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Effects of Avian Eggshell Oiling With Diluted Bitumen Show Sublethal Embryonic Polycyclic Aromatic Compound Exposure

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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 non-monotonic *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. *Environ Toxicol Chem* 2022;41:159–174. © 2021 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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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

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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.

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.

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 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 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 1).

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 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)

TABLE 1: Summary of embryo exposure scenarios and oil applications for embryotoxicity studies with domestic chicken and double-crested cormorant

	Treatment					Sampling		Egg surface oiled (%)[b]		Measured exposure (mg oil g^{-1} egg)	
Species	Substance	Nominal exposure (mg oil g^{-1} egg)[c]	Day	Development (%)[d]	Eggs (no.)[a]	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.

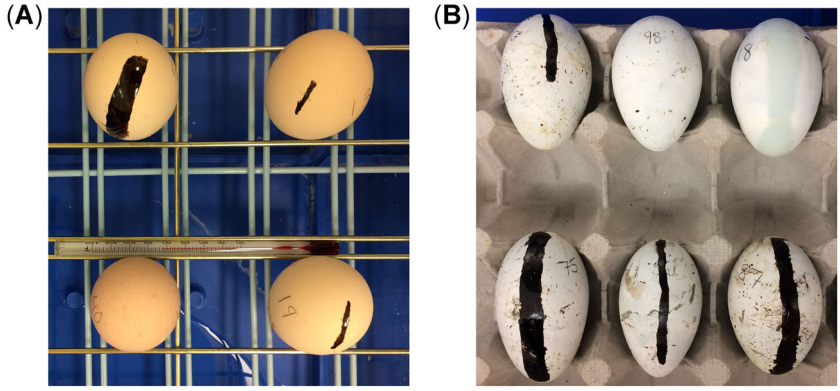

FIGURE 1: Examples of eggshell oiling treatments for eggs from left to right, top to bottom: (**A**) Chicken eggs held vertically in incubators with treatments of 0.15 and 0.015, safflower oil equivalent to 0.15 and 0.015 mg dilbit g^{-1} egg; (**B**) Double-crested cormorant eggs held horizontally at 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.

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 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 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).

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 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) 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 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 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.

RESULTS

Survival and abnormalities

The frequency of embryos that were dead at sampling or had some visible gross deformity or lesion (Figure 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 ($p = 0.684$, $n = 8$).

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). The latter included hematocrit ($F_{4,38.6} = 2.762$, $p = 0.041$) and hemoglobin in chicken ($F_{4,31.4} = 3.974$, $p = 0.010$), 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 ($F_{1,92.1} = 0.895$, $p = 0.347$).

Cyp1a4 mRNA gene expression in cormorant

For cormorant embryo hepatic *Cyp1a4* mRNA gene expression data generated by qPCR (Figure 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).

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 4). Those relative concentrations were 1.8–5.6 times the average concentrations in control embryos untreated or treated only with safflower oil (Figure 4A,B, and G; Supporting Information, Table S3). The most concentrated analytes on a wet weight

TABLE 2: Physiological endpoints related to growth and cardiovascular function were assessed using linear mixed effect models (LME)

			Chicken[a]					Cormorant[b]				
				ANOVA result					ANOVA result			
Physiological role	LME response	LME predictor	LME covariate	*df*	*F*	*p*	Sig. post hoc contrast with control	LME covariate	*df*	*F*	*p*	Sig. post hoc contrast with control
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	
Cardiovascular function	Heart rate	Day	None	2, 86.0	3.367	0.039*	d 12 < d 15, d17	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		—	—	—	—	

When 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.
ANOVA, analysis of variance (Satterthwaite's method for degrees of freedom).
[a]$n \geq 8$ except for hematocrit and hemoglobin where $n \geq 5$. 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]$n \geq 11$ except for heart measurements where $n \geq 6$. To better conform to LME assumptions, outliers were removed (Dixon's test) from heart rate data, data for body mass were transformed by natural logarithm, and data for heart moisture were transformed by arcsine square root.
*$p < 0.05$.

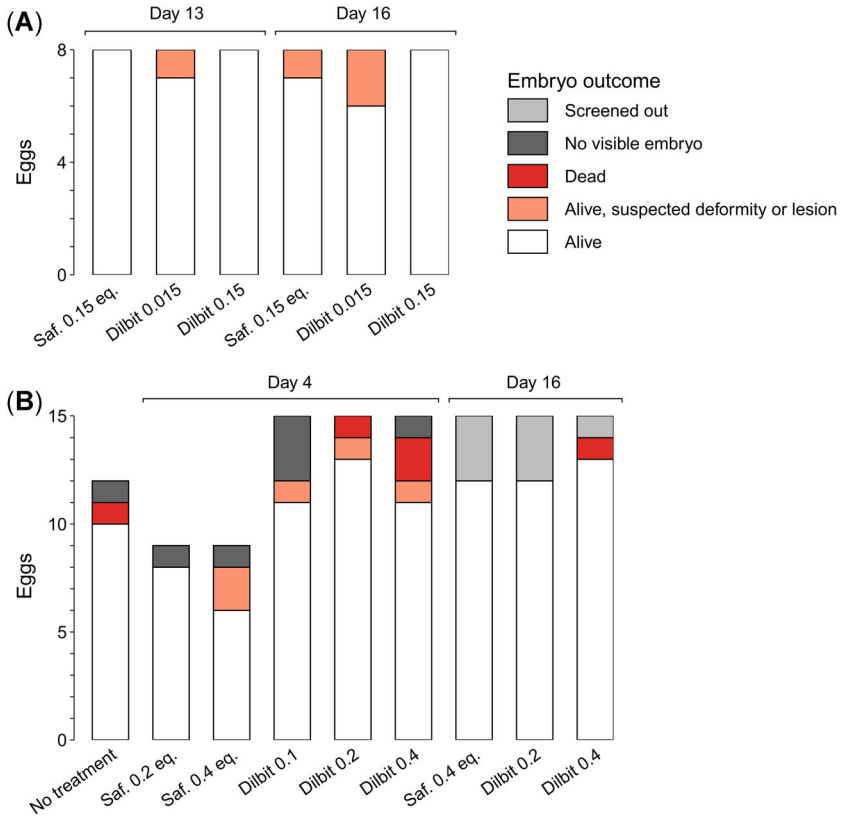

FIGURE 2: Frequency of embryo outcome (see key) at sampling for experimental treatments: (**A**) Chicken eggs dosed on day 13 or 16 with safflower oil (Saf.) over an area equivalent to 0.15 mg dilbit g^{-1} egg or with dilbit amounts of 0.015 or 0.15 mg dilbit g^{-1} egg and sampled on day 18; (**B**) 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).

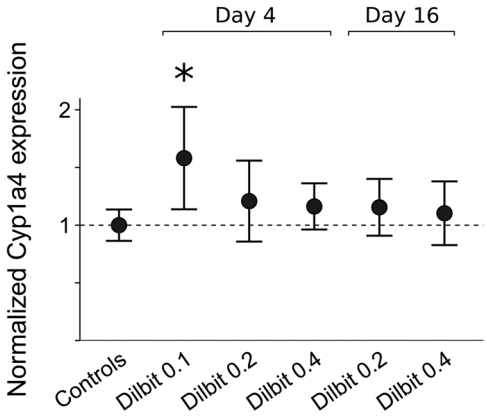

FIGURE 3: Cormorant embryo hepatic *Cyp1a4* mRNA normalized gene expression assayed by quantitative polymerase chain reaction. Dilbit treatments were applied to the eggshell on different days of incubation, and embryos were sampled at day 22. Gene expression is normalized to the control treatments (dashed 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.

basis were C_3 chrysenes (2.55 ng g^{-1}), naphthalenes (non-alkylated, 1-methyl-, 2-methyl-, and C_1–C_3 naphthalenes; 1.02–2.07 ng g^{-1}), phenanthrene (1.40 ng g^{-1}), and C_2 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 4G and H; Supporting Information, Table S3). For example, the C_2 chrysene group was the most abundant PAC analyte in dilbit, yet the amount detected in embryo tissue as a percentage of C_2 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 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 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

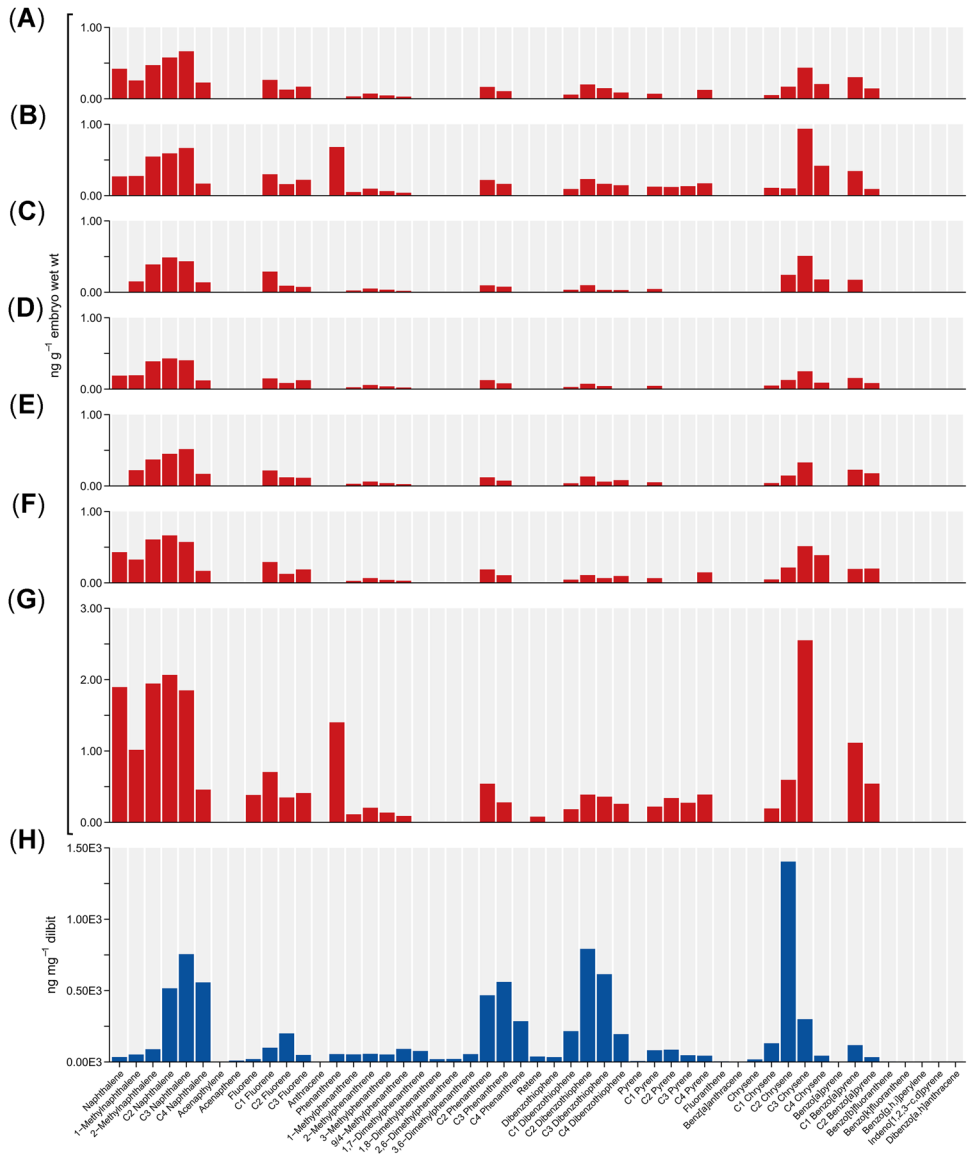

FIGURE 4: Polycyclic aromatic compound (PAC) concentrations (limit of quantitation or higher) in embryo carcass homogenate (ng g^{-1} wet wt) from double-crested cormorant eggs at sampling, incubation day 22. Each homogenate was a composite of six pooled embryos. Control eggs were (**A**) left untreated, 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.

day 4 (Figure 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 4G), albeit to a smaller, less developed embryo, PAC profiles revealed elevated tissue concentrations only when dilbit was applied later on in incubation.

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 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 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.

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 LD*x*-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.

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.

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 C_1 benzo[*a*]pyrenes and C_2 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.

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 C_2–C_3 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 $\sum C_0$–C_3 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.

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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Data Availability Statement—Polycyclic aromatic compound chemistry data are available in the Supporting Information file. Data on the biological effect endpoints evaluated and other study details, associated metadata, and calculation tools are available from the corresponding author (mason_king@sfu.ca).

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