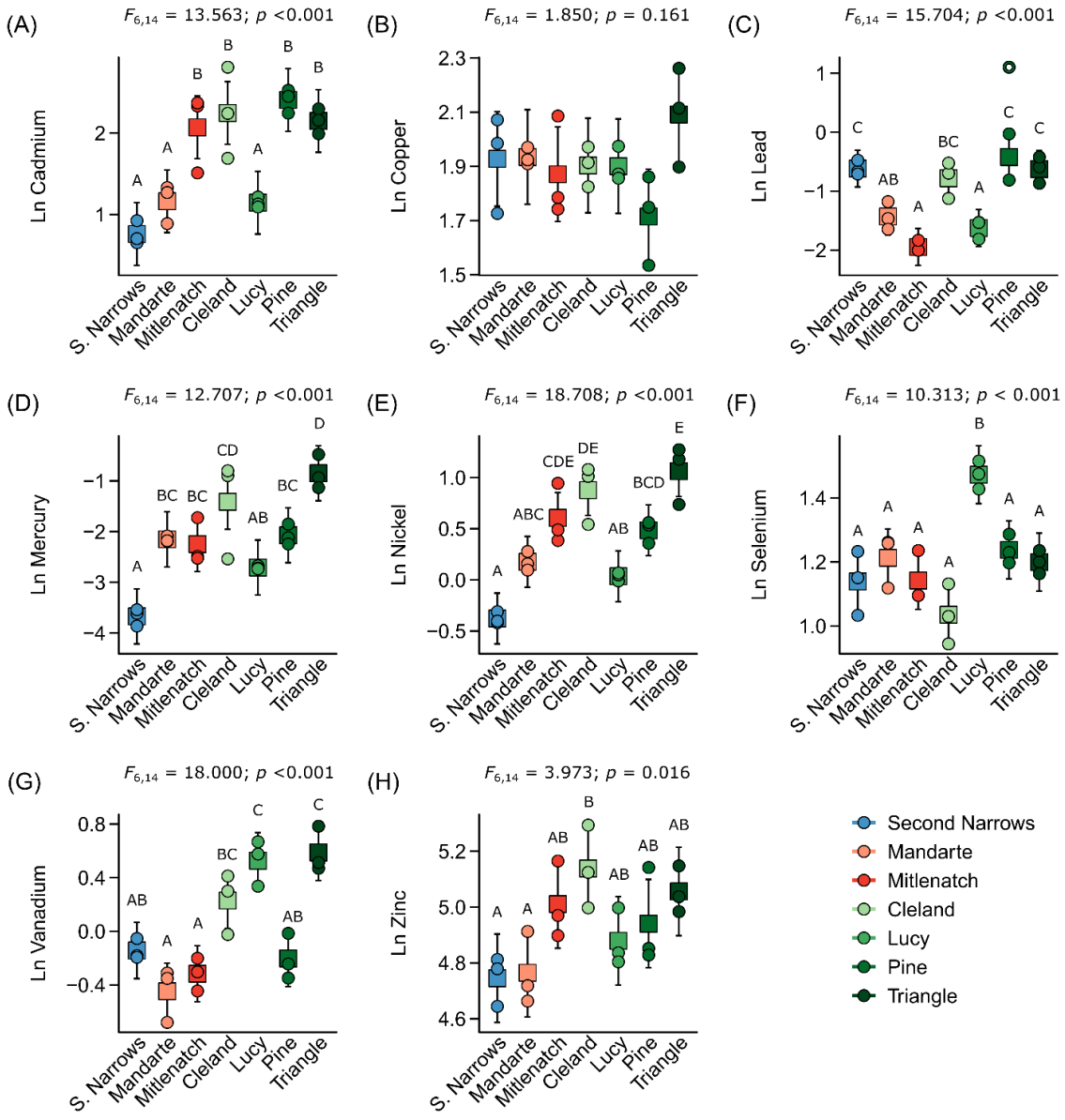

Figure A.4 **Comparison among sites (n = 3) by ANOVA (A–H; means shown as squares [□] with 95% confidence intervals) of natural log-transformed metal concentrations (µg/g dry weight) in mussel soft tissue pools collected from the Pacific coast on Canada in 2018. F test statistic and p values are given above each plot (α = 0.05), and significant pairwise contrasts are labeled with differing letters. An outlier for lead at Pine Island (Dixon's test) was removed and it position on the vertical axis is plotted as an open circle (◎). The Second Narrows site is abbreviated in axes as S. Narrows.**

Appendix B.

Supplemental tables from Chapter 4

Table B.1 Organic chemicals analyzed in rhinoceros auklet embryo carcass and their method limits of detection (MLOD) and reporting (MLOR): flame retardants (FR), organochlorine pesticides (OCP), polychlorinated biphenyls (PCB), and perfluorinated compounds (PFC). Congeners of polybrominated diphenyl ethers (PBDE), perfluorocarboxylic acids (PFCA), and perfluoroalkyl sulfonates (PFAS) were analyzed as sum concentraions.

Analysis group	Summed congeners	Analyte names		Method limits (ng/g wet wt.)	
		Abbreviated [A]	Full [A]	MLOD	MLOR
FR	na	HBB	Hexabromobenzene	0.093	0.279
FR	PBDE	BDE17	2,2',4-Tribromodiphenyl ether	0.041	0.122
FR	PBDE	BDE28	2,4,4'-Tribromodiphenyl ether	0.076	0.227
FR	PBDE	BDE47	2,2',4,4'-Tetrabromodiphenyl ether	0.077	0.232
FR	PBDE	BDE49	2,2',4,5'-Tetrabromodiphenyl ether	0.083	0.250
FR	PBDE	BDE66	2,3',4,4'-Tetrabromodiphenyl ether	0.058	0.173
FR	PBDE	BDE85	2,2',3,4,4'-Pentabromodiphenyl ether	0.009	0.028
FR	PBDE	BDE99	2,2',4,4',5-Pentabromodiphenyl ether	0.024	0.073
FR	PBDE	BDE100	2,2',4,4',6-Pentabromodiphenyl ether	0.050	0.150
FR	PBDE	BDE138	2,2',3,4,4',5'-Hexabromodiphenyl ether	0.030	0.089
FR	PBDE	BDE153	2,2',4,4',5,5'-Hexabromodiphenyl ether	0.036	0.108
FR	PBDE	BDE154/BB153	2,2',4,4',5,6'-Hexabromodiphenyl ether / 2,2',4,4',5,5'-Hexabromobiphenyl	0.059	0.176
FR	PBDE	BDE183	2,2',3,4,4',5',6-Heptabromodiphenyl ether	0.069	0.208
FR	PBDE	BDE190	2,3,3',4,4',5,6-Heptabromodiphenyl ether	0.102	0.307
FR	PBDE	BDE209	Decabromodiphenyl ether	0.468	1.400
FR	na	BB101	2,2',4,5,5'-Pentabromobiphenyl	0.020	0.060
FR	na	α-TBECH	α-1,2-Dibromo-4-(1,2-dibromoethyl)cyclohexane	0.121	0.364
FR	na	β-TBECH/BDE15	β-1,2-Dibromo-4-(1,2-dibromoethyl)cyclohexane / 4,4'-Dibromodiphenyl ether	0.228	0.684
FR	na	HBCD	1,2,5,6,9,10-Hexabromocyclododecane	0.033	0.099
FR	na	anti-DP	anti-Dodecachloropentacyclooctadecadiene	0.096	0.287
FR	na	syn-DP	syn-Dodecachloropentacyclooctadecadiene	0.050	0.151
FR	na	BTBPE	1,2-Bis(2,4,6-tribromophenoxy)ethane	0.016	0.047

OCP	na	α-HCH	α-1,2,3,4,5,6-Hexachlorocyclohexane	0.900	2.600
OCP	na	β-HCH	β-1,2,3,4,5,6-Hexachlorocyclohexane	0.700	2.100
OCP	na	γ-HCH	γ-1,2,3,4,5,6-Hexachlorocyclohexane	0.300	0.900
OCP	na	1,2,3,4-TCB	1,2,3,4-Tetrachlorobenzene	0.900	2.800
OCP	na	1,2,4,5-TCB	1,2,4,5-Tetrachlorobenzene	1.000	2.900
OCP	na	PeCB	1,2,3,4,5-Pentachlorobenzene	0.300	0.900
OCP	na	HCB	Hexachlorobenzene	0.600	1.700
OCP	na	p,p'-DDE	Dichlorodiphenyldichloroethylene	0.800	2.700
OCP	na	p,p'-DDD	Dichlorodiphenyldichloroethane	0.600	1.800
OCP	na	p,p'-DDT	Dichlorodiphenyltrichloroethane	1.200	3.700
OCP	na	Octachloro-styrene	1,2,3,4,5-pentachloro-6-(1,2,2-trichloroethenyl)benzene	0.600	1.700
OCP	na	Dieldrin	(1R,2S,3S,6R,7R,8S,9S,11R)-3,4,5,6,13,13-hexachloro-10-oxapentacyclo[6.3.1.13,6.02,7.09,11]tridec-4-ene	1.100	3.200
OCP	na	Heptachlor Epoxide	1,6,8,9,10,11,11-heptachloro-4-oxatetracyclo[6.2.1.02,7.03,5]undec-9-ene	1.200	3.600
OCP	na	cis-Chlordane	(1R,2S,3R,4S,6S,7S)-1,3,4,7,8,9,10,10-octachlorotricyclo[5.2.1.02,6]dec-8-ene	0.600	1.800
OCP	na	trans-Chlordane	(1S,2S,3R,4R,6S,7R)-1,3,4,7,8,9,10,10-octachlorotricyclo[5.2.1.02,6]dec-8-ene	0.500	1.500
OCP	na	Oxychlordane	1,5,6,8,9,10,11,11-octachloro-4-oxatetracyclo[6.2.1.02,7.03,5]undec-9-ene	0.800	2.500
OCP	na	cis-Nonachlor	(1R,2R,3S,5R,6S,7S)-1,3,4,5,7,8,9,10,10-nonachlorotricyclo[5.2.1.02,6]dec-8-ene	0.500	1.400
OCP	na	trans-Nonachlor	(1S,2R,3S,5R,6S,7R)-1,3,4,5,7,8,9,10,10-nonachlorotricyclo[5.2.1.02,6]dec-8-ene	0.900	2.600
OCP	na	Photomirex	1,2,3,4,5,5,6,7,9,10,10-undecachloropentacyclo[5.3.0.02,6.03,9.04,8]decane	0.500	1.500
OCP	na	Mirex	Perchloropentacyclodecane	0.500	1.400
PCB	PCB	PCB17/18	2,2',4-Trichlorobiphenyl / 2,2',5-Trichlorobiphenyl	3.700	11.000
PCB	PCB	PCB28/31	2,4,4'-Trichlorobiphenyl / 2,4',5-Trichlorobiphenyl	0.200	0.500
PCB	PCB	PCB33	2,3',4'-Trichlorobiphenyl	0.100	0.300
PCB	PCB	PCB44	2,2',3,5'-Tetrachlorobiphenyl	0.500	1.600
PCB	PCB	PCB49	2,2',4,5'-Tetrachlorobiphenyl	0.300	0.800
PCB	PCB	PCB52	2,2',5,5'-Tetrachlorobiphenyl	0.600	1.800
PCB	PCB	PCB70	2,3',4',5-Tetrachlorobiphenyl	0.600	1.800
PCB	PCB	PCB74	2,4,4',5-Tetrachlorobiphenyl	0.700	2.100
PCB	PCB	PCB87	2,2',3,4,5'-Pentachlorobiphenyl	0.500	1.400
PCB	PCB	PCB95	2,2',3,5',6-Pentachlorobiphenyl	0.400	1.200
PCB	PCB	PCB99	2,2',4,4',5-Pentachlorobiphenyl	0.300	0.900

PCB	PCB	PCB101	2,2',4,5,5'-Pentachlorobiphenyl	0.400	1.100
PCB	PCB	PCB105	2,3,3',4,4'-Pentachlorobiphenyl	0.200	0.500
PCB	PCB	PCB110	2,3,3',4',6-Pentachlorobiphenyl	0.200	0.700
PCB	PCB	PCB118	2,3',4,4',5-Pentachlorobiphenyl	0.400	1.200
PCB	PCB	PCB128	2,2',3,3',4,4'-Hexachlorobiphenyl	0.500	1.600
PCB	PCB	PCB138	2,2',3,4,4',5'-Hexachlorobiphenyl	0.500	1.600
PCB	PCB	PCB149	2,2',3,4',5',6-Hexachlorobiphenyl	0.600	1.700
PCB	PCB	PCB151	2,2',3,5,5',6-Hexachlorobiphenyl	0.300	0.900
PCB	PCB	PCB153	2,2',4,4',5,5'-Hexachlorobiphenyl	0.900	2.600
PCB	PCB	PCB156	2,3,3',4,4',5-Hexachlorobiphenyl	0.400	1.100
PCB	PCB	PCB158	2,3,3',4,4',6-Hexachlorobiphenyl	0.100	0.400
PCB	PCB	PCB170	2,2',3,3',4,4',5-Heptachlorobiphenyl	0.600	1.900
PCB	PCB	PCB171	2,2',3,3',4,4',6-Heptachlorobiphenyl	0.400	1.300
PCB	PCB	PCB177	2,2',3,3',4,5',6'-Heptachlorobiphenyl	0.400	1.100
PCB	PCB	PCB180	2,2',3,4,4',5,5'-Heptachlorobiphenyl	0.500	1.500
PCB	PCB	PCB183	2,2',3,4,4',5',6-Heptachlorobiphenyl	0.400	1.100
PCB	PCB	PCB187	2,2',3,4',5,5',6-Heptachlorobiphenyl	0.300	0.800
PCB	PCB	PCB194	2,2',3,3',4,4',5,5'-Octachlorobiphenyl	0.500	1.500
PCB	PCB	PCB195	2,2',3,3',4,4',5,6-Octachlorobiphenyl	0.800	2.400
PCB	PCB	PCB199	2,2',3,3',4,5,5',6'-Octachlorobiphenyl	0.700	2.100
PCB	PCB	PCB205	2,3,3',4,4',5,5',6-Octachlorobiphenyl	0.500	1.600
PCB	PCB	PCB206	2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl	0.800	2.400
PCB	PCB	PCB208	2,2',3,3',4,5,5',6,6'-Nonachlorobiphenyl	1.100	3.300
PCB	PCB	PCB209	Decachlorobiphenyl	1.100	3.300
PFC	PFCA	PFBA	Perfluorobutanoic acid	0.063	0.208
PFC	PFCA	PFPeA	Perfluoropentanoic acid	0.020	0.067
PFC	PFCA	PFHxA	Perfluorohexanoic acid	0.010	0.034
PFC	PFCA	PFHpA	Perfluoroheptanoic acid	0.005	0.016
PFC	PFCA	PFOA	Perfluorooctanoic acid	0.006	0.020
PFC	PFCA	PFNA	Perfluorononanoic acid	0.006	0.021
PFC	PFCA	PFDA	Perfluorodecanoic acid	0.006	0.020
PFC	PFCA	PFUdA	Perfluoroundecanoic acid	0.005	0.013
PFC	PFCA	PFDoA	Perfluorododecanoic acid	0.004	0.012
PFC	PFCA	PFTrDA	Perfluorotridecanoic acid	0.005	0.017
PFC	PFCA	PFTeDA	Perfluorotetradecanoic acid	0.005	0.016
PFC	PFCA	PFHxDA	Perfluorohexadecanoic acid	0.003	0.011
PFC	PFCA	PFODA	Perfluorooctadecanoic acid	0.005	0.015
PFC	PFAS	PFBS	Perfluorobutanesulfonate	0.008	0.025
PFC	PFAS	PFHxS	Perfluorohexanesulfonate	0.004	0.012
PFC	PFAS	PFOS	Perfluorooctane sulfonate	0.010	0.033
PFC	PFAS	PFDS	Perfluorodecanesulfonate	0.008	0.025

[A] "/" indicates coeluting analytes

Table B.2 Primers for qPCR on rhinoceros auklet genes with cDNA from embryo liver tissue mRNA.

Gene symbol	Primer sequence (5' -3')	Amplicon length (bp)	Reaction concentration (nM)
acsl5	Fwd: TGACAAGCCTGAGAAAGCACA Rev: TCTTCCCAGCTCCTCAACCT	169	400
alas1	Fwd: TCATCTCTGGAACGCTTGGC Rev: GCTCTTCAGAGTTCGGACGG	170	200
apob	Fwd: ACCCACACTGCCAAAACTAAGA Rev: CCAGATCTACATGGTAACTGCCAA	173	200
batf3	Fwd: CAGAAAGCAGATAAACTTCACGAGG Rev: GCAGTGCAATAGCGGACAGA	150	200
cyp1a4	Fwd: GCCTTATCCTGGAGCCTTATGT Rev: CTCTCCCAGCCAATGGTCC	92	200
cyp3a37	Fwd: AGCGTATTCCCCAGTGATGC Rev: GTCGGTCAGGGCTTTATCGT	168	200
cyp7b1	Fwd: CCTGGAGAGTGCCTTAAACGAG Rev: TCGGGGTCCATGTGCAAAAT	165	200
eef1a1	Fwd: AGCCAAGCTCTAACATGCCC Rev: CTTGAAGAGGCAGACGCAGA	147	200
fgf19	Fwd: GAGCAGTGCCAAGCAAAGAC Rev: ATGCTGTCCGTTTCCAGAGG	174	400
gpx3	Fwd: GGGCTCCCAGAAGAGGTTTTT Rev: CAGGAACCCGGACAGAACTC	201	200
gstm3	Fwd: CGGTGAGACAGAGGAGGAGA Rev: CCCCGGCAAACCATTTTCTG	191	200
hmgcr	Fwd: TCCACGCTCTCCCTGAAGAA Rev: GCCTTGGTTGATGACTCTGCT	194	200
lbfabp	Fwd: CCGTGAACATGGTAAACGGGA Rev: TTCTGACAAGTGTTACTCCACCAA	120	400
mdm2	Fwd: ACCGGCTGTGGAAGAGAATG Rev: TGGATTCCACGCTTTCCTCC	170	200
mt4	Fwd: CTGCTGCTCCTGCTGCC Rev: GTTCTTGCAGACACAGCCCT	61	200
ncoa3	Fwd: AGGATTCATTGGGACCTCTTTTGT Rev: AAGTCCTTCCCGGTCCTCTT	179	400
p53	Fwd: GTTACCACGACGACGAGACC Rev: CCATGCAGGAACTGTTGCAC	120	400
p53r2	Fwd: TGAGAATGTTCACTCGGAGATGT Rev: ACACAAGGCATTGTTTCAATAGCA	105	400
pah	Fwd: TTCGCCATGCATCCAGACC Rev: TCCCAGAGATGCCAGTCCAA	131	200
pdk4	Fwd: TTCCAAGAAAGCAGTCCATCCC Rev: CGACCATCATCCATCCTCGG	173	200
rgn	Fwd: TCTAATGGGCTGGACTGGTCA Rev: CGCTACCCAGAGTTTGCCTT	195	200
rpl4	Fwd: AGACCAGTGCCGATCTTGGG Rev: CGTCGGGGCAAACATGCGG	133	200
sepp1	Fwd: AGACATAGACACCACCACCG Rev: GAGCAGCGTCTACCTGTTCAT	166	200

ttr	Fwd: AGCTGGCAGGACTTTGCTAC Rev: CCCTTCCAGTAAGAGCTGGTG	125	200
ugt1a1	Fwd: GCTCACCCTAAGACTCGTGC Rev: CTGCTCCCCGTGTCTCTACT	145	200

Table B.3 Rhinoceros auklet gene expression qPCR performance for initial standard curves and subsequent gene arrays. Endpoint PCR provided support for assay specificity. Genes *eef1a1* and *rpl4* were reference genes used to calculate normalized gene expression ($2^{-\Delta Cq}$).

Gene	qPCR standard curve			qPCR gene array							Endpoint PCR	
	E	R^2	Median (T_m)	Amplification curve screen	Dissociation curve screen	No. wells screened out [a]	Reaction failure rate (%) [a]	Maximum intra assay SD (%) [b]	Mean intra assay SD (%) [b]	Inter assay CV [c]	Gel electrophoresis size match, single band	Sanger sequencing BLAST results
acls5	105.2	0.995	80.0	y	y	4	6.1	0.35	0.11	2.1	pass	match
alas1	107.2	0.994	81.5	y	y	2	3.0	0.72	0.19	31.3	pass	match
apob	95.3	0.990	76.0	y	y	1	1.5	0.39	0.11	4.3	pass	match
batf3	110.3	0.981	77.5	y	y	6	9.1	1.09	0.25	7.8	pass	match
cyp1a4	104.8	0.997	- [d]	y	n [d]	0	0.0	1.08	0.19	2.3	fail	match
cyp3a37	110.1	0.993	80.0	y	y	0	0.0	0.43	0.13	4.1	pass	inconclusive
cyp7b1	97.2	0.995	81.0	y	y	0	0.0	0.61	0.15	7.4	pass	match
eef1a1	100.0	0.997	83.0	y	y	0	0.0	0.31	0.11	na	pass	match
fgf19	107.5	0.995	82.5	y	y	4	6.1	0.50	0.14	5.7	pass	match
gpx3	106.5	0.991	84.0	y	y	0	0.0	0.69	0.18	9.8	pass	match
gstm3	98.5	0.986	84.0	y	y	0	0.0	0.77	0.15	3.7	fail	match
hmgcr	108.6	0.986	78.5	y	y	2	3.0	0.44	0.14	7.1	pass	match
lbfabp	101.1	0.996	77.5	y	y	0	0.0	0.25	0.08	6.8	pass	match
mdm2	109.1	0.984	79.5	y	y	0	0.0	0.58	0.12	0.1	pass	inconclusive
mt4	110.7	0.964	- [d]	y	n [d]	1	1.5	1.05	0.17	4.5	pass	inconclusive
ncoa3	107.9	0.988	78.0	y	y	1	1.5	0.61	0.12	14.8	pass	match
p53	110.5	0.978	84.5	y	y	0	0.0	0.74	0.11	5.3	pass	match
p53r2	97.4	0.988	76.0	y	y	11	16.7	0.83	0.24	8.1	pass	match
pah	94.1	0.994	81.0	y	y	0	0.0	0.31	0.09	8.0	pass	match
pdk4	100.6	0.997	80.5	y	y	2	3.0	0.46	0.16	27.0	pass	match
rgn	102.4	0.995	79.5	y	y	1	1.5	0.28	0.10	4.9	pass	match

rpl4	107.0	0.992	84.0	y	y	0	0.0	0.44	0.08	na	pass	match
sepp1	102.0	0.995	81.0	y	y	0	0.0	0.38	0.10	2.5	pass	match
ttr	97.6	0.998	79.0	y	y	0	0.0	0.40	0.10	6.9	pass	match
ugt1a1	107.9	0.991	82.0	y	y	0	0.0	0.46	0.11	7.4	pass	match

[a] Individual well deletions out of 33 duplicate reactions (i.e. 66 wells), usually screened out due to aberrant melt peak

[b] Standard deviation (SD) calculated on Cq (n = 32, excludes the inter assay replicate)

[c] Coefficient of variation (CV) calculated on normalized gene expression for one individual run twice on separate well plates (i.e. n = 2)

[d] Post-amplification phase fluorescence unstable, irregular dissociation curves

Table B.4 Rhinoceros auklet embryo carcass residue (ng/g wet wt.) for chemicals with ≥5 MLOR quantitations (i.e. n = 5 to 32). Analyte names abbreviated here are given in Table B.1.

Analyte	Min	Median	Max
THg	30.45	91.27	366.24
p,p'-DDE	16.1	42.3	199
HCB	3.5	6.85	24.7
Oxychlordane	2.8	3.4	4.3
trans-Nonachlor	2.6	3.3	4.6
β-HCH	2.1	2.85	8.4
Mirex	1.6	2.1	2.7
PCB153	4.6	9.6	29.4
PCB138	2.7	5.15	15.4
PCB180	1.5	3.3	9.8
PCB118	1.7	3.1	8.7
PCB128	1.6	2	3.4
PCB183	1.2	1.6	2.7
PCB187	0.8	1.5	6.6
PCB99	0.9	1.4	4.4
PCB105	0.5	0.9	2.5
PCB28/31	0.5	0.6	1.1
PCB158	0.4	0.5	1.2
PFUdA	12	26.5	57.2
PFOS	6.93	14.5	23.6
PFTrDA	4.65	8.39	21.2
PFDA	2.8	5.67	9.68
PFNA	0.9	5.31	12.8
PFDoA	2.04	3.49	7.84
PFOA	0.13	0.89	3.85
PFTeDA	0.46	0.87	1.64
PFPeA	0.12	0.29	0.46
PFDS	0.08	0.24	0.66
PFHpA	0.03	0.21	0.64
PFHxS	0.03	0.17	0.43
PFHxA	0.04	0.1	0.44
PFHxDA	0.01	0.04	0.12
BDE47	0.26	0.63	1.77
HBCD	0.32	0.63	4.76
BDE100	0.15	0.37	1.25
BDE99	0.09	0.3	0.89
BDE154/BB153	0.2	0.29	0.61
BDE153	0.11	0.24	0.79

Table B.5 Comparison of rhinoceros auklet embryo carcass contaminant residue (Table B.4) with values from previously published studies (ng/g wet wt.). Values are summarized from literature data and should be considered approximations only. They are derived from various available data and represent a combination of no observed effect levels (NOEL), lowest test concentration if no NOEL, or the LD50 of a particularly sensitive species (Hg, indicated by ≥). Where studies differ, we give the lower concentration. While this summary considers all species and endpoints in avian embryos, the majority of data are nominal doses from egg injection studies with domestic chicken (*Gallus gallus domesticus*) rather than measured chemical concentrations. Analyte names abbreviated here are given in Table B.1.

Analyte	This study data			Literature data	
	Min	Median	Max	Approx. effects range	Citation
THg	30.45	91.27	366.24	≥120 (MeHg)	Heinz et al., 2009
BDE47	0.26	0.63	1.77	>100 (∑Penta congeners)	McKernan et al., 2009 Rattner et al., 2013 Fernie et al., 2008, 2009
BDE99	0.09	0.30	0.89		
BDE100	0.15	0.37	1.25		
BDE153	0.11	0.24	0.79		
BDE154/BB153	0.20	0.29	0.61		
HBCD	0.32	0.63	4.76	>50	Crump et al., 2010
HCB	3.50	6.85	24.70	>9.5	de Roode et al., 2002
p,p'-DDE	16.10	42.30	199.00	>1000	Blus, 1996 Quinn et al., 2008
PCB105	0.50	0.90	2.50	>100	Brunström, 1989 Harris & Elliott, 2011
PCB153	4.60	9.60	29.40	>14,000	Harris & Elliott, 2011
PFOA	0.13	0.89	3.85	>500	O'Brien et al., 2009a Norden et al., 2016
PFUdA	12.00	26.50	57.20	>10,000	O'Brien et al., 2009a
PFDS	0.08	0.24	0.66	>10,000	O'Brien et al., 2009a
PFOS	6.93	14.50	23.60	>100	O'Brien et al., 2009b Molina et al., 2009

MeHg = Methylmercury

Table B.5 References

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