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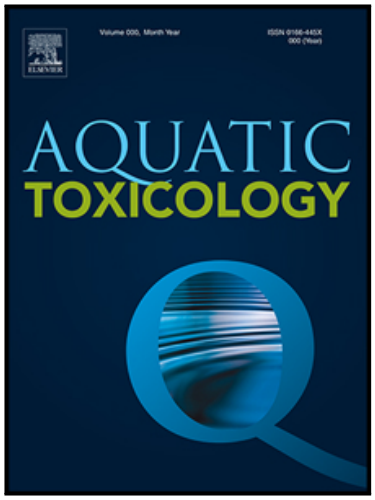

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Resilience of larval wood frogs (*Rana sylvatica*) to hydrocarbons and other compounds released from naturally weathered diluted bitumen in a boreal lake

Samuel A. Patterson[a], Daniel T.J. Denton[b], Caleb T. Hasler[b,*,#] c.hasler@uwinnipeg.ca, Jules M. Blais[c], Mark L. Hanson[d], Bruce P. Hollebone[e], Jose Luis Rodriguez-Gil[c,d,f], Valérie S. Langlois[g], Géraldine Patey[g], Zeyu Yang[e], Diane M. Orihel[a,h,*,#] diane.orihel@queensu.ca

[a]School of Environmental Studies, Queen's University, 116 Barrie Street, Kingston, ON, Canada K7L 3N6

[b]Department of Biology, University of Winnipeg, 515 Portage Avenue, Winnipeg, MB, Canada R3B 2E9

[c]Department of Biology, University of Ottawa, Ottawa, ON, Canada K1N 6N5

[d]Department of Environment and Geography, University of Manitoba, Winnipeg, MB, Canada R3T 2N2

[e]Emergencies Science and Technology Section, Environment and Climate Change Canada, Ottawa, ON, Canada K1A OH3

[f]International Institute for Sustainable Development-Experimental Lakes Area, Winnipeg, MB, Canada R3B 0T4;

[g]Institut National de la Recherche Scientifique, Centre Eau Terre Environnement, 490 rue de la Couronne, Québec, QC, Canada G1K 9A9

[h]Department of Biology, Queen's University, 116 Barrie Street, Kingston, ON, Canada K7L 3N6.

*These authors equally shared leadership on this study.

#Corresponding authors. +1-204-786-9722; 613-533-6000 x79052).

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Data statement: Data sets from this study will be deposited in an online data repository and are available upon request from the corresponding authors.

Highlights

- Impacts on amphibians were characterized during unique experimental dilbit spills
- No significant effects on growth, survival, or development of larval wood frogs
- Significant change in only one of three behavioural metrics was observed
- PAC concentrations in water were too low to induce CYP1A biomarkers in wood frogs
- Dilbit spills may not result in aqueous PAC concentrations that cause direct toxicity to larval amphibians

Abstract

The risks to aquatic wildlife from spills of diluted bitumen (dilbit) into inland waters are poorly understood. In this paper, we describe the response of larval wood frogs (*Rana sylvatica*) to hydrocarbons and other compounds released from experimental spills of dilbit in a temperate boreal lake. To simulate a wide range of environmentally relevant oil spill scenarios, different volumes of Cold Lake Winter Blend dilbit (0, 1.5, 2.9, 5.5, 18, 42, 82, and 180 L) were added to 10-m diameter in-lake limnocorrals. Larvae (n = 360) were reared (from Gosner Stage (GS) 25 to ~42) in land-based aquatic microcosms,

where they were first exposed to clean water during a 2-week baseline phase, and then (at GS ~30), to contaminated water withdrawn from the limnocorrals for 3 weeks. We observed no statistically significant trends in survival, growth, or development of larvae as a consequence of exposure to the chemical compounds released from naturally weathered dilbit. Likewise, cytochrome P450 1A biomarkers or levels of thyroid hormones in wood frogs near metamorphic climax were not related to volume of the oil spills. However, there was a modest statistically significant decrease in larval activity (up to 8.7% relative to the control), but no change in other behavioural metrics (sociality or space use). Our work adds to the limited body of literature on the effects of unconventional oils on aquatic wildlife and helps to inform risk assessments regarding pipeline projects.

Keywords

amphibians, aquatic toxicology, frog development, oil spill, diluted bitumen

1. Introduction

As amphibians are now the most threatened class of vertebrates on Earth (Díaz et al., 2019), identifying drivers of their global decline is an on-going challenge to amphibian conservation. Pollution is often underestimated as a potential threat to at-risk species (McCune et al., 2019), and research is needed on how contaminants affect amphibian health. In particular, the toxicological consequences of oil exposure on amphibians are not well-studied, with only about a dozen studies published over the last four decades (Table S1). Scientific interest in oil toxicity to amphibians was first sparked when a tank barge en route from Canada to the United States ran aground and spilled 300,000 gallons of industrial fuel into the St. Lawrence River (USEPA, 1979). A variety of conventional oils are acutely lethal to early life stages of frogs, toads, and salamanders (Amaeze et al., 2014; Hedtke and Puglisi, 1982; McGrath and Alexander, 1979). Sublethal effects on larval growth are caused by chronic exposure to used engine oils (Lefcort et al., 1997; Mahaney, 1994) and crude oils (Sutuyeva et al., 2019). Developmental delays and abnormalities (e.g. abnormal gut coiling, axial curvature, and edema) have been observed in larval frogs exposed to crude oil (Sutuyeva et al., 2019). Crude oils also affect the expression of genes related to thyroid signaling (Truter et al., 2017) and the structure and function of the thyroid gland (Sutuyeva et al., 2020). Recent studies (Krohn et al., 2021; Lara-Jacobo et al., 2019) have examined the response of amphibians to unconventional oils (that is, oils mined by unconventional means; NAS 2016), which is the focus of the current study.

Bitumen is the highly degraded, extremely heavy unconventional crude oil that is extracted through surface mining and *in situ* recovery from Canada's oil sands. The most

common mode of transporting oil sands products from mine sites to oil refineries is by pipeline, and to a lesser extent, by rail and tanker (King et al., 2020). Because bitumen is a semi-solid, it must be diluted with lighter petroleum products for transport. These blends are referred to as ‘diluted bitumen’ (or simply ‘dilbit’, in the case of blends produced for pipelines) (NAS, 2016). The chemical composition of dilbit is variable, but in general, is abundant in the high-molecular-weight resins and asphaltenes characteristic of heavy crudes, but also contains the highly water-insoluble saturates and aromatics that are most abundant in light crudes (NAS, 2016). Dilbit also contains minor amounts of metals, sulfur, and naphthenic acids (Lee et al., 2015).

Of particular toxicological concern in dilbit and other crude oils are the aromatics, including the monoaromatics that are acutely toxic and the polycyclic aromatic compounds (PACs) that are chronically toxic (Lee et al., 2015). The PACs are a diverse group of compounds, including polycyclic aromatic hydrocarbons (PAHs), their alkylated derivatives (alkyl-PAHs), and hetrocyclic aromatics containing N, S, or O atoms. Alkyl-PAHs and the heterocyclic dibenzothiophenes and benzonaphthathiophenes are predominant in Canadian bitumen (Marvin et al., 2021). In wildlife, PAC exposure can cause embryotoxicity, cardiotoxicity, DNA damage and cancer, oxidative stress, endocrine disruption, and developmental and reproductive impairments (reviewed by Wallace et al. (2020)). The induction of ethoxyresorufin-O-deethylase (EROD), a liver cytochrome P450 (CYP1A) enzyme, has been used for environmental monitoring in the the oil sands as a biomarker of PAC exposure in fish (Culp et al., 2021) and amphibians (Hersikorn and Smits, 2011).

The transport of dilbit across North America has raised concerns about the environmental impacts of accidental spills into freshwater environments. The ecological consequences of dilbit spills are poorly understood and a high priority for research (Lee et al., 2015; NAS, 2016). Much research on how dilbit affects freshwater vertebrates has focused on fish (e.g., Alderman et al., 2017; Everitt et al., 2021; Madison et al., 2015), with only two studies on amphibians. Lara-Jacobo et al. (2019) exposed frog (*Silurana tropicalis*) embryos to water accommodated fractions (WAF) and chemically enhanced WAFs of two dilbits in the laboratory. Only the chemically enhanced WAFs significantly increased the rates of mortality and malformations (at ≥152 and 47 μg/L total of 49 PACs, respectively), but cytochrome P450 1A (*cyp1a*) mRNA expression indicated the activation of phase I detoxification in all dilbit treatments (at μg/L levels). Krohn et al. (2021) examined metal concentrations in larval frogs (*Rana sylvatica*) in shoreline enclosures in which weathered dilbit had been experimentally spilled and cleaned up after 72 h. Larvae raised in these enclosures after clean-up contained higher concentrations of vanadium, molybdenum, and cadmium than controls, but not at levels of toxicological concern.

Clearly, much remains to be learned about how exposure to dilbit affects amphibians across their complex life history, and under ecologically relevant spill scenarios. To study the fate and effects of dilbit in freshwater ecosystems, our team simulated seven environmentally relevant oil spill scenarios in a natural boreal lake (Rodriguez-Gil et al., 2021). The seven scenarios represent the upper end of all onshore pipeline crude oil spills in North America between 2008 and 2019 (see Fig. S1, Rodriguez-Gil et al., 2021). This large-scale, highly realistic oil spill experiment was a

unique opportunity to expose wood frogs (*R. sylvatica*) to dilbit weathered by natural processes. In this paper, we describe a before-after-control-impact study to examine the response of developing wood frogs to environmentally relevant oil spill scenarios. We hypothesized that exposure of wood frog larvae to hydrocarbons and other compounds released from naturally weathered dilbit would: impair survival, growth, and development; induce *cyp1a* mRNA expression and EROD activity; alter levels of thyroid hormones; and depress activity, sociality, and space use.

2. Material and methods

2.1. Limnocorral study in Lake 260.

Wood frogs were exposed to hydrocarbons and other compounds released from dilbit spilled into limnocorrals in a natural, oligotrophic lake at the International Institute for Sustainable Development-Experimental Lakes Area (ELA) in northwestern Ontario, Canada (Fig. 1). The set-up of the limnocorrals and the properties of the added oil are described in Rodriguez-Gil et al. (2021) and Stoyanovich et al. (2021). Briefly, 10-m diameter cylindrical limnocorrals (1.5 m depth; ~100 m^3 volume) open to the atmosphere and lake bottom were installed in ELA Lake 260 (49°41’56.0”N, 93°45’57.9”W). On June 20, 2018, oil was added to seven limnocorrals, each of which received a different volume (1.5, 2.9, 5.5, 18, 42, 82, and 180 L) of Cold Lake Winter Blend dilbit to simulate a broad range of environmentally relevant oil spill sizes ranging from 1:71,353 to 1:505 oil-to-water ratio by volume (Rodriguez-Gil et al., 2021). Test solutions for the wood frog experiment were withdrawn from a water depth of 1 m from the center of seven oil-treated limnocorrals and one far-field control limnocorral (that received no oil).

2.2. Pre-experiment husbandry.

On May 5, 2018, one wood frog egg mass (~750 eggs) was removed from ELA Lake 227 and split into three equal parts. Each mass was suspended in a 70-L tank of clean lake water. Embryos hatched on May 15, 2018 and were reared in these tanks until Gosner Stage (GS) 25 (Gosner, 1960).

2.3. Experimental set-up and design

The wood frog experiment followed a replicated regression design using a before-after-control-impact (BACI) approach (Fig. 1). Experimental treatments (1 control, 7 oil treatments) were assigned (in triplicate) to 24 outdoor microcosms (53 x 32 x 20 cm food-grade stainless steel tanks) using spatially stratified randomization. Microcosms were located in the forested upland of Lake 260. On June 6, 2018 (day 0 of the experiment), microcosms were filled with unfiltered water (16 L) from their respective limnocorral, and the next day, 360 larvae (approximately GS 30) were randomly stocked at a density of 15 individuals per microcosm. Every 3-4 days, 50% (8 L) of microcosm water was removed and replaced with 8 L of new water from the appropriate limnocorral. The experiment consisted of a baseline phase (day 0–14) before oil was applied to the limnocorrals, and an exposure phase (day 15–30) after oil was applied to the limnocorrals (Fig. 1). Microcosms were covered with a wire mesh to deter predators, and contained floating platforms for metamorphs to rest on. Wood frogs were fed organic spinach (10 g per microcosm) every other day, and residual waste was removed daily.

2.4. Water sampling and analyses

Dissolved oxygen, pH, and water temperature were measured daily in microcosms using a Hach HQ40d meter (Hach Company, Loveland, Colorado). Water quality parameters were within acceptable ranges for amphibian health and were similar among treatments (Table S2).

As described in Rodriguez-Gil et al. (2021), water samples for hydrocarbon analyses were collected from the center of each limnocorral through 0.6 cm inner diameter high-density polyethylene tubing connected to a Spectra Field-Pro Peristaltic Pump (flow rate of 1 L/min). Hydrocarbon concentrations in the water column of the limnocorrals were determined ± 1 day of when water was withdrawn for the wood frog experiment (as such, these concentrations represent a conservative upper estimate of those in the microcosms). Additional water samples for hydrocarbon analyses were collected, in triplicate, directly from the microcosms housing wood frogs on day 33 (in conjunction with final lethal sampling of wood frogs; section 2.7).

All water samples were analyzed for total petroleum hydrocarbons (TPH) and 46 individual PACs, including 25 alkylated PAC homologues and other unsubstituted PACs, summarized as the total of all 46 PACs (ΣPAC_{46}), of alkylated PACs (ΣPAC_{alk}), and of 16 EPA-priority PAHs (ΣPAH_{EPA16}). Methods for hydrocarbon determination were described by Rodriguez-Gil et al. (2021) and Stoyanovich et al. (2022). Briefly, samples were collected (with no headspace) in pre-weighed 1-L certified clean amber glass bottle with PTFE-lined caps and processed on the day of collection. Samples were spiked with surrogates (20 µg o-terphenyl, 20 µg d_{50}-tetracosane ($C_{24}D_{50}$) and 1 µg deuterated PAH mixture including five 5 PAH congeners - manufactured by SPEX CertiPrep Labs, NJ, USA) and extracted with 50 mL of dichloromethane (DCM) for 16 h at 8 rpm on a roller

apparatus (DWK Life SciencesWheaton™ Bottom-Drive R2P 2.0 Roller Apparatus). The extracts were then removed and dried by passage through sodium sulphate and stored in the dark in 125 mL precleaned amber glass jars at 2 °C. Extracts were concentrated via rotary evaporation, solvent exchanged to hexane and then evaporated to a volume of 2 mL prior to TPH analysis by GC-FID. Extracts for PAC analysis went through an additional step of silica gel column fractionation. Triplicate laboratory blanks were run on ultra-pure Milli-Q water and PAC concentrations were blank-corrected. Percent recoveries of surrogates ranged from 71 to 92% for water samples. Hydrocarbon analyses were conducted by Environment and Climate Change Canada and Fisheries and Oceans Canada.

2.5. Survival, development, and growth

Survival was monitored daily. Body size and developmental stage were monitored three times during the baseline (days 4, 8, and 14) and exposure (days 18, 23, and 29) phases. At each sampling event, 10 larvae from each microcosm were staged using criteria from Gosner (1960), weighed in a beaker of water, and photographed dorsally. Photographs were analyzed using ImageJ to measure total length (head to tail tip). Due to their biphasic life cycle, the aquatic life stage of wood frogs is characterized by an initial period of rapid growth, followed by a reduction in size as the body restructures during metamorphosis (Riha and Berven, 1991). Thus, growth rates were confined to larval stages (< GS 42) only. In each phase, growth rates were determined over 10 days as the slope of the relationship between day of experiment vs. total length, assuming either a linear (G_{lin}; mm/d) or exponential model (G_{exp}; %/d).

2.6. Behavioral assays and video analysis.

Behavioral assays were performed four times (days 16, 20, 24, and 27) during the exposure phase. Assays consisted of open-arena tests (Carlson and Langkilde, 2013) that were performed on five individuals randomly selected from each microcosm. The subgroup was transferred to a white plastic behavioral arena (24 x 28 x 14 cm) containing treatment water. The arena was only used for one assigned microcosm and wood frogs were acclimated in the arena for 10 min before being filmed for 10 min. Five assays (five arenas, each with five individuals) were completed simultaneously using Activeon CX Gold Plus action cameras (San Diego, CA; 1080 pixels, 60 fps, narrow FOV). Three behavioral metrics were analyzed: activity, sociality, and space use (Sih et al., 2004). Twenty photos representing every 30 s interval were used for analysis. Location of each wood frog was determined using of a 6 x 7 grid overlay. An individual was recorded as being located in a grid square when the majority of its body, excluding the tail, was observed within the grid square. Space use was determined based on the location of an individual in an outer, middle, or inner square (Figure 1F). Activity was quantified as the number of lines crossed over the shortest distance to reach the individual's grid location from its previous location (Figure 1F). Sociality was quantified as the number of wood frogs within one grid unit of the center of an individual's body at each frame (Figure 1F). Two of the three replicate microcosms per treatment were randomly selected for these manual video analyses.

2.7. Final lethal sampling.

On day 35, all wood frogs were euthanized using an overdose of MS-222. Livers (6 individuals per microcosm; GS 37–45) were removed and flash frozen in liquid nitrogen

then stored at -80 °C. Livers from three individuals were used to quantify *cyp1a* and *60S ribosomal protein L8* (*rpl8*; reference gene) mRNA abundance using qPCR (Table S3). A long fragment (361 bp) was first amplified with primers designed from a *cyp1a* sequence of *Lithobates catesbeianus* using similar methods and kits described in Lara-Jacobo et al. (2019). The expression of *cyp1a* was normalized to the average transcript level of *rp18* to determine the relative fold change. The other three livers were used to measure EROD activity, an indicator of *cyp1a* gene activation. Briefly, EROD activity was measured in the post-mitochondrial supernatant "S9 fraction" prepared from homogenates of 15 to 30 mg crushed liver in 1 mL of Hepes Grinding Buffer and centrifuged at 10,000X g for 20 min at 4 ºC. Five wood frogs (GS 41–43) per microcosm were sampled for thyroid hormone analysis. Following euthanasia, whole bodies were flash frozen in Eppendorf tubes. Total 3,5,3'-triodo-L-thyroinine (T_3) and 3,5,3',5'-tetraiodo-L-thyronine (T_4) were quantified using enzyme immunoassay test kits (07BC-1005 for T3, and 07BC-1007 for T4; MP Biomedicals, Solon, Ohio, USA) based on methods adapted from Hersikorn and Smits (2011).

2.8. Data analysis.

Statistical tests were performed using R (R Core Team, 2018) or SigmaPlot Version 14.0. The $\log_{10}$ of oil volume (+1) added to each limnocorral was used as the main effect in all models. Tests for normality and homogeneity of variance were performed, or residuals were visually examined, to ensure model assumptions were met, and data were transformed when required. Linear regression models were used to analyze TPH and ΣPAC_{46} concentrations on day 33 ($\log_{10}$), percent of individuals as metamorphs (GS 42 or higher) on day 35, body size on day 29 (squared), body mass on day 29, relative

mRNA abundance of *cyp1a* on day 35 (log_{10}), EROD activity on day 35 (square root), and whole-body T_4 concentration on day 35. Logistic ordinal regression was used to examine the GS of individuals on day 35. To analyze growth rates, a repeated measures 2-way ANOVA was performed with oil treatment and experimental phase as factors. Whole-body T_3 concentration could not be transformed to meet assumptions of parametric tests, so a Spearman rank order correlation was performed. Restricted maximum likelihood linear mixed effects models were used to examine the relationship between oil volume and activity and sociality. Each microcosm was treated as a statistical unit, therefore, prior to analysis, activity and sociality were totaled for the five individuals monitored in each tank. Sampling day and microcosm number were included in the model as random effects. Microcosm replicate and assessment day were not statistically significant, and thus were not treated as independent variables (i.e., each sampling point represents the behaviour of five individuals in a microcosm on one of the four sampling days). The 'lmer' function in the 'lme4' package was used to create the models and the 'coef' function was used to approximate *P* values for the linear mixed effects model coefficients.

3. Results

3.1 Hydrocarbon concentrations.

Wood frogs experienced increasing concentrations of hydrocarbons over the exposure phase of the experiment, as the spilled oil weathered and compounds from the oil entered the water column of the limnocorrals. As described by Stoyanovich et al. (2021), volatile petroleum hydrocarbons quickly evaporated from the surface oil slicks, greatly increasing the density and viscosity of the oil, and forming tar balls, patties, and mats; depending on

the volume of oil applied, these tar formations began to sink to the lake sediments between 12 to 31 days after oil addition. Petroleum hydrocarbons were detected in water within 12 hr of the oil spills (Stoyanovich et al., 2022). Over the next 2 weeks, concentrations of hydrocarbons in the oil-treated limnocorrals (i.e., the source water for the wood frog experiment) generally increased over time (Fig. 2A-B) and reached maximum concentrations of 276-1,506 μg/L for TPH and 664-2,346 ng/L for ΣPAC_{46}. In contrast, TPH and ΣPAC_{46} concentrations in the control limnocorral remained low (<107 μg/L and <25 ng/L, respectively) and showed little change over time (Fig. 2A-B). PACs in the water column of oil-treated limnocorrals were dominated by C1, C2, and C3 alkylated homologs of naphthalenes, phenanthrenes, fluorenes, and dibenzothiophenes (Stoyanovich et al., 2022).

In conjunction with the final lethal sampling of wood frogs (Section 3.6), a full suite of hydrocarbon chemistry was conducted in all microcosms on day 33. Treatment-mean TPH concentrations in the water of oil-contaminated microcosms ranged from 319-1,018 μg/L (Table 1), similar to those in the limnocorrals (Fig. 2A). Background levels of TPH in the control microcosms (437 ± 57 μg/L, mean ± standard deviation (SD)) were somewhat higher than expected. In all microcosms, the TPH profile was dominated by mid-range *n*-C_{16} to *n*-C_{34} hydrocarbons, with a lesser amount of heavy range (>*n*-C_{34}) compounds and semivolatives (*n*-C_{10} to n-C_{16}), and a negligible amount of volatiles (<*n*-C_{10}) (Table 1). Treatment-mean ΣPAC_{46} concentrations for oil-contaminated microcosms ranged from 37–121 ng/L (Table 1), above concentrations in the control microcosms (23 ± 8 ng/L, mean ± SD), but less than those in oil-treated limnocorrals (Fig. 2B). Similar to the limnocorrals (Rodriguez-Gil et al., 2021), PACs in the microcosms were dominated

by alkylated forms (Table 1), mainly naphthalenes, phenanthrenes, fluorenes, and dibenzothiophenes (Table S4).

Wood frogs were exposed to a gradient of hydrocarbons during the exposure phase that corresponded to the magnitude of the experimental oil spills. On the days water was withdrawn from the limnocorrals and added to the microcosms, concentrations of ΣPAC_{46} in lake water generally increased as a function of the volume of oil applied to the limnocorrals, but this gradient was much stronger later in the exposure phase (Fig. 2C; linear regression; day 15: $F_{(1,6)} = 66$, $P < 0.001$; day 19: $F_{(1,6)} = 0.1$, $P = 0.7$; day 23: $F_{(1,6)} = 7.2$, $P = 0.04$; day 30: $F_{(1,6)} = 17$, $P = 0.006$). Concentrations of TPH and ΣPAC_{46} in the microcosms measured 3 days after the final addition of limnocorral water confirmed the gradient in hydrocarbon concentrations across treatments persisted between water changes (Fig. S1). After normalization to control values, mean concentrations of TPH and ΣPAC_{46} in microcosms on day 33 were significantly related to oil treatment (linear regression; [normalized TPH, µg/L] = -132 + (240 * $\log_{10}$[oil volume + 1, L]); $R^2 = 0.80$, $F_{(1, 21)} = 36$, $P < 0.001$); and $\log_{10}$[normalized ΣPAC_{46} + 10, ng/L] = 1.0 + (0.38 * $\log_{10}$[oil volume + 1, L]); $R^2 = 0.60$, $F_{(1, 21)} = 32$, $P < 0.001$).

3.2. Survival.

Almost all wood frogs survived the experiment, and the few mortalities that did occur were not related to oil treatment. During the baseline phase, survival was 100% in all microcosms. During the exposure phase, survival was 100% in all microcosms except two. One replicate microcosm of the 2.9-L and 5.5-L treatments had 13 and 87% survival, respectively; given the unexplained low survival of the former, this replicate of the 2.9-L treatment was excluded from subsequent analyses.

3.3. Development.

Development of wood frogs through the Gosner (1960) staging system was similar among experimental groups in the baseline (Fig. S2) and exposure (Fig. 3) phases. On day 4, 80% or more of the wood frogs were at GS 26, the first larval stage of the amphibian life cycle, and the variation among individuals was limited (range: GS 26–28). By the end of the baseline phase, a wider range in development (from GS 27–32, on day 14) was observed as hind limbs formed (Fig. S2). During the exposure phase, development spanned across the larval stages on day 23 (from GS 28–39) and day 29 (from GS 30–41) and was a mix of late-stage larvae and metamorphs (i.e., GS 42 or higher) on day 35 (GS 36–45) (Fig. 3). The time to metamorphosis (i.e., from GS 20–42) was <57 days in all treatments. By the end of the exposure phase, 21% of wood frogs were metamorphs; although this percentage varied among treatments (from 14% in the 42-L treatment to 29% in the 82-L treatment) it was not significantly related to oil treatment (Fig. S3; linear regression; [percent metamorphs per microcosm] = 22.7 – (1.2 × $\log_{10}$[oil volume, L + 1]; $F_{(1, 22)} = 0.22$, $P = 0.64$). Furthermore, no statistical differences were found when the GS of wood frogs in each oil treatment on day 35 were compared to the control (Table S5).

3.4. Larval growth.

Exposure to oil-contaminated water did not alter growth rates of larval wood frogs (Fig. 4). During the baseline phase, wood frogs approximately doubled in body size, with growth rates among microcosms ranging from 1.1–2.0 mm/d (G_{lin}) or 4.6–9.4 %/d (G_{exp}). During the exposure phase, growth rates among microcosms ranged from 0.7–1.6 mm/d (G_{lin}) or 1.5–4.1%/d (G_{exp}). Oil treatment had no significant effect on growth rate of

wood frogs, irrespective of whether a linear (2-way repeated measures ANOVA; $F_{(15, 45)}$ = 1.4, P = 0.29) or exponential (2-way repeated measures ANOVA; $F_{(15, 45)}$ = 1.3, P = 0.31) growth model was assumed. Growth rates were significantly lower during the exposure phase in the exponential model (2-way repeated measures ANOVA; $F_{(15, 45)}$ = 148, $P < 0.001$), but this was true for all treatments and the control.

The same conclusion is reached when body size and mass of wood frogs were examined at specific time points. The mean body length of larvae in oil treatments during the exposure phase was within ± 8% of the control, with no apparent trend in relation to oil volume (Fig. S4B). On the last sampling day before metamorphic climax (i.e., day 29), larval body length was not significantly related to oil treatment (linear regression; $[\text{body size, mm}]^2 = 2564 - (30 \times \log_{10}[\text{oil volume, L} + 1]$; $F_{(1, 228)} = 0.274$, $P = 0.60$). Similarly, body mass of larvae approximately doubled (from day 19 to 29) during the exposure phase, but with little variation among treatments (Fig. S4C). Body mass on day 29 was not statistically related to oil treatment (linear regression; $[\text{body mass, g}] = 1.5 - (0.011 \times \log_{10}[\text{oil volume, L} + 1]$; $F_{(1, 228)} = 0.11$, $P = 0.74$).

3.5. Larval behavior.

Exposure to oil-contaminated water influenced larval behavior of wood frogs, but only for one of the three analyzed metrics (Fig. 5). For every 1% increase in oil applied to the limnocorrals, the number of lines crossed during the assay by wood frogs decreased by 0.17 (restricted maximum likelihood linear mixed effects model; fixed effect (Fig. 5A; $\log_{10}[\text{oil volume} + 1]$ estimate = -16.848 ± 8.517 (SE), t = -1.978, p = 0.048, n = 64). The mean fitted values (401 ± 4.58, mean ± SD) for number of lines crossed during the assay by wood frogs exposed to the highest oil treatment (180-L) was 38 less than the

mean fitted values (439 ± 4.58) for control individuals, which corresponds to an 8.7% reduction in activity (Fig. 5A). Treatment had no influence on sociality (Fig. 5B; restricted maximum likelihood linear mixed effects model; fixed effect ($\log_{10}$[oil volume + 1] estimate = -0.695 ± 1.640 (SE), t = -0.424, p = 0.672, n = 64;) nor space use (Fig. 5C). Percentage of time spent in the outer ring of the behavioral arena ranged from 90.1 ± 4.02 % to 93.8 ± 1.04, with the difference between individuals in the highest and lowest oil treatment being less than 1%.

3.6. Final lethal sampling.

When wood frogs were lethally sampled on day 35, treatment means ranged from 43.2–51.3 mm for body length, 0.88–1.25 g for body mass, and 0.026–0.052 g for liver mass. While the control had the highest mean value for these metrics among the experimental groups, no relationship was evident in relation to oil treatment (Fig. S5A-C). Morphological malformations (primarily bent tails) were observed in all experimental groups, but tended to be lower in the control (15.5%, on average) than most oil treatments (Fig. S5D). Gosner stage on day 35 ranged from 36–45, but was dominated (>77%) by GS 39–42 (Fig. 3, black bars).

3.7. Cytochrome P450.

Cytochrome P450 1A enzymes in wood frogs sampled on day 35 were not altered by exposure to oil-contaminated water. Normalized *cyp1a* mRNA expression in liver of wood frogs varied up to 5-fold relative to the control mean, but was not related to oil treatment (Fig. 6A; linear regression; $\log_{10}$[normalized *cyp1a* mRNA, fold-change relative to control] = -0.22 + (0.057 × $\log_{10}$[oil volume, L + 1]); $F_{(1, 58)} = 0.59$, $P = 0.4$).

Likewise, EROD activity in liver of wood frogs ranged from 4.9–78.6 pmol/min/mg protein, and was not related to oil treatment (Fig. 6B; linear regression; square root [EROD, pmol/min/mg protein] = 5.89 + (0.038 × $\log_{10}$[oil volume, L + 1]); $F_{(1, 59)} = 0.0$, $P = 0.9$). We also checked if GS could explain some of the variability in *cyp1a* mRNA expression and EROD activity among experimental groups, but it was not a significant factor ($P > 0.05$) when included in regression models (Fig. S6A-B).

3.8. Thyroid hormones.

Thyroid hormone levels measured in whole-body samples of wood frogs on day 35 were also not influenced by exposure to oil-contaminated water. Whole-body concentrations of T_3 and T_4 of individuals ranged from 18.2–36.7 and 12.2–175.8 ng/g tissue (wet weight), respectively, and did not change as a function of oil treatment for T_3 (Fig. 6C; Spearman rank order correlation; $\log_{10}$[oil volume, L + 1] vs. [whole-body T_3 concentration, ng/g tissue)]; $r = 0.040$, $P = 0.8$, n = 40) or T_4 (Fig. 6D; linear regression; [whole-body T_4 concentration, ng/g tissue] = 78.4 – (4.1 × $\log_{10}$[oil volume, L + 1]); $F_{(1, 38)} = 0.33$, $P = 0.6$). Although no relationship was evident, wood frogs exposed to water from the 180-L limnocorral had the highest mean concentrations of T_3 (35.3 ± 1.1 ng/g) and T_4 (101 ± 48 ng/g) among the experimental groups (Fig. 6C-D). The ratio of T3 to T4 ranged from 0.21–2.1 and was not related to oil treatment, irrespective of whether GS was, or was not, included in the regression model (Fig. S7).

4. Discussion

4.1. Hypothesis 1: Impaired survival, growth, and development.

We found no evidence to support the hypothesis that exposure to hydrocarbons and other compounds released from dilbit as it weathers naturally in a lotic freshwater environment affects the survival, growth, or development of larval wood frogs.

Direct exposure to large quantities of oil in water (especially under high mixing energy conditions) can be acutely lethal to aquatic amphibians, but the water fraction under oil is much less toxic and not likely to be lethal under most spill conditions. When Lefcort et al. (1997) directly exposed larval mole salamanders (*Ambystoma spp.*) to used motor oil at concentrations of 6,000–100,000 μL oil/L water, the highest concentration resulted in 100% mortality after 17 h, and the average 96-h LC_{50} was 19,880 μL oil/L water. In a comprehensive study of the acute lethality of five types of oils (Hedtke and Puglisi, 1982), oil emulsions were the most lethal to wood frogs (96-h LC_{50} from 5–78 μL oil/L water), exposure to a floating layer of oil was less so (96-h LC_{50} ranged 1,500–6,300 μL oil/L water), and exposure to water-accommodated fractions in static tests were the least toxic (96-h LC_{50} from 342,000–561,000 μL oil/L water for No. 2 fuel oil and >250,000 μL oil/L water for mixed blend sweet crude). In comparison, we applied between 14–1,982 μL oil/L water to limnocorrals in a boreal lake with low mixing energy and exposed wood frogs to water from these limnocorrals. Thus, even the highest oil concentration in our study was two orders of magnitude less than published LC_{50} values for wood frogs exposed to water accommodated fraction of oils.

Growth and development are ubiquitously used as a measure of fitness in amphibian ecotoxicology and previous studies suggest that petroleum products may negatively influence these fundamental processes in larval amphibians. For example, in a

60-day chronic exposure to the water-accommodated fraction of Kazakhstan crude oil (at concentrations of 0.05, 0.5, and 1.5 mg/L), marsh frog (*R. ridibunda*) larvae exhibited a concentration-dependent reduction in body weight, body size, and developmental stage (Sutuyeva et al., 2019). Similarly, green treefrog (*Hyla cinerea*) larvae housed in artificial ponds had substantially lower body weights when exposed to a high concentration of used crankcase oil (100 mg/L), but this difference in body weight relative to controls was not observed at lower concentrations (10 and 55 mg/L) (Mahaney, 1994). Although no significant differences in the number of metamorphs among treatments were observed in the study by Mahaney (1994), none of the larvae exposed to 100 mg oil/L water reached metamorphosis. Further, larval mole salamanders held in mesocosms contaminated by motor oil and silt, but not motor oil alone, had lower body mass and length at metamorphosis, but in contrast to the previous studies, oil-exposed individuals reached metamorphosis earlier than controls (Lefcort et al., 1997). In our study, we found no evidence that exposure to oil-contaminated water across a broad range of realistic spill scenarios had any influence on larval growth or development, even though we explored this rigorously using multiple metrics of growth (i.e., body length and body mass measurements at several time points, and a BACI comparison of growth rates across 10-day periods in the baseline and exposure phase) and development (i.e., frequency histograms of GS at several time points, and percent of individuals reaching metamorphic climax at the end of the experiment).

4.2. Hypothesis 2: Elevated cyp1a mRNA expression and EROD activity.

The cytochrome P450 enzymes are a superfamily of heme-containing proteins that play a vital role in the phase 1 detoxification pathway in vertebrates. As CYP1A enzymes are

strongly induced when xenobiotics bind to the aryl hydrocarbon receptor, the induction of CYP1A is commonly used as a biomarker of exposure to many organic contaminants, including dioxins, polychlorinated biphenyl ethers, and PAHs. In this study, we examined whether spills of dilbit across a broad and realistic range of oil:water ratios result in aqueous hydrocarbon concentrations at levels high enough to induce CYP1A in wood frogs (near metamorphic climax), which we measured through two, independent methods.

First, we measured the abundance of *cyp1a* mRNA in wood frog livers. While *cyp1a* mRNA levels were, on average, the greatest in the highest oil treatment (about 2-fold the control), we did not observe a significant relationship between oil treatment and *cyp1a* mRNA levels in wood frogs. Prior to our work, only one previous study measured *cyp1a* mRNA levels in any amphibian species after exposure to dilbit. Lara-Jacobo et al. (2019) reported *cyp1a* mRNA levels in de-jellied frog (*S. tropicalis*) embryos were elevated after exposure to dilbit WAFs, with estimated ΣPAC concentrations near 10,000 ng/L resulting in ~10-fold increase in *cyp1a* mRNA relative to controls. The ΣPAC_{46} concentrations in the microcosms around the time wood frogs were sampled for *cyp1a* mRNA expression (121 ng/L and below; Table 1) are less than the threshold (i.e., approximately 700 ng/L) where Lara-Jacobo et al. (2019) reported elevated *cyp1a* mRNA in *S. tropicalis* embryos, and therefore, it is not surprising that *cyp1a* mRNA expression was not induced in wood frogs in the present experiment.

Second, we measured EROD activity (a direct measure of CYP1A) in wood frog livers. Laboratory studies support the efficacy of this assay as a biomarker of PAH exposure in amphibians. For example, some, but not all, PAHs increase EROD activity in

primary hepatocytes of *R. esculenta* adults (Rouhani-Rankouhi and Van Den Berg, 2005), and EROD activity responds in a concentration-dependent manner to the PAH benzo(*a*)pyrene in *X. laevis* larvae (Gauthier et al., 2004). EROD activity measured in our study (5–79 pmol/min/mg protein) overlaps with values reported for *Lithobates pipiens* adults captured from Green Bay, USA, a site contaminated by dioxins, furans, and PCBs (~90–400 pmol/min/mg protein; Huang et al. (1999)), as well as values reported for *X. laevis* larvae exposed to water samples from France's Dadou River, a site impacted by a fluorspar mine (~12–154 pmol/min/mg protein; Gauthier et al. (2004)), but less than those measured in *R. sylvatica* larvae held in oil sands mining-impacted wetlands (<1 pmol/min/mg protein; Hersikorn and Smits (2011)). Notably, in the latter study, there was no significant difference in EROD activity in wood frogs caged in artificial wetlands on former mine sites versus reference wetlands. Likewise, we found no difference in EROD induction in wood frogs across oil treatments, despite a statistically significant gradient in PAC exposure concentrations.

We suggest CYP1A enzymes in wood frogs were not induced in our experiment because: i) very little of the PACs in the spilled oil ended up in the water column of the limnocorrals (Stoyanovich et al, 2022), and ii) larval wood frogs are known to efficiently metabolize and eliminate many parent and alkyl-substituted PACs (Bilodeau et al., 2019). Considering our study simulated an environmentally relevant range of oil spill sizes, it is unlikely *cyp1a* mRNA or EROD activity would be reliable biomarkers of PAC exposure in larval amphibians in all but the most extreme dilbit spill situations.

4.3. Hypothesis 3: Altered thyroid hormone levels.

Petroleum products, or wastes created from their production, may disrupt the thyroid system of amphibians, as suggested by altered expression of important genes in the thyroid cascade or direct measurements of thyroid hormone levels in amphibian tissues. For example, exposure of *X. laevis* to water accommodated fractions of crude oil causes downregulation of thyroid hormone receptor beta (TRβ), a gene that is directly modulated by thyroid hormone, although this result was only significant at one of the four oil concentrations tested (i.e., 0.25 g/L) (Truter et al., 2017). More convincingly, whole-body T_3 and T_4 concentrations of tadpoles chronically exposed to the water-accommodated fraction of crude oil or diesel fuel showed significant, concentration-dependent, reduction in two frog species (Sutuyeva et al., 2020). Whole-body T_4 concentrations in wood frogs caged in wetlands in Canada's oil sands were highest in young (<7 year old) reclamation wetlands on old mine sites, yet these T_4 concentrations were not significantly different from those measured in larvae in older reference wetlands (Hersikorn and Smits, 2011).

Given the potential for dilbit to contain endocrine-disrupting compounds, we hypothesized that exposure to oil-contaminated water from a dilbit spill during the critical period of prometamorphosis would alter levels of thyroid hormones in wood frogs near metamorphic climax. We found, however, no evidence to support this hypothesis; neither whole-body T_3 concentrations, whole-body T_4 concentrations, nor the ratio of whole-body T_3:T_4 (which indicates how much thyroid hormone is present in the more active form) was related to oil treatment. A similar lack of sensitivity of T_3 and T_4 concentrations was reported when larval wood frogs were exposed to different levels of

Hg in their diet, despite Hg tissue levels above known toxicity thresholds for other species (Wada et al., 2011).

4.4. Hypothesis 4: Depressed activity, sociality, and space use.

Larval amphibians rely on activity to compete for and obtain food, to avoid predators, and to seek out optimal habitat conditions. Lower foraging effort reduces growth in of frog larvae and can lengthen development (Skelly and Werner, 1990). The reduction in activity we observed in larval wood frogs exposed to oil-contaminated water may have been due to motor neuron failure caused by inhibition of acetylcholinesterase activity, although future study is necessary to confirm this hypothesis. Effects on larval activity following exposure to dilbit or PACs have not previously been quantified; however, experiments with fishes have shown that exposure to crude oil result in inhibition of acetylcholinesterase activity (e.g., Akaishi et al., 2004). Another possible mechanism for the observed reduction in activity was a change in the structure or function of the heart. In juvenile fish, exposure to crude oil can cause cardiac remodeling (Alderman et al., 2017) and alter heart rate and stroke volume (Nelson et al., 2017). These changes to the heart are thought to be correlated to reduced burst and sustained swimming performance in fishes because lower cardiac activity cannot sustain the respiratory demands required for swimming (Alderman et al., 2017; Nelson et al., 2017). Future studies should consider investigating neuromuscular physiology and cardiac function in response to dilbit exposure and how this might influence the activity of larval amphibians.

Only one of three measured behaviors of wood frogs in our study changed in response to exposure to oil-contaminated water. Sociality and space use have been found to be repeatable in amphibian larvae, suggesting that these risk-taking personality traits

should be related to activity (Carlson and Langkilde, 2013; Urszan et al., 2015). Perhaps the sample size did not provide sufficient statistical power to detect the effect of oil exposure on sociality and space use. Further, the absence of predators in our study may have contributed, as repeatable behaviors are more likely to exhibit high interindividual variation in the presence of predator cues (Urszan et al., 2015).

4.5. Conclusions and future research. We found that experimental spills of dilbit, spanning the range of onshore spills in North America over the last decade, in a temperate, boreal lake did not result in hydrocarbon concentrations in water that affected the survival, development, growth of larval wood frogs, or levels of thyroid hormones at metamorphic climax. In other words, even the worst oil spill scenario in our experiment (similar to the Kalamazoo River spill, by oil-to-volume ratio) resulted in hydrocarbon contamination of the water column below the no-observed-adverse-effects level. The only significant result in our study was a modest reduction in activity levels of wood frogs (but with no concurrent change in space use or sociality). Such a behavioral change could have fitness consequences for amphibians, and further research is justified to corroborate this finding. Why did our study find little effect of dilbit on larval wood frogs when several earlier studies have reported adverse effects of this unconventional oil on aquatic vertebrates (mainly, fish)?

First, this lack of congruence could be explained, in part, by species and life stage of study organisms. Interspecific differences in sensitivity to chemicals could have played a role, as amphibians tend to be less sensitive than fish to most chemicals (Weltje et al., 2013). In addition, the timing of oil exposure in this study (i.e., spanning the larval stage

of the amphibian life cycle) may have occurred after many of the early-life developmental milestones previously shown to be affected by dilbit (Alsaadi et al., 2018).

Second, our study was designed to simulate a real-world spill of dilbit in a freshwater environment, which is not the goal of most aquatic toxicology studies of dilbit. We added dilbit to a boreal lake, then allowed the oil to weather naturally under ambient environmental conditions. Based on diagnostic ratios of *n*-alkanes, isoprenoids, and PAHs, the major processes acting on the oil slick in the limnocorrals were evaporation, dissolution, and photooxidation, but not likely biodegradation (Stoyanovich, 2021). Only a small percentage (1% or less, by mass) of the hydrocarbons in the spilled oil was present in the water column of the limnocorrals (Stoyanovich et al., 2022). Our approach is quite different than creating oil solutions in the laboratory, which attempts to mix oil and water mechanically, sometimes with the help of chemical dispersants or high energy to force more hydrocarbons into the water phase (Lee et al., 2015).

Third, we simulated an environmentally-relevant range of oil-to-water ratios in the limnocorrals based on previous on-shore oil spills in North America (Rodriguez-Gil et al., 2021). Again, this is quite different from most toxicological studies, which usually attempt to expose organisms to a broad range of concentrations to yield complete concentration-response curves so toxicological parameters can be calculated with confidence. Aquatic toxicological studies show us the potential for dilbit to harm aquatic organisms, whereas field-based ecotoxicological experiments, such as ours, test what harm from dilbit is likely under real-world spill conditions.

Lastly, we point out that wood frogs in our study were not in physical contact with dilbit which is likely the situation for larvae in real-world spills, as they are mobile

and could presumably avoid a surface oil slick or sunken bitumen. However, this may not be the case for other life stages (e.g., egg masses suspended near the water surface, or metamorphs as they emerge onto land), and thus we recommend future research explore the potential effects of physical contact with dilbit on these vulnerable life stages.

5. Authorship contribution statement

Conceptualization (CTH, DMO, DTJD, JMB, SAP), methodology (CTH, DMO, DTJD, GP, SAP), formal analysis and investigation (CTH, DMO, DTJD, GP, JLRG, SAP), original draft preparation (CTH, DMO, DTJD, SAP), reviewing and editing (all authors), funding acquisition (BH, CTH, DMO, JMB, MLH, VSL), resources (BH, CTH, DMO, JMB, MLH, JLRG, VSL), supervision (CTH, DMO, JLRG).

Supplemental information

The online supplementary information contains five tables (Table S1 – S5) and seven figures (Fig. S1 – S7).

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics and permitting

All animal collection and handling procedures were done in accordance with the animal care protocol approved by Queen's University (Protocol No. 2018-1831). Permission to collect wood frog was authorized by the Ontario Ministry of Natural Resources (Wildlife Scientific Collector's Authorization No. 1089584). Permission to conduct oil spills in ELA Lake 260 was authorized by the Ontario Ministry of the Environment and Climate Change (authorization letter dated June 13, 2018).

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References

Akaishi, F.M., de Assis, H.C., Jakobi, S.C., Eiras-Stofella, D.R., St-Jean, S.D., Courtenay, S.C., Lima, E.F., Wagener, A.L., Scofield, A.L., Ribeiro, C.A., 2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax* sp.) after waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Archives of Environmental Contamination and Toxicology 46, 244-253.

Alderman, S.L., Lin, F., Farrell, A.P., Kennedy, C.J., Gillis, T.E., 2017. Effects of diluted bitumen exposure on juvenile sockeye salmon: From cells to performance. Environmental Toxicology and Chemistry 36, 354-360.

Alsaadi, F., Hodson, P.V., Langlois, V.S., 2018. An embryonic field of study: the aquatic fate and toxicity of diluted bitumen. Bull Environ Contam Toxicol 100, 8-13.

Amaeze, N.H., Onadeko, A., Nwosu, C.C., 2014. Comparative acute toxicity and oxidative stress responses in tadpoles of *Amietophrynus regularis* exposed to refined petroleum products, unused and spent engine oils. African Journal of Biotechnology 13, 4251-4258.

Carlson, B.E., Langkilde, T., 2013. Personality traits are expressed in bullfrog tadpoles during open-field trials. Journal of Herpetology 47, 378-383.

Culp, J.M., Droppo, I.G., di Cenzo, P.D., Alexander, A.C., Baird, D.J., Beltaos, S., Bickerton, G., Bonsal, B., Brua, R.B., Chambers, P.A., Dibike, Y., Glozier, N.E., Kirk, J.L., Levesque, L., McMaster, M., Muir, D.C.G., Parrott, J.L., Peters, D.L., Pippy, K., Roy, J.W., 2021. Ecological effects and causal synthesis of oil sands activity impacts on river ecosystems: Water synthesis review. Environmental Reviews 29, 315-327.

Díaz, S., Settele, J., Brondízio, E.S., Ngo, H.T., Agard, J., Arneth, A., Balvanera, P., Brauman, K.A., Butchart, S.H.M., Chan, K.M.A., Garibaldi, L.A., Ichii, K., Liu, J., Subramanian, S.M., Midgley, G.F., Miloslavich, P., Molnár, Z., Obura, D., Pfaff, A., Polasky, S., Purvis, A., Razzaque, J., Reyers, B., Chowdhury, R.R., Shin, Y.-J., Visseren-Hamakers, I., Willis, K.J., Zayas, C.N., 2019. Pervasive human-driven decline of life on Earth points to the need for transformative change. Science 366, eaax3100.

Everitt, S., Fujita, K.K., MacPherson, S., Brinkmann, M., Pyle, G.G., Wiseman, S., 2021. Toxicity of weathered sediment-bound dilbit to early life stages of zebrafish (*Danio rerio*). Environmental Science & Technology 55, 1721-1729.

Gauthier, L., Tardy, E., Mouchet, F., Marty, J., 2004. Biomonitoring of the genotoxic potential (micronucleus assay) and detoxifying activity (EROD induction) in the River Dadou (France), using the amphibian *Xenopus laevis*. Science of the Total Environment 323, 47-61.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16, 183-190.

Hedtke, S.F., Puglisi, F.A., 1982. Short-term toxicity of five oils to four freshwater species. Archives of Environmental Contamination and Toxicology 11, 425-430.

Hersikorn, B.D., Smits, J.E., 2011. Compromised metamorphosis and thyroid hormone changes in wood frogs (*Lithobates sylvaticus*) raised on reclaimed wetlands on the Athabasca oil sands. Environmental Pollution 159, 596-601.

Huang, Y.-W., Karasov, W.H., Patnode, K.A., Jefcoate, C.R., 1999. Exposure of northern leopard frogs in the Green Bay ecosystem to polychlorinated biphenyls, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans is measured by direct chemistry but not hepatic ethoxyresorufin-o-deethylase activity. Environmental Toxicology and Chemistry 18, 2123–2130.

King, T.L., Robinson, B., Ryan, S., Clyburne, J.A.C., 2020. Canadian bitumen is engineered for transport, but the type of product produced can affect spill contingency planning. Environmental Science Processes & Impacts 22, 863-872.

Krohn, R.M., Palace, V., Smits, J.E.G., 2021. Metal changes in pre- and post-metamorphic wood frog (*Lithobates sylvaticus*) tadpoles: Implications for

ecotoxicological studies. Archives of Environmental Contamination and Toxicology 80, 760-768.

Lara-Jacobo, L.R., Willard, B., Wallace, S.J., Langlois, V.S., 2019. Cytochrome P450 1A transcript is a suitable biomarker of both exposure and response to diluted bitumen in developing frog embryos. Environmental Pollution 246, 501-508.

Lee, K., Boufadel, M., Chen, B., Foght, J., Hodson, P., Swanson, S., Venosa, A., 2015. Expert panel report on the behaviour and environmental impacts of crude oil released into aqueous environments, Royal Society of Canada, Ottawa, ON.

Lefcort, H., Hancock, K.A., Maur, K.M., Rostal, D.C., 1997. The effects of used motor oil, silt, and the water mold *Saprolegnia parasitica* on the growth and survival of mole salamanders (genus *Ambystoma*). Archives of Environmental Contamination and Toxicology 32, 383–388

Madison, B.N., Hodson, P.V., Langlois, V.S., 2015. Diluted bitumen causes deformities and molecular responses indicative of oxidative stress in Japanese medaka embryos. Aquatic Toxicology 165, 222-230.

Mahaney, P.A., 1994. Effects of freshwater petroleum contamination on amphibian hatching and metamorphosis. Environmental Toxicology and Chemistry 13, 259-265.

Marvin, C.H., Berthiaume, A., Burniston, D.A., Chibwe, L., Dove, A., Evans, M., Hewitt, L.M., Hodson, P.V., Muir, D.C.G., Parrott, J., Thomas, P.J., Tomy, G.T., 2021. Polycyclic aromatic compounds in the Canadian environment: Aquatic and terrestrial environments. Environmental Pollution 285, 117442.

McCune, J.L., Colla, S.R., Coristine, L.E., Davy, C.M., Flockhart, D.T.T., Schuster, R., Orihel, D.M., 2019. Are we accurately estimating the potential role of pollution in the decline of species at risk in Canada? FACETS 4, 598-614.

McGrath, E., Alexander, M., 1979. Observations on the exposure of larval bullfrogs to fuel oil. Transactions of the Northeast Section of the Wildlife Society 36, 45-51.

NAS, 2016. Spills of diluted bitumen from pipelines: A comparative study of environmental fate, effects, and response. National Academies of Sciences, Engineering, and Medicine. The National Academies Press, Washington, DC.

Nelson, D., Stieglitz, J.D., Cox, G.K., Heuer, R.M., Benetti, D.D., Grosell, M., Crossley II, D.A., 2017. Cardio-respiratory function during exercise in the cobia, *Rachycentron canadum*: The impact of crude oil exposure. Comparative Biochemistry and Physiology, Part C 201, 58-65.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

Riha, V.F., Berven, K.A., 1991. An analysis of latitudinal variation in the larval development of the wood frog (*Rana sylvatica*). Copeia 1, 209-221.

Rodriguez-Gil, J.L., Stoyanovich, S., Hanson, M.L., Hollebone, B., Orihel, D.M., Palace, V., Faragher, R., Mirnaghi, F.S., Shah, K., Yang, Z., Black, T.A., Cederwall, J., Mason, J., Patterson, S., Timlick, L., Seguin, J., Blais, J.M., 2021. Simulating diluted bitumen spills in boreal lake limnocorrals - Part 1: Experimental design and responses of

hydrocarbons, metals, and water quality parameters. Science of the Total Environment 790, 148537.

Rouhani-Rankouhi, T., Koomen, B., Sanderson, J.T., Bosveld, A.T.C., Seinen, W., , Van Den Berg, M., 2005. Induction of ethoxy-resorufin-O-deethylase activity by halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons in primary hepatocytes of the green frog (*Rana esculenta*). Environmental Toxicology and Chemistry 24, 1428-1435.

Sih, A., Bell, A., Johnson, J.C., 2004. Behavioral syndromes: An ecological and evolutionary overview. Trends in Ecology and Evolution 19, 372-378.

Skelly, D.K., Werner, E.E., 1990. Behavioral and life-historical responses of larval American toads to an Odonate predator. Ecology 71, 2313-2322.

Stoyanovich, S., 2021. Chapter 4: Chemical weathering patterns of diluted bitumen spilled into freshwater limnocorrals. In: The fate and behaviour of diluted bitumen and its chemical constituents in freshwater systems following simulated spills, PhD Thesis, Department of Biology. University of Ottawa, Ottawa, Ontario, Canada, p. 322.

Stoyanovich, S., Yang, Z., Hanson, M., Hollebone, B.P., Orihel, D.M., Palace, V., Rodriguez-Gil, J.R., Mirnaghi, F., Shah, K., Blais, J.M., 2022. Fate of polycyclic aromatic compounds from diluted bitumen spilled into freshwater limnocorrals. Science of the Total Environment.

Stoyanovich, S.S., Rodriguez-Gil, J.R., Hanson, M., Hollebone, B.P., Orihel, D.M., Palace, V., Faragher, R., Mirnaghi, F.S., Shah, K., Yang, Z., Blais, J.M., 2021.

Simulating diluted bitumen spills in boreal lake limnocorrals - Part 2: Factors affecting the physical characteristics and submergence of diluted bitumen. Science of the Total Environment 790, 148580.

Sutuyeva, L., Shalakhmetova, T.M., Trudeau, V., 2020. Histological structure of thyroid gland and level of thyroid hormones in tadpoles exposed to oil and petroleum products. International Journal of Biology and Chemistry 13, 24-32.

Sutuyeva, L.R., Trudeau, V.L., Shalakhmetova, T.M., 2019. Mortality of embryos, developmental disorders and changes in biochemical parameters in marsh frog (*Rana ridibunda*) tadpoles exposed to the water-soluble fraction of Kazakhstan crude oil and O-Xylene. Journal of Toxicology and Environmental Health, Part A 82, 200-215.

Truter, J.C., van Wyk, J.H., Oberholster, P.J., Botha, A.M., Mokwena, L.M., 2017. An evaluation of the endocrine disruptive potential of crude oil water accommodated fractions and crude oil contaminated surface water to freshwater organisms using in vitro and in vivo approaches. Environmental Toxicology and Chemistry 36, 1330-1342.

Urszan, T.J., Garamszegi, L.Z., Nagy, G., Hettyey, A., Torok, J., Herczeg, G., 2015. No personality without experience? A test on *Rana dalmatina* tadpoles. Ecology and Evolution 5, 5847-5856.

USEPA, 1979. Damage assessment studies following the NEPCO 140 oil spill on the St. Lawrence River. United States Environmental Protection Agency, EPA-600/7-79/256.

Wada, H., Bergeron, C.M., McNabb, F.M., Todd, B.D., Hopkins, W.A., 2011. Dietary mercury has no observable effects on thyroid-mediated processes and fitness-related traits in wood frogs. Environmental Science & Technology 45, 7915-7922.

Wallace, S.J., de Solla, S.R., Head, J.A., Hodson, P.V., Parrott, J.L., Thomas, P.J., Berthiaume, A., Langlois, V.S., 2020. Polycyclic aromatic compounds (PACs) in the Canadian environment: Exposure and effects on wildlife. Environmental Pollution 265, 114863.

Weltje, L., Simpson, P., Gross, M., Crane, M., Wheeler, J.R., 2013. Comparative acute and chronic sensitivity of fish and amphibians: A critical review of data. Environmental Toxicology and Chemistry 32, 984-994.

Bilodeau J.C., Gutierrez Villagomez J.M., Kimpe L.E., Thomas P.J., Pauli B.D., Trudeau V.L., Blais J.M. 2019. Toxicokinetics and bioaccumulation of polycyclic aromatic compounds in wood frog tadpoles (*Lithobates sylvaticus*) exposed to Athabasca oil sands sediment. Aquatic Toxicology, 207: 217-225

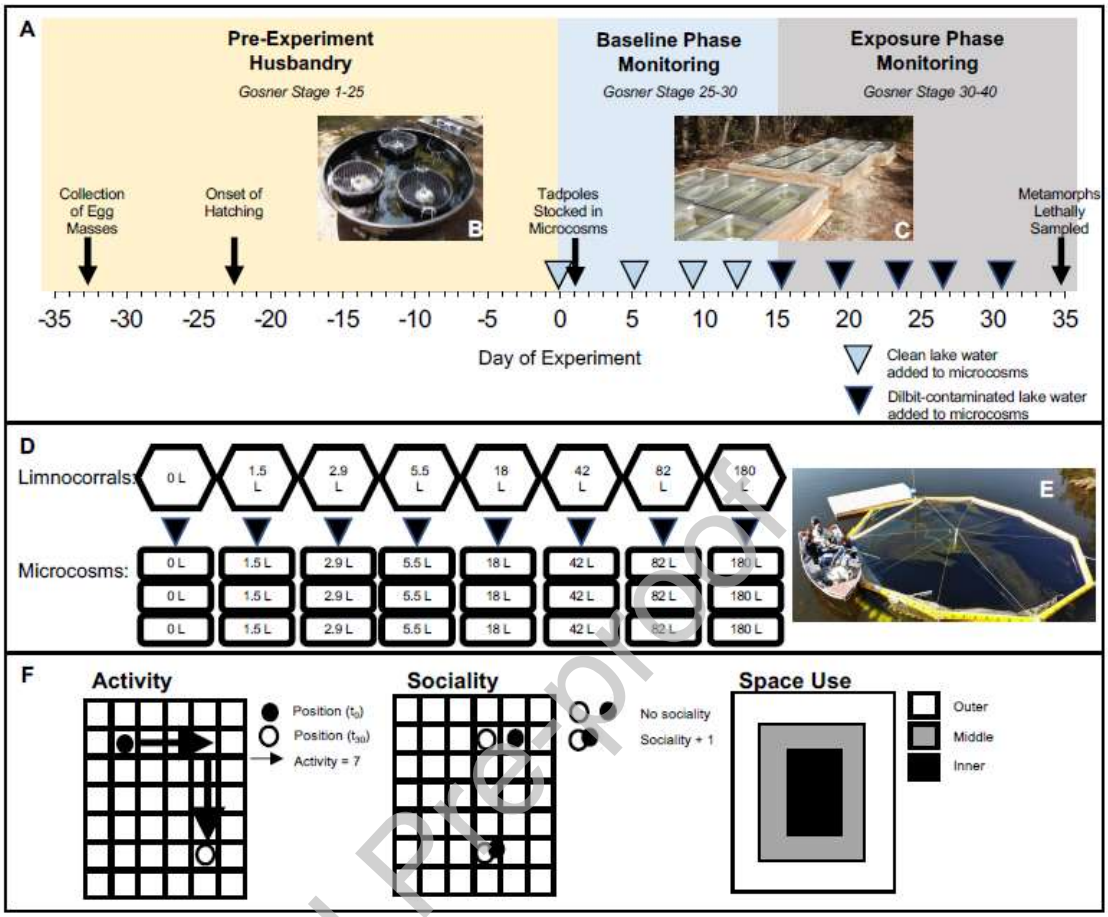

Fig. 1. [COLOR] Timeline of study (panel A) showing pre-experiment husbandry of wood frog embryos in rearing tanks (panel B), as well as baseline and exposure phases of the experiment where tadpoles were housed in outdoor aquatic microcosms (panel C). Experimental design of study (panel D) illustrating how three replicate microcosms received lake water from each of eight in-lake limnocorrals (panel E), seven of which received different volumes of diluted bitumen (from 1.5 to 180 L of Cold Lake Winter Blend). Diagrams of the three behavioural tests (i.e., for activity, sociality, and space use) performed on tadpoles during the exposure phase of the experiment (panel F).

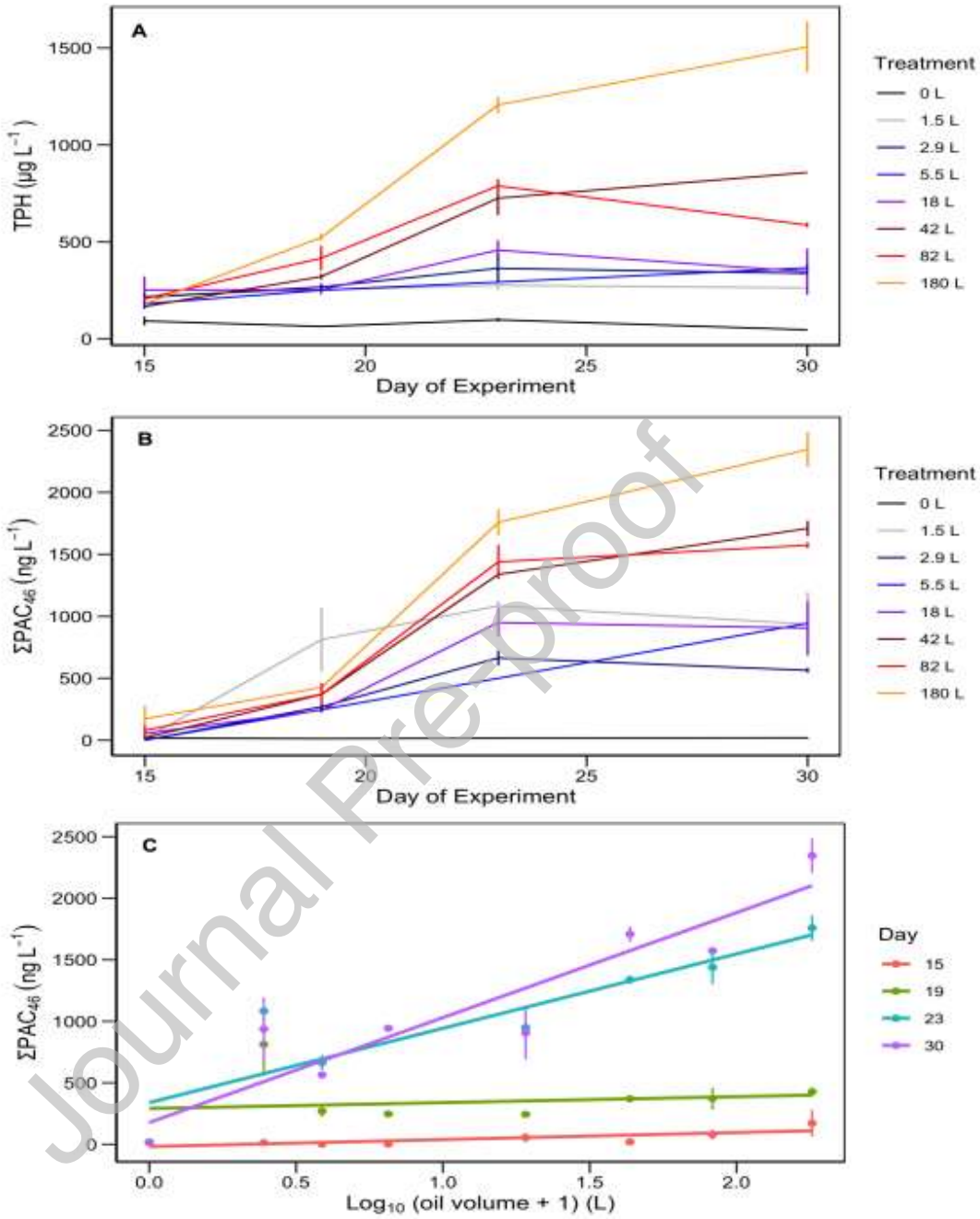

Fig. 2. [COLOR] Hydrocarbon concentrations in the water column of the limnocorrals (i.e., source of water for the microcosms housing larval wood frogs) during the exposure phase of the experiment. Temporal changes in total petroleum hydrocarbons (TPH; panel A) and total polycyclic aromatic compounds (ΣPAC_{46}; panel B) in the limnocorrals.

Relationship between the volume of oil applied to limncorrals and TPH and ΣPAC_{46} concentrations on experimental days (panel C). Each point represents the average of 2 water samples. Data from Rodriquez et al. (2021).

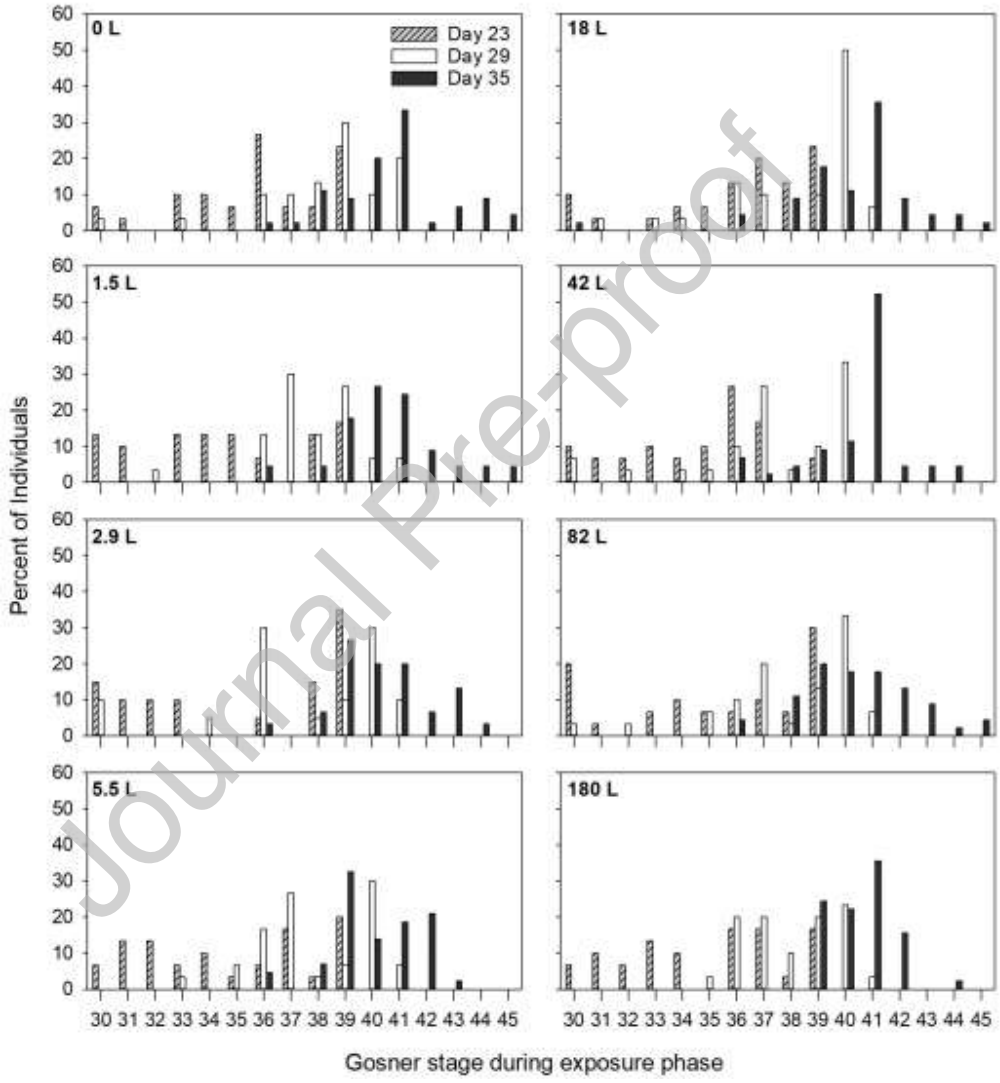

Fig. 3. Histogram of Gosner (1960) stages of wood frogs on three sampling days during the exposure phase of the experiment. Each panel is one treatment (n = 30 individuals). Gosner stage of wood frogs during the baseline phase is shown in Fig S2.

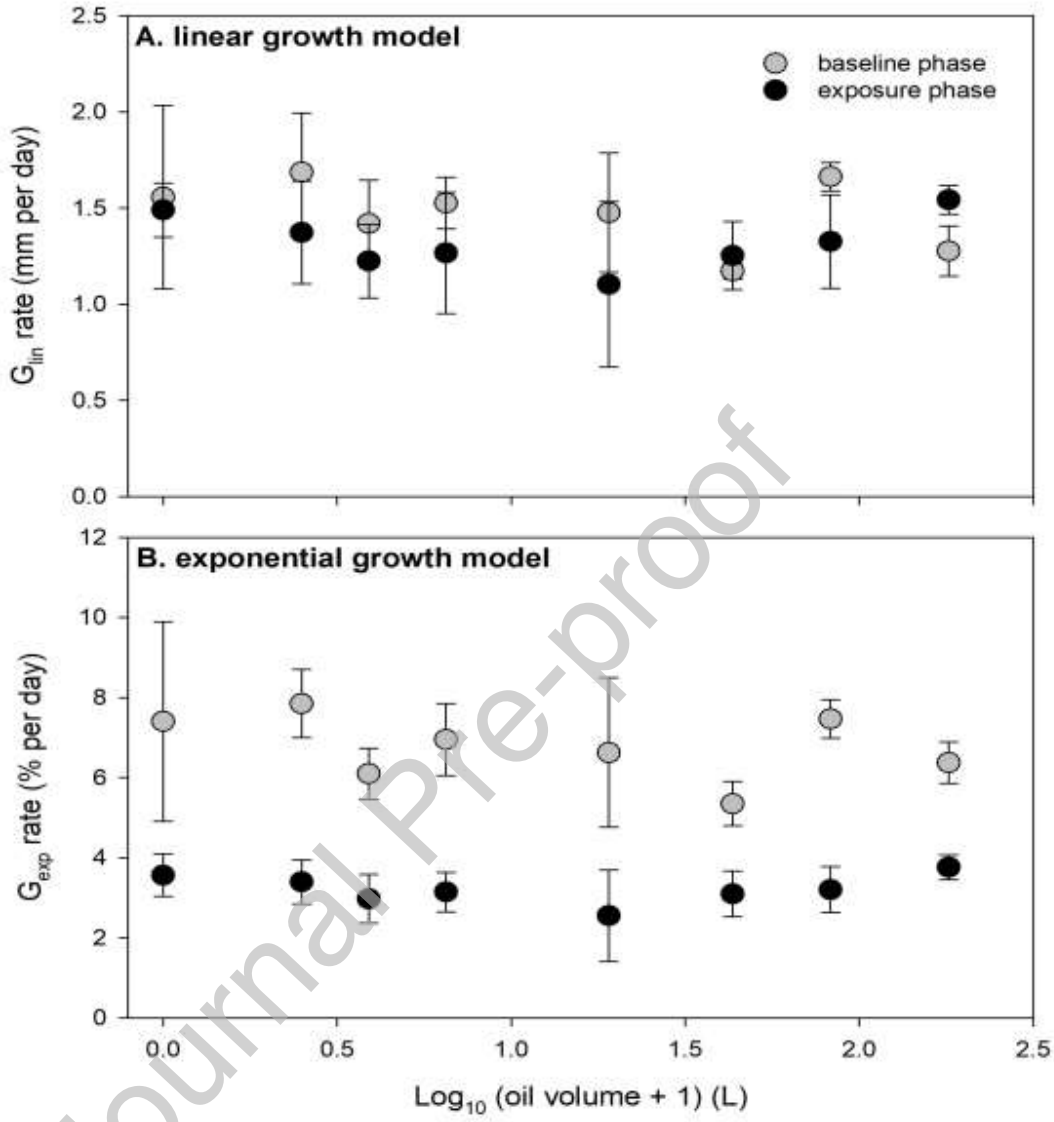

Fig. 4. Growth rate (i.e., change in total body length over 10 days) of larval wood frogs during the baseline (grey symbols) and exposure (black symbols) phases as a function of oil volume added to limnocorrals. A linear model was assumed to model growth rate (G_{lin}) in panel A, and an exponential model was assumed to model growth rate (G_{exp}) in panel B. Total body length was measured in 30 wood frogs on three sampling days per phase. Total body length (and body mass) on specific sampling days are shown in Fig S4.

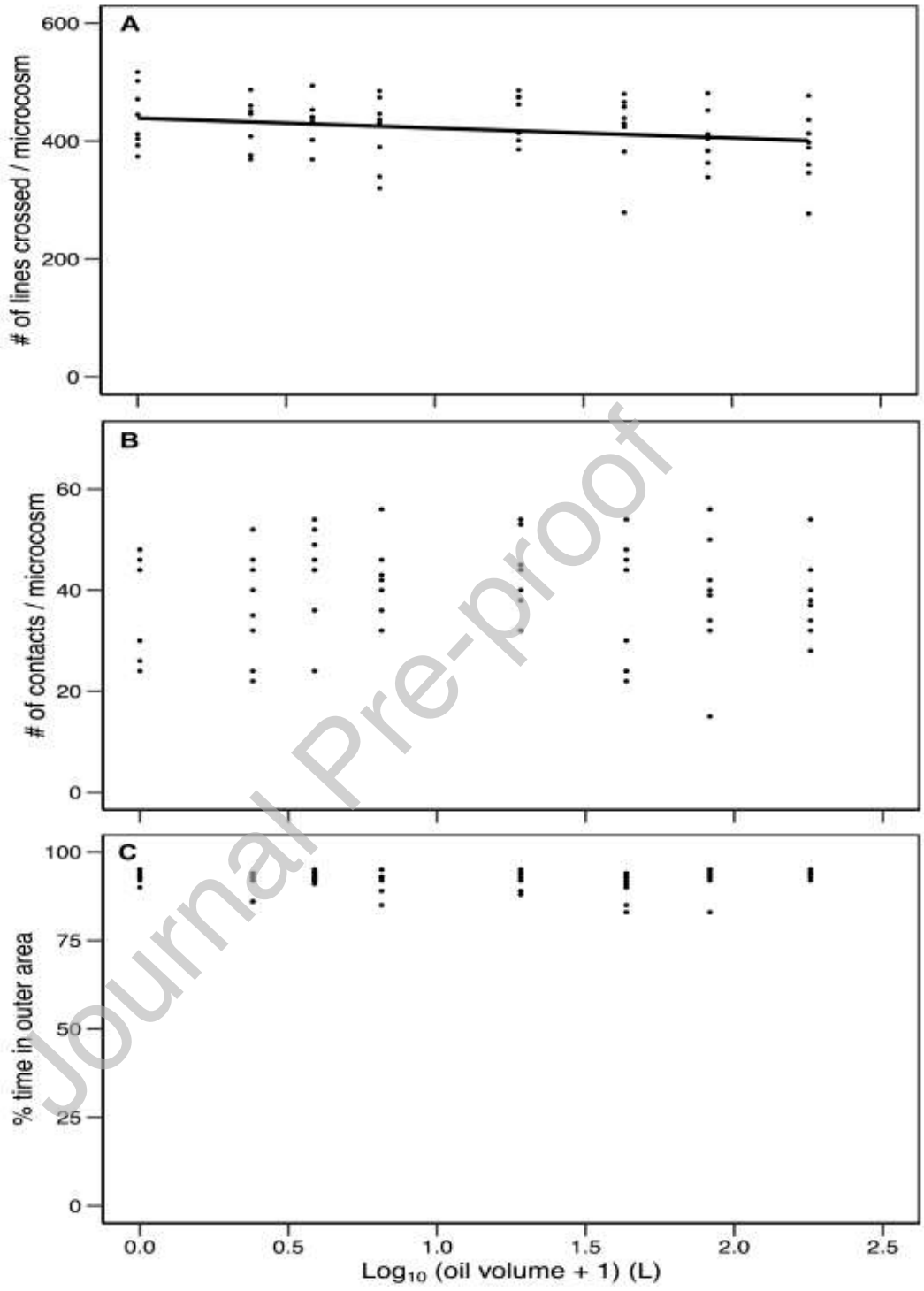

Fig. 5. Behavioural metrics calculated from five random wood frogs per microcosms.

Microcosm replicate and assessment day were not statistically significant, and thus were

not treated as independent variables (i.e., each dot represents the total number of lines crossed by five tadpoles in a microcosm on one of the four sampling days). Volume (L) of oil applied (+1) was log transformed to present and analyze data on a linear scale. (A) Number of lines crossed per microcosms (activity) exposed to lakewater from limnocorrals where various volumes of oil were added. The black line represents a significant linear fit of the fitted values from a linear mixed effects model (log base 10(volume of oil applied + 1) estimate = -16.848 ± 8.517 (SE), t = -1.978, p = 0.048, n = 64). (B) Number of contacts per microcosm (sociality) treated with water from mesocosms where various volumes of oil were added. Sociality did not statistically relate to oil volume. (C) Percent time in the outer area of the behavioural arena (location) treated with water from mesocosms where various volumes of oil were added. Location did not statistically relate to oil volume.

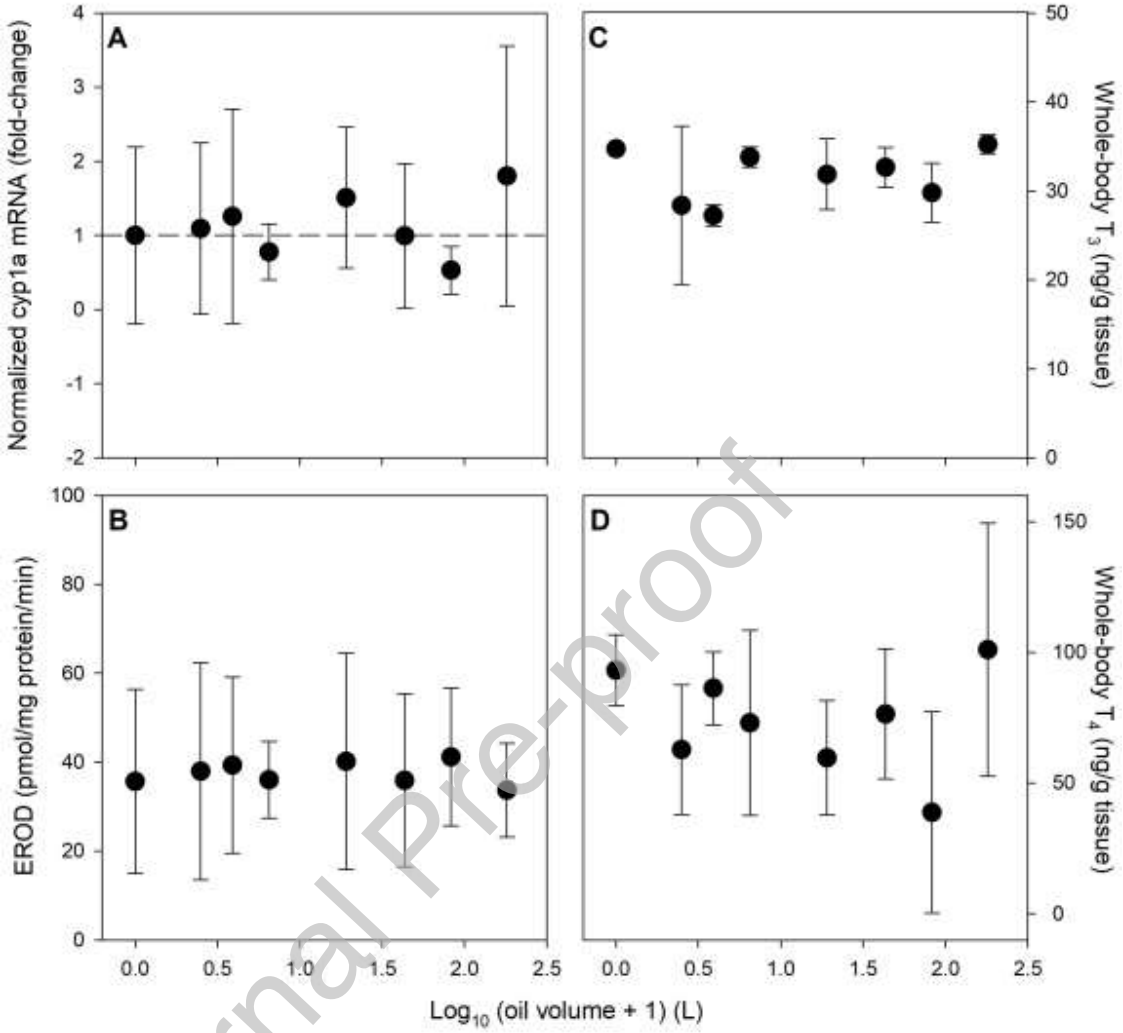

Fig. 6. Cytochrome P450 1A enzymes and thyroid hormones (mean ± standard deviation of each treatment) in wood frogs sampled on the final day of the experiment (day 35) as a function of oil volume. Normalized (relative to reference gene *rpl8*) *cyp1a* mRNA expression (fold-change relative to the control mean, shown by dashed line; panel A) and ethoxyresorufin-O-deethylase (EROD; panel B) activity measured in liver (n = 9 per treatment). Concentrations of 3,5,3'-triodo-L-thyroinine (T_3; panel C) and 3,5,3',5'-tetraiodo-L-thyronine (T_4; panel D) in whole-body samples (n = 5 per treatment).

Table 1. Concentrations of total petroleum hydrocarbons (TPH) and polycyclic aromatic compounds (PAC) in experimental microcosms housing wood frogs. Water samples were collected at the end of the experiment (on day 33), in conjunction with final lethal sampling of wood frogs. Shown are the mean (± standard deviation) of 3 microcosms per treatment.

Treatment	TPH[a] (µg/L)					PAC[b] (ng/L)		
(L oil)	F1	F2	F3	F4	ΣTPH	ΣPAC_{alk}	ΣPAH_{EPA16}	ΣPAC_{46}
Control	-	41	270	126	437	21	1.7	23
1.5 L	-	33	211	76	319	33	3.8	37
2.9 L	-	52	253	111	417	30	3.9	34
5.5 L	-	79	364	110	553	43	1.7	45
18 L	-	49	318	101	468	37	4.2	41
42 L	-	89	444	120	654	61	2.7	64
82 L	-	109	487	101	697	51	5.3	56
180 L	-	181	737	99	1,018	118	3.4	121

[a] Fraction (F)1: <*n*-C_{10}; F2: <*n*-C_{10}-n-C_{16}; F3: *n*-C_{16}-n-C_{34}; F4 >*n*-C_{34}; ΣTPH: sum of all fractions;

[b] ΣPAC_{alk}: sum of alkylated PACs; ΣPAH_{EPA16}: sum of 16 EPA-priority s; ΣPAC_{46} : sum of 46 PACs.