Biologic monitoring of environmental contaminants in marine (killer whale) and terrestrial (caribou, moose, and wolf) wildlife populations using scat samples

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Abstract

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Wildlife are intimately associated with contaminated waters and polluted landscapes, serving as sentinels to the health of our shared environment. This project optimized trace analytic techniques for measuring toxicants in scat samples and evaluated contamination levels among Southern Resident killer whales (SRKWs; *Orcinus orca*) in the Salish Sea and Woodland caribou (*Rangifer tarandus caribou*), moose (*Alces americanus*), and Grey wolf (*Canis lupus*) in the Alberta Oil Sands (AOS). Scat sampling provides an unprecedented opportunity to non-invasively monitor marine and terrestrial wildlife across broad geographic landscapes.

Exposure to persistent organic pollutants (POPs) is a primary risk factor for the endangered SRKWs. POPs are lipophilic toxicants associated with adverse health effects including endocrine disruption and reproductive toxicity. Scat samples collected from 2010-2013 demonstrated that contaminant levels are highest, from endogenous lipid stores, and have the greatest potential for toxicity when the whales are nutritionally compromised. Toxicant exposures may contribute to increased mortality and decreased fecundity previously associated with limited prey abundance. Accumulation patterns showed an expected age-related increase, excepting nulliparous females that may have toxicant offloading from unrecorded neonate loss. Mobilization from endogenous lipid stores for milk production and associated transfer of POPs

was apparent, particularly for first-born calves with diminished transfer to subsequent calves. POPs were not associated with disruption of thyroid hormone levels as expected.

The AOS are third largest international oil reserve; oil production is projected to more than double from 2008-2018. Polycyclic aromatic hydrocarbons (PAHs), well-established carcinogens and mutagens, are a component of crude oil (petrogenic) and form during combustion events (pyrogenic). Scat samples collected in 2009 from areas of the AOS with varying degrees of *in situ* oil production activity were evaluated for PAH levels. Samples were from Woodland caribou, moose, and Grey wolf, terrestrial species with markedly different dietary preferences and resource utilizations. The high oil production area demonstrated petrogenic (oil) source PAH ratios in moose samples and increased PAHs in wolf samples. Caribou samples from the area of historical forest fire indicated pyrogenic (combustion) source PAHs.

The results from these studies should help promote conservation goals to keep our marine and terrestrial environments healthy.

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Permits

Fecal samples from Southern Resident killer whales were collected in United States waters under National Marine Fisheries Service permit 10045. Samples were collected in Canadian waters under Marine Mammal License numbers 2010-09 and 2012-08, as well as Species at Risk Act permits numbered 109 and 155.

CHAPTER 1. Persistent organic pollutant determination in killer whale scat samples: optimization of a gas chromatography/mass spectrometry method and application to field samples

ABSTRACT

Biologic sample collection in wild cetacean populations is challenging. Most information on toxicant levels is obtained from blubber biopsy samples; however sample collection is invasive and strictly regulated under permit, thus limiting sample numbers. Methods are needed to monitor toxicant levels that increase temporal and repeat sampling of individuals for population health and recovery models. The objective of this study was to optimize measuring trace levels (parts per billion) of persistent organic pollutants (POPs), namely polychlorinated-biphenyls (PCBs), polybrominated-diphenyl-ethers (PBDEs), dichlorodiphenyltrichloroethane (DDTs), and hexachlorocyclobenzene in killer whale scat (fecal) samples. Archival scat samples, initially collected, lyophilized, and extracted with 70% ethanol for hormone analyses, were used to analyze POP concentrations. The residual pellet was extracted and analyzed using gas chromatography coupled with mass spectrometry. Method detection limits ranged from 11-125 ng/g dry weight. The described method is suitable for *p,p*'-DDE, PCBs-138, 153, 180, and 187, and PBDEs-47 and 100; other POPs were below the limit of detection. We applied this method to 124 scat samples collected from Southern Resident killer whales. Scat samples from 22 adult whales had known POP concentrations in blubber and demonstrated significant correlations ($p<0.01$) between matrices across target analytes. Overall, the scat toxicant measures matched previously reported patterns from blubber samples of decreased levels in reproductive-age females and a decreased *p,p'*-DDE/$\sum$PCB ratio in J-pod. Measuring trace levels of toxicants in scat samples provides an unprecedented opportunity to non-invasively evaluate contaminants

levels in wild cetacean populations, as well as monitor temporal and annual trends across broad geographic landscapes.

INTRODUCTION

The Salish Sea is an area of Pacific inland waterways that includes Puget Sound and the San Juan Islands of Washington state, and southern British Columbia. This diverse and productive ecosystem is riddled with human impacts. The Southern Resident killer whales (SRKW; *Orcinus orca*), which consist of 3 family groups, or pods, J, K, and L, subsist in these waters during the summer months (May-October) (Hanson et al. 2010). This killer whale eco-type experienced an unexplained 20% decline in their population census in the late 1990s, which alarmed the public and scientists alike to the possibility that this ecosystem may no longer be able to sustain these carnivores at the top of the food web. The SRKWs were listed as endangered in 2001 and 2005 by Canada and the U.S., respectively. Excessive exposure to environmental contaminants was identified as one of the primary risk factors that may be contributing to the population decline by the National Marine Fisheries Service in the Southern Resident Killer Whale Recovery Plan (National Marine Fisheries Service 2008); other risk factors included decreased prey availability, vessel effects and sound, and oils spills.

Persistent organic pollutants (POPs) are a group of toxic chemicals that are ubiquitous in the environment and have been associated with adverse health effects (e.g., endocrine disruption) in wildlife populations (Tyler et al. 1998), including studies specific to marine mammals (Reijnders 1986, Lahvis et al. 1995, Schwacke et al. 2002, Jepson et al. 2005). Organochlorine pesticides, such as dichlorodiphenyltrichloroethanes (DDTs), were banned in the United States during the

1970s and 1980s, and polychlorinated biphenyls (PCBs), used in capacitors, transformers, and consumer products such as house paint, were banned from open source use in the United States in the 1970s. Polybrominated diphenyl ethers (PBDEs) have been widely used as a flame retardant in consumer products. The industrial production and importation of two of the three technical mixtures of PBDEs (penta- and octaBDEs) was stopped in the United States in 2004, and United States manufactures and importers of the remaining form (decaBDE) committed to terminate all uses by the end of 2013 (Environmental Protection Agency 2014). Substantial human and ecological exposures to these POPs continue to occur due to the large quantities that were used historically and continue to be used in certain global regions, as well as their persistence in the environment, resistance to biodegradation, and bioaccumulation in the food chain (Li et al. 2006, Letcher et al. 2010).

Blubber biopsy samples previously collected on the SRKWs demonstrated that levels of POPs in this population exceed a health-effects threshold developed by Kannan et al. (2000) through risk characterizations extrapolated from studies of immunologic and reproductive effects in seals, otters, and mink (Kannan et al. 2000, Ross et al. 2000, Krahn et al. 2009). Additionally, risk assessments from other cetaceans suggest reproductive impairment is apparent at the current levels of contaminants (Schwacke et al. 2002, Hall et al. 2006).

Pod, age, and sex-class differences in POP levels have been previously reported in SRKWs based on measures from blubber biopsy samples (Ross et al. 2000, Krahn et al. 2007, Krahn et al. 2009). Specifically, ratios of DDTs to PCBs in blubber samples, used to provide insight into whales' foraging locations, were demonstrated to be higher in K- and L- pods compared to the J-

pod. This POP ratio pattern in K- and L-pods is often referred to as the 'California signature' due to alleged seasonal foraging off of the central California coast where high levels of DDT are still found in the marine environment (Sericano et al. 2014). PCB concentrations are higher in J-pod (male, 1 sample, 41,000 ng/g lipid) compared to the other two pods of SRKWs (K- and L-pod males, 4 samples, range 24,000 - 39,000 ng/g lipid) (Krahn et al. 2009). This is considered part of an 'urban signature' and is believed to be associated with the more frequent foraging of J-pod in Puget Sound. The concentration of these contaminants generally increase with age, particularly the POPs more resistant to biotransformation (Boon et al. 1994, Wolkers et al. 2007). Decreased toxicant levels in reproductive-age females (Krahn et al. 2009) have been attributed to mobilization of these compounds from adipose tissue in response to the high-energy needs of lactation (Pomeroy et al. 1996, Ross et al. 2000, Debier et al. 2003, Krahn et al. 2009). Krahn et al. demonstrated maternal transfer of a large portion of the mother's POPs burden during gestation and through breast milk in two mother-offspring pairs of SRKWs; the resulting POP concentrations in the nursing calves were higher than their mother's (Krahn et al. 2009). This same study also demonstrated female whales re-accumulate POPs between pregnancies once lactation ended and transfer of POPs to a calf ceased. Males have been shown to accumulate toxicants throughout their lifetime (Ross et al. 2000).

To date, collection of biological samples for toxicant analyses in wild killer whale populations has been limited to analysis of blubber biopsy samples (Ross et al. 2000, Ylitalo et al. 2001, Krahn et al. 2007, Krahn et al. 2009) and tissues (e.g., blubber, liver) collected during necropsies of whales that have washed ashore (Kannan et al. 1993, Kajiwara et al. 2006). While the toxicant information obtained from biopsy samples has been invaluable (Hickie et al. 2007,

Alava et al. 2012), collection is invasive and strictly regulated under permit. As such, this sampling method restricts sample size, and the cross-sectional study design generally only includes one sample per whale over all sampling periods.

Numerous modeling efforts have been made to evaluate population consequences in cetacean populations associated with exposures to pollutants, or other factors such as prey abundance (Hall et al. 2006, Hickie et al. 2007, Ward et al. 2009). Ward et al. (2009) quantified the effect of prey abundance on killer whale reproduction, and reported SRKW fecundity was highly correlated with Chinook salmon abundance. The authors stated, "It remains unclear what effects contaminants have on killer whale fecundity because appropriate data do not exist." Hall et al. (2006) estimated the population consequence of PCB exposure in bottlenose dolphins (*Tursiops truncates*) using PCB levels reported in bottlenose dolphins from Sarasota Bay, Florida (Wells et al. 2005), coupled with dose-response data (Reddy et al. 2001) and age-specific fecundity and survival data from a mark recapture study (Wells and Scott 1990), and reported decreased calf survivorship associated with maternal PCB accumulation. The authors acknowledged the predictions were limited by model naivety and parameter uncertainty and emphasized the need for more data collection.

Current methods to monitor temporal and annual trends of toxicant levels in the SRKW population include stable tissue archives (Becker et al. 1997), but these reserves are limited and new methods would help ensure comprehensive contaminant data are available for population monitoring and health assessment models. Non-invasive measurement of POPs in killer whale feces provides one solution, owing to its availability in the ecosystem and relative ease of

collection (Hanson et al. 2010, Ayres et al. 2012). A total of 266 scat samples were collected between 2010 and 2013; the limiting factor in this study was not sample availability but funds to evaluate the samples. The similar congener profiles of PCBs demonstrated in the blood, internal fat, and feces of bottlenose dolphins (captive) and otters (Marsili et al. 1995, Van den Brink and Jansman 2006), and the detection of specific POP congeners and metabolites in the feces of river otters, rats, mice, and sheep (Vrecl et al. 2005, Elliott et al. 2008, Hakk et al. 2009), indicate that circulating POP concentrations can be reliably measured in feces. However, to our knowledge, no study has evaluated toxicant levels in scats of killer whales or in any wild cetacean population.

The primary objective of the current study was to optimize a gas chromatography/mass spectrometry method to measure trace levels of POPs (PCBs, PBDEs, DDTs, and HCB) in killer whale scat samples. The second objective was to apply these methods to samples collected from SRKWs in the Salish Sea to evaluate whether scat POP levels are reflective of measures from blubber samples, thereby indicating biologic accumulation of these compounds.

MATERIALS AND METHODS

Sample collection

SRKWs appear with regularity in the areas around the San Juan Islands and Puget Sound between spring (mid-May) and fall (mid-October). Sample collection occurred in these inland waters of Washington State and British Columbia, collectively referred to as the Salish Sea, between May and October from 2010-2013.

SRKWs were located and identified by the field team based on dorsal fin shape and saddle patch pattern (CWR 2015). Detection dogs were trained to locate SRKW scat floating on the water's surface (Hunt et al. 2004, Rolland et al. 2006, Ayres et al. 2012) taking advantage of the dog's remarkable ability to locate SRKW scat samples at distances compatible with the "Be Whale Wise" guidelines (>200 yards away). Samples were scooped off of the surface of the water using a 1L polypropylene beaker, collected in a 50mL polypropylene tube, immediately centrifuged using a small field centrifuge, and all sea-water was decanted. The remaining pellet was frozen on dry ice and remained frozen until processed in the lab (within 6 months of collection). In the lab, samples were thawed, homogenized by combining multiple collection tubes from the same sample in an acetone-rinsed glass beaker and stirring thoroughly with an acetone rinsed stainless steel spatula, centrifuged at 1092 relative centrifugal force (RCF), decanted, and swabbed for genetic analysis to identify species, sex, pod, and individual identification by National Oceanic and Atmospheric Association Northwest Fisheries Science Center (NOAA NWFSC) (Ford et al. 2011). Genetic material was not compromised by undergoing a freeze/thaw cycle. As such, samples were swabbed in the lab, instead of the field, to ensure an adequate material was collected and sterile technique was employed. One genetic match per sample assured sample collection was from one animal.

All samples analyzed were originally collected to evaluate a combination of fecal hormone measures (thyroid and glucocorticoid) to assess inadequate prey and increased vessel traffic on this endangered population (Ayres et al. 2012). As such, following homogenization samples were freeze-dried, weighed to 80 mg dry weight, and hormones were extracted from the pellet using 70% ethanol (15 mL). The ethanol extract was decanted and stored at -20°C for hormone

analyses (Wasser et al. 2000, Wasser et al. 2010, Ayres et al. 2012). The remaining fecal pellet was stored at -20 °C for toxicant analyses. A total of 263 scat samples were collected May through October from 2010 to 2013. Analysis of all samples was cost prohibitive; as such, 126 samples were selected for analysis of toxicant concentrations. Selection of samples was restricted to those with a mass ≥ 0.07 g that were also successfully genotyped to confirm individual identity (n=165) (two additional samples were selected from suspected pregnant females, based on progesterone profiles). The final sample selection was based on *a priori* hypotheses to evaluate seasonal and annual changes in toxicant concentration, and variation based on reproductive status with no consideration of whether data was available on POP levels in blubber.

The Southern Resident killer whale population, including births and deaths, has been well documented since 1976 by the Center for Whale Research (CWR 2013). This data was used to evaluate age and sex class characteristics of individual whales.

Analysis for persistent organic pollutants

Chemicals and reagents

All chemicals, solvents and reagents used were pesticide-grade. All glassware, aluminum foil, stainless steel spatulas, and other materials used were triple rinsed in acetone (Fisher Scientific, Pittsburgh, PA) or methylene chloride (Fisher Scientific, Pittsburgh, PA) prior to use to remove trace organics. Florisil and sodium sulfate were triple rinsed in methylene chloride, then baked at 130 °C for 24 hours. Florisil was deactivated by adding 1.2% of conditioned deionized water based on dry weight. Silica (Mallinckrodt, 100 mesh powder) was rinsed 3 times with methylene

chloride followed by drying in an oven at 160 °C for 3 hours. Acidic (concentrated sulfuric acid, 44% w/w) and basic (1N sodium hydroxide, 23% w/w) silica were prepared from the cleaned silica (Environmental Protection Agency 2007).

The individual PBDE and PCB congeners reported in this study follow the International Union of Pure and Applied Chemistry (IUPAC) numbering system for PCBs. A stock solution of native compounds was prepared by dissolving the individual congeners PBDE-28, 47, 99, 100, 153, 154 (Wellington Laboratories, Ontario, Canada), PCB-95, 99, 149, 187 (Accustandard, New Haven, CT), *p,p*'-dichlorodiphenyldichloroethylene (*p,p'*-DDE; a metabolite of DDT) (Supelco, St. Louis, MO), and hexachlorobenzene (HCB) (Accustandard, New Haven, CT), and a PCB mixture including PCB-101, 118, 138, 153, 180 (PCB Congener Standard #2, Restek, Bellefonte, PA), in toluene (Omnisolv ®). Calibration curve solutions were made by dilution in toluene, with concentrations ranging from 0.5 to 100 ppb. Target analyte selection was based on pilot sample analyses performed by NOAA NWFSC using a gas chromatography coupled with mass spectrometry (GC/MS) method described in Sloan et al. (Sloan et al. 2014).

A surrogate standard mixture (250 ng/mL of each compound) was prepared in toluene from individual isotope-labelled standards PCB-118 (Wellington Laboratories, Ontario, Canada), *p,p'*-DDE (Cambridge Isotope Laboratories, Andover, MA), and HCB (Cambridge Isotope Laboratories, Andover, MA), a PCB mixture including isotope-labelled PCB-101, 138, 153, 180 (Cambridge Isotope Laboratories, Andover, MA), and individual native congeners PBDE-30, 77, 105, 140 (Wellington Laboratories, Ontario, Canada). This standard was used to monitor analyte recoveries and for internal standard quantification using isotope dilution methods. Native

standards with the same number of bromines but different substitutions were selected as surrogates for PBDE internal standards rather than labelled compounds because NCI fragments both to ^{79}Br and ^{81}Br (Eljarrat et al. 2002). The PBDE native surrogates were selected to have different retention times on the GC system, allowing them to be distinguished from the target analytes. A similar fragmentation to halogen occurred with PCB-52 and 95. As such, PCB-52 was dropped from the analysis because an appropriate internal standard surrogate was not available, and PCB101-$^{13}C_{12}$, an isotope-labelled homologue for PCB-95, was selected as the new surrogate for PCB-95.

Extraction and purification

Scat sample extraction, purification, and quantification methods were developed using procedures modified from EPA methods 3630C and 1614 (Environmental Protection Agency 1996, Environmental Protection Agency 2007). Frozen fecal pellets from the hormone extraction were thawed and spiked with 20 µL surrogate standard (250 ng/mL) prior to extraction. Sodium sulfate (500 mg) and florisil (500 mg) were added to the samples to remove residual water and polar compounds, respectively, along with 20 mL of methylene chloride. The sample extracts were sonicated in a water bath at room temperature for 30 minutes, centrifuged for 10 minutes at 2910 RCF, and decanted into a glass collection tube. This extraction step was repeated with an additional 20 mL of methylene chloride, and the two extracts were combined. The extract volume was reduced to approximately 1 mL at 45 ^{0}C under a gentle stream of nitrogen, and solvent-exchanged to hexane (Fisher Scientific, Pittsburgh, PA).

The extract was loaded onto a 5 mL disposable polypropylene column (Thermo Scientific, Rockford, IL) packed from bottom to top with 200 mg basic silica (23% w/w), 50 mg silica, and 300 mg acidic silica (44% w/w). The target compounds were eluted using 11 mL of hexane:methylene chloride (50:50, v/v). The final sample was solvent exchanged to 100 uL toluene under a gentle stream of nitrogen and stored in the dark at -20 ^{0}C until analysis.

Analysis

All sample extracts and standards were analyzed using GC/MS (Agilent 7890A) operating in the negative chemical ionization (NCI) mode, using methane as the reagent gas, with selected ion monitoring (SIM) to enhance sensitivity prior to instrumental analysis. The instrument was fitted with a DB-XLB column (30 m length, 0.25 mm film thickness, 0.25 um i.d., with Duraguard, 10 m) (Agilent, Santa Clara, CA). The port was operated in splitless mode, fitted with a glass liner, and delivered 1 µL by auto-injection. The carrier gas was helium. Chromatographic separation was achieved using an initial temperature of 60 °C with a 2 minute hold, ramping to 240 °C at a rate of 30 °C per minute with a 2 minute hold, then ramping to 340 °C at a rate of 10 °C per minute with a 5 minute hold. The total run time per sample was 25 minutes.

Each analytical batch (two total) contained: a ten-point standard calibration curve at the beginning and end of each run; a repeat run of one point from the calibration curve every 15 samples to check instrument maintenance (calibration check vial); a laboratory procedure blank for each extraction group (every 11 samples) to monitor contamination (i.e., the presence of artifact POPs arising from laboratory associated procedures); one sample was repeated in each analytical batch to test the instrument for accuracy and precision; and a toluene blank to detect

solvent contamination. Sample concentrations were calculated for the individual POPs using the standard calibration curve. The reproducibility of the response factors in the calibration check vials were checked for each series of samples analyzed, and were typically ±10% the expected values. Any measured responses in the procedure lab blanks were subtracted from the responses measured in samples from the same extraction group. All final values are reported as ng/g dry fecal weight.

Ethanol extracts

Eight archived 70% ethanol extracts were analyzed for POPs to ensure that previous extraction of the fecal pellet with 70% ethanol for hormone analyses (Ayres et al. 2012) would not affect the POP concentrations obtained from the subsequent methylene chloride extraction of the fecal pellet (i.e., extraction method optimized in this study). Four archived ethanol extracts from methylene chloride extracted fecal pellets with low POP concentration levels, and 4 ethanol extracts from samples with high POP concentrations, were selected to test that POPs extracted by the ethanol were relative across a range of sample toxicant concentrations. A total of 5 mL of ethanol extract per sample was spiked with 20 µL of 250 ng/mL surrogate standard to monitor recovery and for quantification using isotope dilution methods. The volume of each ethanol extract was reduced to approximately 1 mL at 45 ^{0}C under a gentle stream of nitrogen. Sodium sulfate was added to thoroughly cover and saturate the remaining liquid, which was assumed to be mostly water, and 5 mL of hexane were added. The resulting hexane extract was run through a 5 mL Pasteur pipet column packed with sodium sulfate to ensure complete removal of water. The sample extract volume was reduced to 1 mL under a gentle stream of nitrogen. Final extracts were loaded onto solid-phase extraction columns and analyzed by NCI-GC/MS using the

protocol outlined above for the fecal pellets. Analyte concentration measures in the procedure blank were subtracted from the sample measures for all compounds except for PCBs, which were not representative of the scat samples. PCB levels were negligible in all procedure blanks for the extraction and purification of the sample pellets, therefore laboratory contamination was not a concern. All final values are reported as ng/mL.

Blubber biopsy samples

Toxicant levels previously measured in 22 blubber biopsy samples from adult (≥10 years old) SRKWs, and associated whale identification data, were provided by NOAA NWFSC. Blubber biopsy samples were collected in the United States and Canada between May 2006 and January 2013. All samples were analyzed using techniques described elsewhere (Krahn et al. 2007, Sloan et al. 2014). All blubber biopsy samples were analyzed for the same target analytes as measured in the scat samples. The average period of time between collection of blubber and scat samples was 3 years, 2 months (range: 0 year, 1 month to 7 years, 2 months).

Method detection limit and limit of quantification

The method detection limit and percent recovery experiment included three standards covering the range of expected values in the samples based on the pilot sample analyses performed by NOAA NWFSC (described above): 0.5, 5, and 50 ppb. In total, 100 uL of each standard was added (i.e., spiked) to an 80 mg fecal pellet made of pooled fecal samples representing all age-sex classes and pods in the SRKW population, previously extracted in 70% ethanol, and stored at -20 °C. Each standard across the expected range was measured with six replicates; six matrix blanks were also evaluated. Coefficient of variation (%CV) was calculated as the percent of

dividing the standard deviation by the mean. Method detection limit (MDL) and limit of quantification (LOQ) were calculated by multiplying the standard deviation by 3.365 and 10, respectively. MDL, LOQ, and mean recovery (with matrix subtracted out) were quantified using an external standard calibration method because the internal standard method was not available at the time of this experiment.

Statistical methods

Toxicant concentrations measured in eight ethanol extracts (ng/mL) were compared to the toxicant concentration measured in the methylene chloride extracted fecal pellet (ng/g dry scat) from the same sample using linear regression analysis. All values were log-transformed to achieve normality. This was the only analysis using the toxicant measures in the ethanol extracts. All other analyses only use the toxicant measures in the methylene chloride extracted fecal pellets.

Toxicant concentrations were measured in 126 scat samples, 124 of these samples had a genotype confirmed individual identity representing 52 unique whales. Twenty-two unique adults whales had both blubber and scat toxicant measures available. Blubber and scat toxicant measures of individual congeners and compounds were log-transformed to achieve normality, and compared using mixed effects linear regression models adjusted for repeat sampling on individual whales by modeling individual whales as a random effect. This comparison was restricted to adult whales to minimize variation due to the growth dilution effect of toxicants in juvenile whales (Hickie et al. 2007) which would be exacerbated by the variable period of time between collection of blubber and scat samples. Toxicants in scat were evaluated by individual

congener and class of compound (∑PCBs; ∑PBDEs; and *p,p'*-DDE), as well as by ratio of *p,p'*-DDE/∑PCBs. The adjusted mean and standard error of toxicant measures were compared by year of sample collection, pod, and age-sex class. Age-sex class was defined as: juveniles (<10 years old; either sex), adult males (≥10 years), reproductive age females (≥10-<40 years), and post-reproductive age female whales (≥40 years). Effects were estimated using a mixed effects model, to account for repeat sampling on individual whales by modeling individual whales as a random effect. Potential covariates included sex, age, pod, and Julian date of sample collection. Final model selection was based on AICc (Akaike Informational Criterion) score.

All statistical analyses were performed using SAS v9.3 (SAS Institute Inc, Cary, NC).

RESULTS AND DISCUSSION

The target analytes, retention times, and quantification and confirmation ions are listed in Table 1. PCB95 and PCB99, each using PCB101-$^{13}C_{12}$ as a surrogate standard, were dropped as target analytes because the %CV for recovery of the 5ppb spike was 22.7% and 21.4%, respectively, indicating this was not an appropriate surrogate for either compound. An external standard method was also tested, but the %CV for recovery of the 5ppb spike was still high, 23.0% and 21.8%, respectively, indicating there was too much matrix interference for these compounds to be quantified at 5ppb, the concentration level in the middle of the range of expected values.

The mean recoveries for the remaining target analytes (7 PCBs, 6 PBDEs, *p,p'*-DDE, and HCB) ranged from 61-101% with a 50 ppb spike, and 84-135% with a 5 ppb spike for all compound except for HCB, which had a 224% recovery (Table 2). The recovery for the 0.5 ppb spike was

inflated (15-835%) likely due to high variability in the recovered compounds due to matrix interference on the GC system. The MDL ranged from 11-20 ng/g for individual PCBs, 16-37 ng/g for individual PBDEs, and was 124 ng/g for *p,p'*-DDE and 125 ng/g for HCB. Low toxicant concentrations in these mass limited samples as well as matrix interference may limit the usefulness of this method for whales with a low toxicant burden. Future studies would benefit from a larger sample mass.

Significant correlations were demonstrated when comparing the POP concentrations in the eight archived hormone ethanol extracts with the associated fecal pellet extracted with methylene chloride for *p,p'*-DDE, PCB-118, 149, 138, 153, 180, 187, and PBDE-47 and 100 ($R^2 > 0.80$; p-value < 0.05; Table 3). This finding indicates that extraction of the fecal pellet with 70% ethanol, prior to the subsequent methylene chloride extraction of the fecal pellet for toxicant analysis, is proportional to the total contaminant level thus the effect of contaminant removal from the ethanol extraction step is consistent between samples. Therefore, no additional ethanol extracts were analyzed for toxicant concentrations. HCB, PCB101, PBDE28, PBDE99, PBDE153, and PBDE154 did not demonstrate a significant linear association and were excluded from the final analyses out of concern of non-uniform extraction by the ethanol extraction step across different toxicant concentration levels. PCB149 was retained, although the 28.1 %CV indicates a possible non-linear association across varying levels of toxicant concentrations.

Killer whale fecal toxicant concentrations (ng/g dry weight) measured in the methylene chloride extracted fecal pellets were compared to concentrations of cumulative and individual congeners of PCBs (∑6PCBs: PCB118, 149, 138, 153, 180, 187), PBDEs (∑2PBDEs: PBDE47, 100) and

p,p'-DDE in blubber biopsy samples (ng/g lipid adjusted) from the same whale (n=22 unique pairs; Figure 1). The linear associations, adjusted for multiple scat samples collected per whale, were significant ($p<0.01$) for *p,p'*-DDE (slope estimate, 1.0176; standard error (SE), 0.1896), both PBDE congeners (∑2PBDE slope estimate, 0.4773; SE, 0.1595), and all PCB congeners measured except for PCB118 ($p=0.0673$) (data not shown). The finding for PCB118 may be explained by this congener being more readily metabolized than the other PCBs measured therefore blubber samples may not reflect bioaccumulation of this compound (Boon et al. 1994). A new metric, ∑4PCBs (cumulative measure of PCBs138, 153, 180, 187), was created excluding PCB118 based on the non-significant correlation with the blubber biopsy samples analysis and PCB149 based on the possible non-linear association across toxicant concentration levels in the ethanol extract analysis. A comparison of the resulting ∑4PCBs metric in the blubber and scat samples from the same whale was statistically significant ($p<0.001$) (slope estimate, 0.8961; SE, 0.1810) (data not shown; Figure 1). Inclusion of years between blubber and scat sample collection from the same whale did not improve the fit of the data to the model.

Sample analyses performed by NOAA NWFSC were on 'unprocessed' samples that had not undergone lyophilization or ethanol treatment. Linear associations of 39 samples that were analyzed using both the protocol at NOAA NWFSC (Sloan et al. 2014) and the protocol outlined in this paper were significant for ∑4PCB, ∑2PBDEs, and *p,p'*-DDE (all $p<0.001$; data not shown). This demonstrates any loss to volatilization during lyophilization or through the ethanol treatment does not affect the relative amount of toxicants in the samples. Nonetheless, unprocessed (fresh) samples should be used when possible to minimize concern of altered

measurement of toxicant levels; in the least, an internal standard should be added to the sample prior to processing to correct for potential loss of target analytes.

Application of the optimized methods to measure POPs in methylene chloride extracted fecal pellets in 124 SRKW scat samples from 52 unique whales collected between May and October, 2010-2013, showed a statistically higher ratio of *p,p'*-DDE/∑4PCBs in K pod (geometric mean (GM), 2.7; 95% Confidence interval (CI), 2.2-3.4) and L pod (3.3; 2.8-3.9), compared to J pod (1.2; 1.0-1.4) (Figure 2). This ratio was previously used to demonstrate pod differences in toxicant levels measured in blubber samples to provide insight into whales' foraging locations, and matches the reported findings in scats (Krahn et al. 2007). There was no statistical difference in ∑4PCBs, ∑2PBDEs, or *p,p'*-DDE concentrations between years or pods, adjusted for Julian day, age-sex class, and repeat sampling on individual whales (Table 4). However, there was a suggested increase in PCBs in J pod and *p,p'*-DDE in K- and L-pod similar to previous reports on blubber biopsy samples (Krahn et al. 2007). Due to the persistent nature of these compounds a difference in toxicant level by year was not expected over a 4-year study. Statistically lower levels of POPs in reproductive age females (age ≥10-<40), compared to juveniles, adult males, and post-reproductive age females, was apparent when evaluating the concentration by age-sex class. The GM for ∑4PCBs in females age ≥10-<40 was 41.7 ng/g dry wt (95% CI, 27.0-64.5), whereas the GM for juveniles was 123.4 ng/g dry wt (95% CI, 68.8-221.3). This pattern is similar to the previously reported finding from blubber biopsy samples and is likely related to the maternal transfer of these toxicants during gestation and lactation (Pomeroy et al. 1996, Ross et al. 2000, Debier et al. 2003, Krahn et al. 2009). Likewise, the elevated levels in post-reproductive females (≥40 years) (GM, 237.2; 95% CI, 117.4-479.4) and

adult males (≥10 years) (136.5, 82.0-227.2) also fit the expected values based on the bio-accumulation of these pollutants. These reported values exhibit measures in the scat samples that have undergone lyophilization and ethanol extraction treatments. Any loss from these treatments has been demonstrated to be proportional to the total contaminant levels in the samples, therefore between sample evaluations are considered acceptable comparisons of these relative values. However, any application of these measures to metrics outside of this study should be made with caution.

POPs are lipophilic compounds that are resistant to biological degradation. As top-level predators, cetaceans are highly susceptible to POPs due to bio-magnification from contamination in the food chain (Hickie et al. 2007; USEPA. 2002). Accumulated POPs are stored predominantly in the adipose tissue, as measured by blubber biopsy samples. The significant linear association ($p<0.01$) of toxicant measures in the blubber and measures in the scat for all classes of compounds indicate measures in the scat reflect accumulated levels of these contaminants. The SRKW population feed almost exclusively on salmonids, particularly Fraser River Chinook salmon (*Oncorhynchus tshawytscha*), between spring and fall (Ford and Ellis 2006; Ford et al. 1998; Hanson et al. 2010). Chemical analyses have revealed the current tissue levels of POPs in Fraser River Chinook are present at concerning levels (O'Neill et al. 2006, Kelly et al. 2011). The extent toxicant measures in the scat are also reflecting elimination of toxicants in the prey is not known. Further study evaluating the toxicant profiles of Fraser River Chinook salmon and other potential prey sources would allow for the calculation of a Metabolic Index that would quantify relative bioaccumulation in the food chain to indicate whether the toxicants measured in the scat are from a prey or blubber source (Wolkers et al. 2004).

CONCLUSIONS

Measuring trace levels of toxicants in scat samples provides an unprecedented opportunity to non-invasively evaluate contaminant levels in wild cetacean populations, as well as monitor temporal and annual trends in toxicant levels across broad geographic landscapes. Using our current sample collection method (a single detection dog team), 39-80 scat samples were successfully collected each year. The collection of multiple scat samples per whale across seasons and years allow for the evaluation of important questions such as how prey availability affects the concentration and profile of circulating toxicants in the endangered SRKW population. Additionally, the concurrent measurement with other physiologic indices obtained from these scat samples, such as nutritional, stress, and reproductive hormones (Ayres et al. 2012), may enable the assessment of associated physiologic impacts of toxicant exposures on population health. The incorporation of these data into population health assessment models has the prospect to help characterize current health risk and be available for vital management decisions for use in population recovery, risk assessment, and remediation efforts.

TABLES AND FIGURES

Table 1. List of persistent organic pollutant target analytes quantified by NCI-GC/MS-SIM with surrogate standard, retention time (RT), quantification ion (Quant Ion), primary confirmation ion (Primary conf ion), and secondary confirmation ion (Secondary conf ion)

Target analyte	Surrogate standard	RT	Quant ion	Primary conf ion	Secondary conf ion[a]
HCB	HCB-^{13}C6	9.13	284	286	282
PCB101	PCB101-$^{13}C_{12}$	11.84	326	328	324
p,p'-DDE	*p,p'*-DDE-d8	12.32	318	316	.
PCB118	PCB118-$^{12}C_{12}$	13.08	326	328	324
PBDE28	PBDE30	13.15	79	81	.
PCB153	PCB153-$^{13}C_{12}$	13.33	360	362	358
PCB149	PCB153-$^{13}C_{12}$	12.82	360	362	358
PCB138	PCB138-$^{13}C_{12}$	13.86	360	362	.
PCB180	PCB180-$^{13}C_{12}$	15.08	394	396	398
PCB187	PCB180-$^{13}C_{12}$	14.04	394	396	392
PBDE47	PBDE77	15.31	79	81	.
PBDE100	PBDE105	16.87	79	81	.
PBDE99	PBDE105	17.34	79	81	.
PBDE154	PBDE140	18.52	79	81	.
PBDE153	PBDE140	19.30	79	81	.

a: . = not applicable

Table 2. Method detection limit and percent recovery of persistent organic pollutants measured in killer whale scat samples

Target analyte	PCB 101	PCB 118	PCB 138	PCB 149	PCB 153	PCB 180	PCB 187	PBDE 28	PBDE 47	PBDE 99	PBDE 100	PBDE 153	PBDE 154	*p,p'*-DDE	HCB
Spike (ng/mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	5	5	5	5	0.5	0.5
Mean[a]	0.0959	0.0562	0.0908	0.0080	0.1109	0.0354	0.0284	0.0239	0.0750	0.2841	0.3356	0.1771	0.2742	0.6498	0.8387
Standard Deviation[a]	0.0072	0.0033	0.0057	0.0008	0.0073	0.0020	0.0012	0.0030	0.0070	0.0226	0.0373	0.0231	0.0343	0.0900	0.0739
Coefficient of Variation (%)	7.5	6.0	6.2	9.9	6.6	5.6	4.3	12.5	9.3	8.0	11.1	13.0	12.5	13.8	8.8
MDL (ng/g)	19.3	15.8	19.0	15.8	20.3	13.4	11.4	16.1	22.5	26.5	37.1	33.5	34.1	124.4	125.0
LOQ (ng/g)	37.9	25.1	35.8	41.0	39.9	18.9	15.5	26.4	45.3	68.1	102.5	86.5	79.1	361.3	356.3
mean, 0.5ppb spike (ng/mL)[b]	0.49	0.73	0.67	0.07	0.94	1.16	1.15	1.48	1.48	1.01	0.83	2.08	0.54	1.06	4.17
mean, 5ppb spike (ng/mL)[b]	4.52	4.19	4.26	5.38	4.51	4.58	5.56	5.16	5.60	5.98	6.76	5.01	5.64	5.72	11.19
mean, 50ppb spike (ng/mL)[b]	32.64	32.50	32.91	46.50	32.82	33.13	46.25	35.25	41.64	50.49	61.01	33.23	38.71	30.66	31.74
%Recovery, 0.5ppb spike	99%	147%	133%	15%	188%	232%	230%	296%	295%	203%	166%	416%	107%	212%	835%
%Recovery, 5ppb spike	90%	84%	85%	108%	90%	92%	111%	103%	112%	120%	135%	100%	113%	114%	224%
%Recovery, 50ppb spike	65%	65%	66%	93%	66%	66%	93%	70%	83%	101%	122%	66%	77%	61%	63%

a: Response values; not quantified using calibration curve; b: concentration levels calculated using an external standard curve

Table 3. Comparison of target analytes measured in eight fecal samples extracted first with 70% ethanol (extract for hormone level analyses), followed by methylene chloride extraction of the fecal pellet (method optimized in this paper to measure POP levels)

Target analyte	R^2	Coefficient of variation (%)	p-value
HCB	0.002	10.1	0.911
p,p'-DDE	0.98	5.6	<0.001
∑6PCB[c]	0.95	7.7	<0.001
∑4PCB[d]	0.95	7.6	<0.001
PCB101	0.02	50.9	0.734
PCB118[a]	0.89	9.9	0.002
PCB149[a]	0.86	28.1	0.003
PCB138	0.98	5.5	<0.001
PCB153	0.98	6.3	<0.001
PCB180	0.97	8.1	<0.001
PCB187	0.96	8.7	<0.001
∑2PBDE[e]	0.82	8.6	0.002
PBDE28	0.24	157.3	0.216
PBDE47	0.81	8.9	0.002
PBDE99	0.21	50.1	0.260
PBDE100[b]	0.95	13.7	<0.001
PBDE153	0.20	122.5	0.264
PBDE154	0.06	56.0	0.570

a: One outlier excluded from statistical model; b: Two outliers excluded from statistical model; c: ∑6PCB = PCB118, PCB149, PCB138, PCB153, PCB180, PCB187; d: ∑4PCB = PCB138, PCB153, PCB180, PCB187; e: ∑2PBDE = PBDE47, PBDE100

Figure 1. Matched comparison of POP levels in blubber (ng/g lipid) and scat (ng/g dry weight) samples from 22 unique adult Southern Resident killer whales; ∑4PCB (slope estimate, 0.8961; p-value <0.001), ∑2PBDE (slope estimate, 0.4773; p-value <0.01), and p,p'-DDE (slope estimate, 1.0176; p-value <0.001)

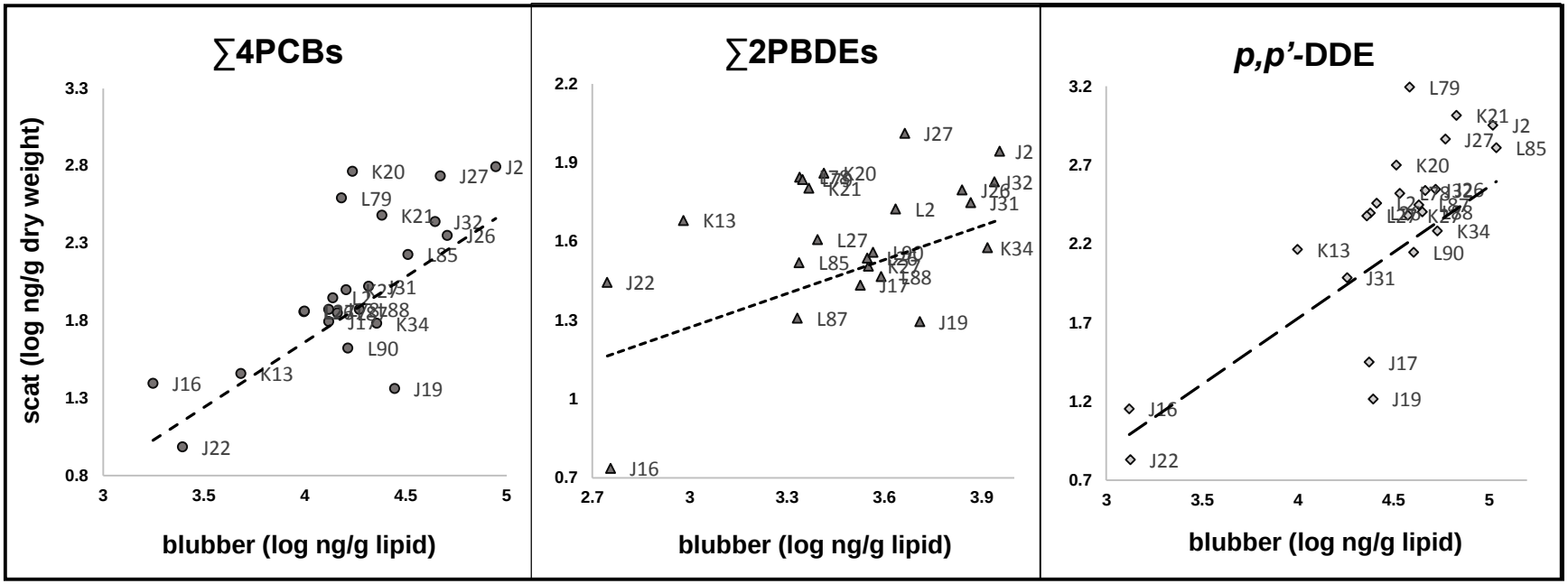
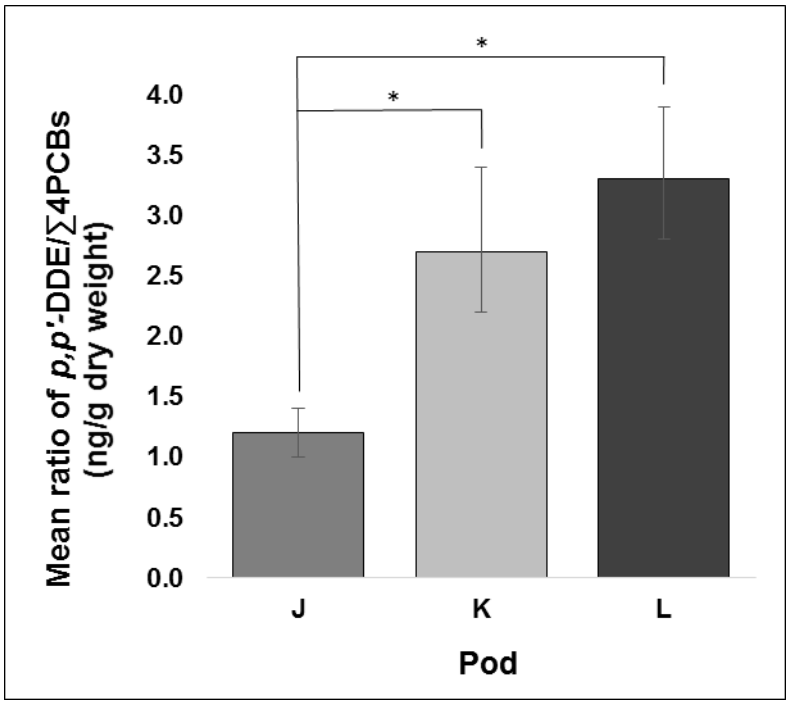


Table 4. Descriptive data of 124 killer whale scat samples from 52 unique whales collected between May and October, 2010-2013, analyzed for persistent organic pollutant concentrations

		Number of samples	Number of unique whales	∑4PCBs[b] (ng/g dry wt)			∑PBDEs[c] (ng/g dry wt)			*p,p'*-DDE (ng/g dry wt)		
				Geometric mean[a]	95% CI		Geometric mean[a]	95% CI		Geometric mean[a]	95% CI	
Year	2010	18	16	130.0	77.0	219.5	26.7	20.1	35.5	243.7	140.5	422.7
	2011	47	38	129.5	90.2	185.9	64.0	52.8	77.5	321.1	220.2	468.4
	2012	33	23	115.9	76.1	176.7	43.9	35.0	55.0	218.8	140.7	340.1
	2013	26	22	75.0	47.5	118.4	34.9	27.3	44.6	156.0	96.6	251.8
Pod	J	58	19	146.0	93.6	227.6	56.2	44.5	71.0	174.3	109.9	276.3
	K	27	13	120.4	68.5	211.5	43.2	31.8	58.8	320.6	178.2	577.0
	L	39	20	86.9	55.7	135.4	37.6	29.5	48.0	288.9	181.8	459.3
Age-sex class[d]	Juvenile	22	12	123.4	68.8	221.3	55.3	39.7	77.1	291.9	158.5	537.8
	Reproductive age female	46	20	41.7	27.0	64.5	29.0	22.8	37.0	55.4	35.2	87.3
	Post-reproductive age female	22	7	237.2	117.4	479.4	60.8	41.4	89.2	596.2	287.3	1237.7
	Male	34	15	136.5	82.0	227.2	43.3	32.6	57.6	363.8	213.7	619.3

a: All geometric means adjusted for Julian day and age-sex class when not as a main effect, and repeat samples from individual whale; b: ∑4PCBs = PCB138, PCB153, PCB180, PCB187; c: ∑PBDEs = PBDE47, PBDE100; d: 2 whales switched from Juvenile to Male category during the 4-year study

Figure 2. Ratio of *p,p'*-DDE/∑4PCBs in n=124 killer whale scat samples collected 2010-2013, adjusted for Julian day, age-sex class, and repeat samples collected from the same whale



*p<0.001

CHAPTER 2. Persistent Organic Pollutants (POPs) in Southern Resident killer whale scat samples: a longitudinal evaluation of contaminant accumulation and mobilization patterns, and modulation in contaminant level and profile by predicting factors such as prey availability and reproductive status

ABSTRACT

Excessive exposure to persistent organic pollutants (POPs) is listed as a current threat to the endangered Southern Resident killer whale (SRKW; *Orcinus orca*) population. POPs are considered endocrine disruptors and have been associated with compromised reproductive success and suppression of the immune system. These biological effects would impact the growth potential and long-term viability of this small population. This study aimed to broaden the understanding of POP accumulation and mobilization, modulation by prey availability, toxicant-related endocrine disruption, and associated deleterious biological effects in SRKWs. In total, 140 killer whale scat samples collected from May-October, 2010-2013, were analyzed for concentrations of POPs [namely, polychlorinated-biphenyls (PCBs), polybrominated-diphenylethers (PBDEs), and dichloro-diphenyl-trichloroethanes (DDTs)]. Sample data was linked to individual (genotyping analysis) and life-history data in this well-studied population. Biologic metrics such as pregnancy status and nutritional stress were also measured in the scat samples using hormone analyses. This study demonstrated the contaminant levels are the highest when prey availability is the lowest. These contaminants have a high potential for toxicity compared to the profile of compounds during high prey availability, and the source is likely endogenous lipid stores. Accumulation patterns showed expected age-related increases, with the exception of nulliparous females that may have toxicant offloading from fetal or neonate loss that was never recorded. The rate of re-accumulation of toxicants by parous females occurred at a faster rate than lifetime accumulation in adult males of a similar age. Mobilization of

endogenous lipid stores for milk production, and subsequent transfer of POPs to a calf, occurred in the first year of lactation particularly for first born calves with diminished transfer to subsequent calves. PBDE and PCB levels did not demonstrate an associated disruption of thyroid hormone, as expected. However, PBDE did demonstrate an inverse association with estrogen hormone levels. Preliminary analysis suggests potential deleterious biologic effects associated with PCB levels, however application of toxicant measures in the scat to health metrics should be interpreted with caution until further investigation on the excretion rates and patterns of these toxicants is better established. This empirical data will be available for application in biologically appropriate models and risk assessments to provide meaningful information to improve and maintain the health of our shared marine environment.

INTRODUCTION

The Southern Resident killer whale (SRKW) population is important to the ecology, culture and economy of the Pacific Northwest. These top-level carnivores are sentinels for the health of Puget Sound and the Salish Sea, important in folklore of First Nations, and the basis for a multi-million dollar tourist industry in Washington State and British Columbia. The SRKWs frequent the Salish Sea between May and October, earning them the title of Residents (Hanson et al. 2010). Female whales reach sexual maturity around 10 years of age, with a gestational period around 18 months; the youngest known SRKW mother (J37) was 11.5 years old (Robeck et al. 2004, CWR 2015). Females produce calves approximately every 5 years with a maximum production peak around 20-22 years, followed by a decline in reproductive performance until reproductive senescence around age 40 (Ward et al. 2009, CWR 2015).

The SRKWs experienced an unexplained 20% decline in their population in the late 1990s. This alarmed the public and scientists alike to the possibility that this ecosystem could no longer sustain these carnivores at the top the food-web. The National Marine Fisheries Service published a Recovery Plan for the SRKW following their listing as an endangered population in 2005 (National Marine Fisheries Service 2008). Excessive exposures to environmental contaminants, particularly persistent organic pollutants (POPs), namely polychlorinated-biphenyls (PCBs), polybrominated-diphenylethers (PBDEs), and dichloro-diphenyl-trichloroethanes (DDTs), and decreased prey availability were indicated as major proposed risk factors to this population in the Recovery Plan, and are still listed as current threats (NOAA Fisheries 2015).

POPs are lipophilic compounds that are resistant to biological degradation. As top-level predators, cetaceans are highly susceptible to POPs due to bio-magnification from contamination in the food chain (Hickie et al. 2007; USEPA. 2002). The SRKW population feed almost exclusively on salmonids, particularly Fraser River Chinook salmon (*Oncorhynchus tshawytscha*), between spring and fall (Ford and Ellis 2006; Ford et al. 1998; Hanson et al. 2010). Levels of POPs in the Puget Sound food web have been demonstrated to exceed guidelines for wildlife consumption (Cullon et al. 2005). The SRKW diet has been highlighted as their most likely source of exposure to POPs (Cullon et al. 2009, Alava et al. 2012).

POPs have been associated with endocrine disruption; specifically reproductive impairment, thyroid dysfunction, and increased risk of infection in laboratory animals, human epidemiologic

studies, and in wildlife populations (Brouwer et al. 1998, Tyler et al. 1998, Rolland 2000, Guillette 2006, Ropstad et al. 2006, Costa et al. 2008). Studies specific to wildlife populations include reproductive sterility in bald eagles of Florida, poor reproductive success in mink and herring gulls in Michigan, demasculinization of alligators in Florida, and reproductive failure in seals from the Baltic Sea (Colborn et al. 1996, Tyler et al. 1998). Deleterious biological effects, such as disruption and/or suppression of the immune (Beland and DeGuise 1993, Lahvis et al. 1995, de Swart et al. 1996, Jepson et al. 2005, Hall et al. 2006) and reproductive systems (Reijnders 1986, Schwacke et al. 2002, Wells et al. 2005, Pierce et al. 2008), have specifically been studied in marine mammals. Blubber biopsy samples previously collected on the SRKWs demonstrated that levels of POPs in this population exceed a health-effects threshold developed by Kannan et al. (2000) through risk characterizations extrapolated from studies of immunologic and reproductive effects in seals, otters, and mink (Kannan et al. 2000, Ross et al. 2000, Krahn et al. 2009). Additionally, risk assessments from other cetaceans suggest reproductive impairment is apparent at the current levels of contamination (Schwacke et al. 2002, Hall et al. 2006).

POPs are stored predominantly in the adipose tissue. The concentration of these contaminants generally increase with age, particularly the POPs more resistant to biotransformation (Boon et al. 1994, Wolkers et al. 2007). Systemic levels of these compounds, and their associated bioavailability to target organs, may increase when fat-metabolism occurs during food shortages (i.e. a decline in prey availability) (Aguilar et al. 1999). Similarly, nursing mothers have been demonstrated to transfer POPs to their offspring in killer whales (Krahn et al. 2009), harp seals (*Phoca groenlandica*) (Wolkers et al. 2002), gray seals (*Halochoerus gryous*) (Addison and Brodie 1987, Pomeroy et al. 1996, Debier et al. 2003), and polar bears (*Ursus maritimus*) (Knott

et al. 2012), among others. The mobilization of endogenous lipid stores (i.e., free fatty acids and lipoproteins) to synthesize milk also mobilize (co-transport) contaminants stored in the lipids. It has been estimated that 2-4 kg of milk converts to 1 kg of mass gain (Oftedal 1997), thus efficiently transporting and depositing contaminants to the new population recruits. Consequently, rapidly developing newborns, arguably the most vulnerable members of a population, are exposed to high-levels of contaminants that may affect their immune and reproductive systems.

A better understanding of POPs in SRKWs, including modulation by prey availability, accumulation and mobilization patterns, and association with deleterious biologic effects, is needed. To date sampling has been constrained by traditional tissue biopsy methods or by tissue collection from necropsied animals. We used measures of trace levels of toxicants in killer whale scat samples non-invasively located by scent using specially-trained detection dog team (Ayres et al. 2012). The longitudinal collection of multiple scat samples per whale across seasons and years presents the unprecedented opportunity to monitor temporal trends in toxicant levels across a broad geographic landscape. The first objective of this study was to evaluate how prey availability modulates the concentration and profile of circulating toxicants in the endangered SRKW population. The second objective was to broaden the understanding of contaminant accumulation and mobilization patterns related to age-sex class and reproductive factors such as pregnancy and lactation. This study also aimed to evaluate the existence of endocrine disruption associated with toxicant levels as measured using hormone measures from the same scat sample. Lastly, preliminary analyses evaluated the association of contaminant levels and deleterious biological effects. These measures will provide scientific data for

recovery strategies to help identify where management efforts can be directed to support recovery of this enigmatic species and ensure the health of our coastal ecosystems.

METHODS

Sample collection

SRKWs appear with regularity in the areas around the San Juan Islands and Puget Sound of Washington state, collectively referred to as the Salish Sea, between spring (mid-May) and fall (mid-October). These selected dates coincide with the Chinook salmon returning to the Fraser River watershed, the preferred seasonal prey of the SRKWs (Hanson et al. 2010).

Our study site is within the inland waters of Washington State. SRKW scat samples were located by detection dogs trained to locate SRKW scat floating on the water's surface (Wasser et al. 2004, Rolland et al. 2006, Ayres et al. 2012). The dog method greatly increases sample size and reduces sampling bias compared to blubber biopsy sampling due to the dog's remarkable ability to locate SRKW scats. Samples were scooped off of the surface of the water using a 1 L polypropylene beaker, collected in a 50 mL polypropylene tube, immediately centrifuged, and all sea-water decanted. The remaining pellet was frozen on dry ice and remained frozen until processed in the lab the following fall (within 6 months of sample collection).

Life history data

Annual population census data has been collected through photo-identification and health assessments since 1976 by the Center for Whale Research (CWR 2013). Census data includes information on births and deaths. As such, age, sex, family lineage, and reproductive status was known on most whales sampled for this study. Age-sex class was defined as juveniles (either

sex, <10 years), adult males (≥10 years), reproductive-age females (≥10-<40 years), and post-reproductive females (40+ years); age-sex class was also considered with reproductive-age females separated by parity (nulliparous and parous). Reproductive status was defined in reproductive-age females as confirmed pregnancy ["Preg (conf)"] (defined below), possible pregnancy ["Preg (poss)"; i.e., apparent miscarriages] (defined below), length of lactation (<1 year, and 1-2 years), and neither pregnant nor lactating. Length of lactation was defined as number of days following estimated date of birth of calf, up to two years.

Laboratory methods

Fecal DNA and hormone measures

In the lab, samples were thawed, homogenized, sub-sampled for high-mass unprocessed toxicant analysis (if wet volume >15mLs) (see details below), and swabbed for DNA using a synthetic tip. SRKW fecal samples were genotyped for species, sex, pod, and individual identification in collaboration with NOAA NWFSC (Ford et al. 2011). Samples were refrozen at -20°C, freeze-dried, and 80 mg (dry fecal weight) (smaller mass samples 40-80 mg were also included) was extracted in 15 mL of 70% ethanol for hormone analyses. Fecal hormone metabolites measured include: glucocorticoid (GC), thyroid (free triiodothyronine, T3; and total thyroxine, T4), testosterone (T), progesterone (P4), and estrogen (E). All hormone metabolites were extracted using methods described previously (Wasser et al. 2000, Wasser et al. 2010, Ayres et al. 2012). Measures of GC, T3, and T4 concentrations in the scat were used to evaluate nutritional stress, as previously demonstrated (Ayres et al. 2012). Briefly, levels of GCs rise quickly in response to poor nutrition and psychological stressors, mobilizing glucose to provide energy to deal with the immediate emergency (Sapolsky et al. 2000). T3, the active form of thyroid hormone, declines

in response to persistently poor nutritional conditions, lowering metabolism to protect the body from exhausting all its reserves (Douyon and Schteingart 2002, Porterfield and White 2007, Wasser et al. 2010). T4, the storage form of thyroid hormone, is excreted in the feces in proportion to its availability in circulation thus reflecting long-term modulation in T3 levels (Porterfield and White 2007, Wasser et al. 2010). The ratio of T3/T4 may provide an indication of conversion of T4 to T3 to provide a more sensitive measure of nutritional stress.

Pregnancy was defined as P4 concentration above 2000 ng/g dry fecal weight concurrent with a T concentration above 50 ng/g (Wasser et al. in prep). Confirmed pregnancy ["Preg (conf)"] was defined as samples from a reproductive-age female (age ≥10-<40) with a hormone profile of pregnancy confirmed by the subsequent appearance of a live birth. Possible pregnancy ["Preg (poss)"; i.e., apparent miscarriages] was defined as a sample with a hormone profile of pregnancy followed by a subsequent sample(s) in the non-pregnancy concentration range and/or failure to observe that female with a live calf within the 18-month gestation period.

Analysis of persistent organic pollutants

Analyses of POPs in killer whale scat samples were performed using two different protocols: high-mass unprocessed samples and low-mass freeze-dried samples. High-mass samples (>15 mL of wet volume) are rarely located in the field; only about 20 collected samples per year were large enough for this analysis, thus selection was considered opportunistic. The thawed and homogenized samples from the field were subsampled prior to freeze-drying for analysis of POP concentrations in collaboration with NOAA NWFSC. All samples collected during a field season (ranging from 39-80 samples per year) qualified for inclusion in the low-mass freeze-

dried sample analysis. These samples ranged from small to large (0.5 - >15 mL wet volume), and had been previously freeze-dried (lyophilized) and extracted in 70% ethanol for hormone analyses. The analysis of all qualifying samples for toxicant measures was cost prohibitive. The final sample selection was based on *a priori* hypotheses to evaluate temporal changes in toxicant concentrations, and variation based on reproductive status.

High-mass unprocessed scat POP analysis methods

Unprocessed scat collected from killer whales were analyzed for POP concentrations using the procedures modified from Sloan et al. (Sloan et al. 2014). Briefly, a homogenized subsample approximately 2 g in wet weight was mixed with sodium and magnesium sulfates to remove water, then extracted with an accelerated solvent extractor using 50 mL of methylene chloride at 100 ^{0}C and 2000 psi. Polar compounds were removed from each sample extract on a gravity flow glass column packed with silica gel/alumina. Bulk lipids and other biogenic materials such as proteins were removed by high-performance size exclusion liquid chromatography. Samples were analyzed using a gas chromatography coupled with mass spectrometry (GC/MS) system equipped with a 60-m DB-5 GC capillary column. The instrument was calibrated using a set of 10 multi-level calibration standards of known concentrations. In total, 40 PCB congeners (PCBs 17, 18, 28, 31, 33, 44, 49, 52, 66, 70, 74, 82, 87, 95, 99, 101, 105, 110, 118, 128, 138, 149, 151, 153, 156, 158, 170, 171, 177, 180, 183, 187, 191, 194, 195, 199, 205, 206, 208, and 209), 11 PBDE congeners (PBDEs 28, 47, 49, 66, 85, 99, 100, 153, 154, 155, and 183), and 24 chlorinated pesticides including DDTs (*o,p'*-DDD, *o,p'*-DDE, *o,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, and *p,p'*-DDT) and hexachlorobenzene (HCB) were analyzed. Percent lipid content in killer whale scat samples was determined gravimetrically (Sloan et al. 2014). All related POP

concentrations were reported on a wet weight (ng/g) basis and were lipid-normalized (lipid-adjusted: la).

Low-mass freeze-dried scat POP analysis methods

Freeze-dried scat sample extraction, purification, and quantification methods were performed as described in Chapter 1 (Lundin et al. in review). Briefly, the methods were developed using modified procedures from EPA Methods 3630C and 1614 (Environmental Protection Agency 1996, Environmental Protection Agency 2007). All samples were previously lyophilized, weighed out to 80 mg dry fecal weight, and extracted with 70% ethanol for hormone analysis. The residual pellet was extracted with 20 mL methylene chloride, and purified with florisil and sulfuric acid-treated silica. All sample extracts and standards were analyzed using a GC/MS system operating in the negative chemical ionization (NCI) mode. The instrument was fitted with a DB-XLB column (30 m length, 0.25 µm film thickness, 0.25 mm i.d.) (Agilent, Santa Clara, CA). The instrument was calibrated with a ten-point standard calibration curve at the beginning and end of each run. Due to the small sample mass, fewer final target analytes were above the limit of detection compared to the high-mass unprocessed samples. As such, the final target analytes included 4 PCB congeners (PCBs 138, 153, 180, 187), 2 PBDE congeners (PBDEs 47, 100), and *p,p'*-DDE. All related POP concentrations were evaluated based on dry fecal weight (ng/g dry weight).

Blubber biopsy samples

Eighteen Southern Resident killer whale blubber biopsy samples were evaluated for this study. Samples were collected in the United States and Canada between May 2006 and January 2013. Samples were obtained and analyzed using techniques described elsewhere (Krahn et al. 2007, Krahn et al. 2009, Sloan et al. 2014). All blubber biopsy samples were analyzed for the same target analytes as measured in the high-mass unprocessed scat samples. Toxicant and associated whale identification data (Ford et al. 2011) were provided by NOAA NWFSC.

PCB structural groups

PCB structural groups were examined to evaluate PCB profiles grouped by shared potential for toxicity and resistance towards metabolic breakdown. Individual PCBs congeners were classified by 5 structural groups based on the reported results from previous studies on seals and cetaceans (Boon et al. 1994, Wolkers et al. 2007). The structural groups were based on the chlorine substitution pattern on the *ortho, meta,* and *para* positions. Group 1 comprised congeners without any vicinal H atoms (e.g., PCB153). Group 2 comprised congeners with vicinal H atoms in *ortho* and *meta* positions and ≥2 *ortho*-chlorine atoms (e.g., PCB138). Group 3 comprised congeners with vicinal H atoms in *ortho* and *meta* positions and 1 *ortho*-chlorine atom (e.g., PCB118). Group 4 comprised congeners with vicinal H atoms in *meta* and *para* position and ≤2 *ortho*-chlorine atoms (e.g., PCB110). Group 5 comprised congeners with vicinal H atoms in *meta* and *para* position and ≥3 *ortho*-chlorine atoms (e.g., PCB149). In total, an average of 51% of all PCBs were in structural Group 1 (28%; PCBs 153, 158, 180, 183, 187, 199) or Group 2 (25%; PCBs 99, 128, 138, 170, 171, 177). Groups 3 (PCBs 28, 31, 66, 70, 74, 105, 118), 4 (PCBs 18, 44, 49, 52, 87, 101, 110), and 5 (PCBs 95, 149, 151) made up 13, 12, and 14% respectively (STable 5).

Data management

Multiple imputation analysis of toxicant data

In total, 70 high-mass unprocessed samples (n=56 with genotype confirmation of individual identity) and 126 low-mass freeze-dried samples (n=124 with genotype confirmation of individual identity) were analyzed; 39 samples were analyzed using both methods (STable1). Linear associations of the 39 samples analyzed both as high-mass unprocessed samples and low-mass freeze-dried samples were significant for ∑4PCBs, ∑2PBDEs, *p,p'*-DDE (all $p<0.001$ using mixed effects linear regression models adjusted for repeat sampling on individual whales; STable 2).

Multiple imputation, the process of estimating values for missing data using known data, was used to extrapolate high-mass unprocessed scat values (ng/g la) for ∑4PCBs, ∑2PBDEs, and *p,p'*-DDE based on measures obtained from the low-mass freeze-dried scat samples (ng/g dry weight). This extrapolation allowed samples from both protocols, high-mass unprocessed samples and low-mass freeze-dried samples, to be analyzed as one combined dataset, thus avoiding a reduction in sample size by performing two separate analyses. The ∑4PCBs ng/g la metric represents, on average, 39% (range: 14-48%) of the ∑40PCB ng/g la metric, based on the 56 high-mass samples. Similarly, the ∑2PBDE ng/g la measure represents, on average, 77% (range: 37-98%) of the ∑11PBDE ng/g la. For the 6 DDTs measured in the 56 high-mass samples, *p,p'*-DDE represents an average of 93% of the summed DDTs (range: 85-100%).

In total, 55.4% (87/157) of samples with toxicant measures from the freeze-dried scat sample analysis needed extrapolated measures to match the high-mass unprocessed scat measures. Separate imputations were run for each congener of interest (PCBs 138, 153, 180, 187; PBDEs 47, 100; and *p,p'*-DDE). In total, 200 values for each missing record were imputed using Markov chain Monte Carlo imputation methods with a separate chain for each imputation, thereby creating two hundred complete datasets. The single point estimate for each variable of interest was attained by averaging the imputed values. All data were normally distributed. Two hundred burn-in iterations were run before the first imputation in each chain to ensure the iterations converged to the stationary distribution before the imputation.

Prey data

Albion test fishery data was used to estimate the number of Chinook salmon returning to the Fraser River watershed (Government of Canada 2014). The Fraser River Chinook salmon collectively consist of three timing groups: early/spring run, before July 15; summer run, between July 15 and September 1; and fall run, after September 1 (DFO 1999). The test fishery uses gill nets to index abundance of Chinook salmon. The nets are deployed two times a day from April through August, to coincide with the daily high tide, and every other day in September and October. Data is reported as catch and effort. For this analysis, catch data was smoothed to create a continuous predictor variable by year. This was accomplished using a general linear model with catch at the response, Julian day (polynomial) as the explanatory variable, and effort, log-transformed, as the offset. The effort data is variable from day-to-day, and changes throughout the season. This approach accounts for variability in the sampling effort while smoothing the fluctuations in the catch data that may be due to measurement error and/or

small sample size. The distribution of the catch data was modeled as a negative binomial, and run as a zero-inflation probability regression model due to the high number of zero catch days. The best fit polynomial for each year, based on AICc (Akaike information criterion with a correction for small sample sizes) (ΔAICc>2), was: 2010, 6th; 2011, 3rd; 2012, 3rd; and 2013, 3rd. The resulting metric, smoothed Fraser River Chinook salmon catch data (Predicted catch), henceforth referred to as "FR Chinook," was used for evaluations of prey abundance (SFigure 1).

The Albion test fishery is an estimated 140 km from the west side of San Juan Island, the whales' primary feeding area where the majority of our samples were collected (Ayres et al. 2012). The travel time for a fish to swim from the whale foraging grounds off the west side of San Juan Island to the Albion test fishery was approximated based on the range of average ground speeds reported for adult Chinook salmon, 0.7-2.7 km/h, and the estimated 140 km distance from San Juan Island to the Albion test fishery (Candy and Quinn 1999, Ayres et al. 2012). Based on these documented Chinook salmon swim speeds, the catch data was lagged by 0 to 14 days. All selected lags were evaluated using a mixed effects model with GC as the predictor [selected because this hormone demonstrates immediate response to changes in metabolic stress (i.e., nutritional status) (Sapolsky et al. 2000)], lagged fish data as the explanatory variable, and year and T3 data as covariates. Fish run data lagged by 12-days was the best-fit model (ΔAIC>2), and was the lag subsequently used in our analyses.

Principal Component Analysis

Principal Component Analysis (PCA) was performed as a sub-analysis on the congener- and metabolite-specific data obtained from the high-mass unprocessed samples with confirmed identity by genotype (n=56 samples). PCA is a statistical approach to minimize the number of variables by recognizing their inherent collinearity. PCA creates a smaller number of uncorrelated variables called principal components, which are linear combinations of the input variables that together explain a portion of variance in the data. All individual PCB congeners were normalized by dividing by ∑40PCBs, all PBDE congeners were divided by ∑11PBDEs, and all DDT metabolites were divided by ∑6DDTs. PCBs with a total percent contribution of <0.5% (PCBs 17, 33, 82, 156, 191, 194, 195, 205, 206, 208, 209) were not included in the analysis. Similarly, PBDEs 85 and 183 were not included. All DDT metabolites had a percent contribution greater than 0.5% and were included. PCAs were run by class of compound. All normalized congeners were log-transformed to achieve normal distribution and entered into the PCA with varimax (orthogonal) rotation. Components needed to have an eigenvalue >1.0 to be retained. At least 70% of the variance of individual variables needed to be explained by the component to be retained. The retention of components was confirmed by a broken stick model, which is based on the concept that the total variance is a shared resource among the principal components. A component was retained if its associated eigenvalue was larger than the broken stick distribution value, which was calculated in terms of the number of total components in a given solution (Cangelosi and Goriely 2007).

Statistical analysis

Comparison of toxicant measures in scat samples and blubber biopsy samples

In total, eighteen unique whales had toxicant measures from both blubber biopsy and high-mass unprocessed scat samples; 16 of these were from adult whales (≥10 years old). Toxicant values were normalized as percent of total contaminant (i.e., percent contribution) by class of compound per ∑40PCBs or ∑11PBDEs, as appropriate. Final comparisons were congener specific. Percent contribution by congener was compared between blubber biopsy and high-mass unprocessed scat samples using mixed effects linear regression models. Linear associations were also evaluated for cumulative levels by class of compound: ∑40PCBs, ∑11PBDEs, *p,p'*-DDE, and HCB using mixed effects linear regression models adjusted for repeat sampling on individual whales. This comparison was restricted to adult whales (n=16 matched pairs) to minimize variation due to the growth dilution effect of toxicants in juvenile whales (Hickie et al. 2007) which would be exacerbated by the variable period of time between collection of blubber and scat samples. Inclusion of years between blubber and scat sample collection was tested as a covariate in the final models.

Evaluation of toxicant levels and prey availability

The evaluation of toxicant levels and prey availability used the combined dataset (n=140 samples). A mixed effects multiple linear regression model was performed using FR Chinook lagged for 12-days as the main effects predictor. After lagging, four samples were outside of the range of dates with associated prey availability data and were excluded. Three additional samples were excluded due to missing hormone (covariate) data. The final analytic dataset contained 133 samples. Cumulative toxicant measure was the response variable, including

individual models by class of compound, ∑4PCBs, ∑2PBDEs, *p,p'*-DDE, and total POPs (tPOPs; defined as the summation of the individual classes). Sub-analyses modeled congener- and metabolite-specific associations using factors from the PCA analysis (high-mass unprocessed scat data) (n=53).

Covariates of interest included year of sample collection, season, pod, age-sex class, birth order, fecal thyroid (T3, T4, and T3/T4 ratio) and GC hormones. Season was defined by the Fraser River Chinook salmon timing groups adjusted for 12-day time lag between sampling site and Albion test fishery site. The main effects were tested for linearity, as well as interactions with the retained covariates.

Contaminant accumulation and mobilization patterns

Number of years of toxicant accumulation was calculated as age for juveniles, males, and nulliparous females. For parous females, years of accumulation was calculated as years since last calf minus 2 years for lactation. If the calf did not survive 2 years, the date was truncated accordingly. The evaluation of toxicant accumulation used the combined dataset (n=140). Mixed effects multiple linear regression modelling was performed using years of accumulation as the predictor and age-sex class with parity as a covariate. Years of accumulation was tested for linearity and as an interaction with age-sex class.

Toxicant level was evaluated by reproductive status in reproductive-age females and by birth order in juvenile whales. Analysis of birth order was restricted to juvenile whales to ensure the measured levels were reflective of maternal burden offload moreso than subsequent

accumulation through diet (Wells et al. 2005). The associations of toxicant level and number of calves (reproductive-age females) and birth order (juvenile whales) were evaluated using mixed effects multiple linear regression models. Cumulative PCB (∑40PCBs ng/g la) level and relative abundance of PCB structural groups were evaluated (high-mass sample data), as well as ∑4PCBs, ∑2PBDE, and *p,p'*-DDE (combined data).

Endocrine disruption

The association of contaminant concentration and hormone level was evaluated. Individual mixed effects multiple linear regression models were performed using level of contaminant (∑4PCBs, ∑2PBDEs, and *p,p'*-DDE) as the response variable and level of hormone (T3, T4, T3/T4, GC, P4, E, and T) as the predictor. Year, season, pod, FR Chinook, age-sex class, age-sex class with parity, and years of accumulation were considered as covariates. Models with reproductive hormones (P4, T, and E) were also stratified by sex. Hormone responses were tested for linearity as well as interactions with the retained covariates.

Individual whale was included as a random effect in all models to account for repeat sampling from individual whales. Final model selections were based on the smallest value of AICc (ΔAICc>2). All statistical analyses were performed in SAS v9.3 (SAS Institute Inc., Cary, NC).

RESULTS

A total of 266 unique samples with a dry fecal weight greater than 0.040 g were collected during the 2010-2013 study period. Of these, 193 were successfully genotyped to confirm individual whale identity. Hormone analyses were performed on 263 samples, regardless of genotype

confirmation of individual identity. A total of 157 killer whale scat samples collected during the 2010-2013 study period were analyzed for toxicant measures, with confirmed genetic identity on 140 samples from 54 unique whales (STable 1). Of these, 70 samples were high-mass unprocessed samples (n=56 with genotype confirmation of individual identity), allowing for sub-analyses that were congener- and metabolite-specific.

Eighteen unique whales had toxicant measures on both blubber biopsy and high-mass unprocessed scat samples. Congener-specific analyses demonstrated no significant difference of percent contribution of individual congeners between samples collected from the same whale (SFigure 2). Linear associations of toxicants concentrations in 16 adult whales with toxicant measures on both blubber biopsy and high-mass unprocessed scat samples were significant for all target compounds evaluated (*p,p'*-DDE and ∑40PCBs, $p<0.001$; ∑11PBDEs, $p<0.01$) (STable 2). HCB was not a significantly associated between the two sample matrices (blubber and scat) ($p = 0.0751$) (STable 2). This was not surprising given the high volatility of this compound. Loss was likely due to metabolism in the scat not experienced in the blubber, laboratory processes, or another unexplained reason. The concentration of ∑40PCBs ng/g la in the scat was 0.14% (median) in relation to the amount per gram lipid in matched blubber samples (10^{th}-90^{th} percentile, 0.03-0.32). Twenty-two unique adult whales had toxicant measures on both blubber biopsy and low-mass freeze-dried scat samples. As previously reported, linear associations of toxicants concentrations were significant for all target compounds evaluated (*p,p'*-DDE, ∑2PBDEs, and ∑4PCBs; $p<0.01$) (Lundin et al. in review) (STable 2). Again, HCB levels in the scat and the blubber were not significantly associated ($p=0.4114$) (STable 2). The average time period between collection of blubber and scat samples was 3 years, 2 months (range: 0 year, 1 month to 7 years, 2 months). These correlations exist despite the time gap

between blubber and scat sampling of the same individual whale. Inclusion of years between blubber and scat sample collection from the same whale did not improve the fit of the data to the model.

Analysis of lipid excretion (percent lipid per gram feces) was performed in the high-mass unprocessed samples (n=56) to examine the relationship between fecal contaminant levels and associated lipids. A mixed effects linear regression model, adjusted for age-sex class with parity, demonstrated a significant positive association of percent lipids per gram of feces with cumulative toxicant concentration per gram of feces (sum of ∑40PCBs, ∑11PBDEs, and *p,p'*-DDE; ng/g with no adjustment for lipid content) (coefficient, 0.8406; SE, 0.1424; p-value, <0.0001) (data not shown). This provided evidence the fat-soluble contaminants are excreted with the lipids. A second analysis of lipid excretion tested if the patterns of toxicant excretion were reflective of prey fat content, which may vary by seasonal timing groups that collectively comprise the Chinook salmon returning to the Fraser River watershed. The association of percent lipid per gram of feces and FR Chinook, run as a mixed effects linear regression model adjusted for age-sex class with parity, was not significant (coefficient, -0.4579; SE, 0.6467; p-value, 0.4822) (data not shown). Likewise, a similar analysis by Julian day, adjusted for year, was also not significant (coefficient, -0.0104; SE, 0.0109; p-value, 0.3467) (data not shown). As such, any demonstrated effect of toxicant excretion and prey availability is not likely due to the concentration of lipids in the diet.

Descriptive data

Samples were representative of each SRKW pod, with 35% of samples from unique members of J pod (19/54), 24% from K pod, and 40% from L pod. Likewise, age-sex class was well represented with 22% of samples from unique juvenile whales (12/54), 39% from reproductive-age females, 13% from post-reproductive females, and 30% from adult males. Samples were represented across seasons, as defined by the 3 FR Chinook timing groups; in 2010 the 23 total samples included 2 from Spring, 11 from Summer, and 10 from Fall. Likewise, total samples in 2011 (Spring, Summer, Fall; 9, 16, 22), 2012 (12, 11, 16), and 2013 (10, 7, 14) were also distributed across seasons (STable 3).

There was no statistical difference in ∑4PCBs, ∑40PCBs, ∑2PBDE, or *p,p'*-DDE values between year or pod (Table 1). Season was not statistically different for any class of toxicant compounds, however Spring was consistently higher; the Spring levels for ∑40PCBs were twice as high as the other two seasons (Spring: mean, 95% CI, 73.9, 39.6-137.7; Summer: mean, 95% CI, 32.0, 18.3-56.0; Fall: mean, 95% CI, 36.3, 22.6-58.1). All toxicant measures demonstrated a significant decrease in levels for parous females [∑4PCBs: mean, 95% Confidence Interval (CI); 6.1, 4.5-8.3] compared to all other age-sex class groups (juveniles, 17.2, 11.7-25.1; females, nulliparous; 15.0. 8.7-26.0; females, 40+, 23.4, 15.2-36.1; and adult males, 20.7, 15.1-28.3). Further evaluation by reproductive status in reproductive-age females (age ≥10 to <40) demonstrated decreased levels of ∑4PCBs in whales lactating at least one year (mean, 95% CI, 2.3, 1.0-5.3) compared to whales neither confirmed nor suspected to be pregnant nor lactating (7.8, 5.5-11.3) (Table 1). Similar findings were evident for *p,p'*-DDE, but not ∑40PCBs or ∑2PBDEs. There were no statistically significant associations by number of calves (modeled as a categorical variable) in reproductive-age females, however there was a suggestive decrease in

toxicant level with each successful calf. Similarly, there was a suggestive decrease in toxicant levels when evaluating by birth order (modeled as a categorical variable) in juvenile whales, with a significant decrease in level of ∑4PCBs in fourth born calves (mean, 95% CI; 7.6, 4.5-12.6) compared to the first born calves (32.2, 22.3-46.3); similar findings were found for *p,p'*-DDE (4^{th} born, 11.6, 5.4-24.9; 1^{st} born, 90.6, 48.5-169.0).

Prey abundance

The rate of change of the conditional mean of tPOP (n=133) with respect to prey (FR Chinook) abundance when T3 (log T3, mean value: 2.197 ng/g dry fecal weight) and age-sex class (adult male) were fixed is estimated to be between -0.196 and -0.017 (95% CI) (Table 2; Figure 1; STable 4). Individual models of ∑4PCBs, ∑2PBDEs, and *p,p'*-DDE also demonstrated significant inverse estimations of the slope value (all metrics, $p<0.05$); the rate of change of the conditional mean of ∑40PCBs (n=53) with respect to FR Chinook was estimated to be between -0.376 and 0.050 (95% CI) (Table 2).

Toxicant profiles were examined using congener- and metabolite-specific data by class of compound (PCBs, PBDEs, and DDTs) with Principal Components Analysis (high-mass samples only; n=56). There were three extracted components for the PCB congeners, two for PBDE congeners, and three for DDT metabolites (STable 6-8). PCBs 171 and 199 accounted for <70% of the variance and were excluded from the final analysis; similarly, PBDEs 100 and 153 were also excluded. Factor 1 of the PCB components accounted for 63.1% of the total variance in the dataset and readily differentiated PCBs by structural group. Factor 1 positive loadings included all PCBs in groups 1, 2, and 5 and negative loadings included all PCBs in groups 3 and 4 (Figure

2). One exception to this was PCB52, which has 2 *meta*, *para* vicinal hydrogen bonds and 2 *ortho*-chlorine bonds qualifying it for Group 4. However PCB52 has a positive factor loading that could be explained by stability and resistance to metabolism accounted for by the symmetry of the compound. This clustering of PCB structural groups 1, 2, and 5 and groups 3 and 4 correspond with previously reporting findings of these structural groups based on their capacity for biotransformation as determined by the organization of hydrogen and chlorine atoms on the biphenyl ring (Boon et al. 1994). PCB structural groups 1, 2, and 5 are described as the "Persistent PCBs" (resistant to metabolism and degradation) and PCB structural groups 3 and 4 as "Readily Metabolized PCBs." Factor 2 of the PCB components accounted for 14.6% of the total variance in the dataset. This Factor separated out clusters of congeners by those having 6 or 7 chlorines substituents (Figure 2). Factor 3 accounted for 10.0% of the total variance in the dataset and distinguished clusters of homologues with 3, 4, or 5 chlorines (Figure 2). Regression model estimates for the rate of change of the conditional mean of PCB Factor 1 with respect to FR Chinook abundance for adult males when year was fixed was -0.5523 (95% CI, -1.0610 to -0.0436) (Table 3, STable 9). Age-sex class was fixed for adult males because their accumulated POP levels are not subject to fluctuations due to parturition and lactation. Factor 2 and Factor 3 were also run as conditional response variables, but were not significantly associated with prey availability (Table 3). In a similar analyses for PBDEs, factor 1 accounted for 68.2% of the variance in the dataset, and factor 2 accounted for 20.5% (STable 7). Factor 1 demonstrated a positive loading for PBDEs 28, 49, 66, 154, and 155 (accounting for 12% of measured PBDEs), and negative loading for PBDEs 47 and 99 (accounting for 74% of measured PBDEs) which have both been described to have high bio-magnification in fish-eating mammals (STable 5) (de Wit 2002). Nonetheless, neither factor was significantly associated with prey availability

(STable 10). PCA factors for DDT metabolites were also not associated with prey availability (STable 10), although 93% of total measured DDTs were *p,p'*-DDE, with 4% *p,p*-DDD, and all other metabolites ≤1% (STable 5; STable 8).

Contaminant accumulation and mobilization patterns

Number of years to accumulate persistent organic pollutants was evaluated in the combined dataset (n=140) by age-sex class with parity. As mentioned above, toxicant levels were highest in post-reproductive females (tPOPs: mean, 95% CI; 85.2, 52.9-137.0) and adult males (73.4, 52.0-103.3), followed by juveniles (60.3, 39.9-90.9) and nulliparous females (44.4, 23.9-81.5), with parous females significantly lower than all other groups (15.5, 10.9-21.9) (data not shown). The estimated rate of change of the conditional mean of tPOP with respect to years of accumulation was estimated to be between 0.014 and 0.082 (95% CI) for parous females and between 0.003 and 0.010 (95% CI) for post-reproductive females (Figure 3; STable 11-12). There was a suggested (non-significant) inverse slope of the conditional mean of tPOP with respect to years of accumulation for juveniles (estimated rate of change, -0.069; 95% CI, -0.159 to 0.021) and nulliparous females (estimated rate of change, -0.027; 95% CI, -0.084 to 0.030), and suggested increase for adult males (estimated rate of change, 0.019; 95% CI, -0.005 to 0.043) (Figure 3; STable 11-12). A similar pattern was retained when individual models were run for ∑4PCBs, ∑40PCBs, ∑2PBDEs, and *p,p'*-DDE (STable 12).

Relative abundance profiles of PCBs by structural group category demonstrated elevated levels of Persistent PCBs in juveniles, nulliparous females, post-reproductive females, and adult males (range: 66%-71%), compared to 53% (95% CI, 46-60) in parous females (high-mass unprocessed

samples, n=56) (STable 13). Upon closer examination of the fecal toxicant levels in reproductive-age females (n=24 samples), all samples qualifying as apparent miscarriages were from nulliparous females (J31, J32, and L90); the fourth nulliparous female had no known calves or pregnancies (J36). The parous females were either confirmed pregnant (J16, J28, and L55), lactating (J17, J28, J35, K12, and L54), or were neither pregnant nor lactating at the time of sampling (J14, J19, K14, K16, and L82). The highest geometric marginal mean concentration of ∑40PCBs ng/g la fecal contaminants was in females with apparent miscarriages (13.7; 95% CI, 2.3-81.9), followed by females lactating less than 1 year (12.1; 3.9-37.3), females neither pregnant nor lactating (11.7; 4.7-29.2), females with confirmed pregnancies (10.0; 2.6-37.4), and females lactating between 1 and 2 years (6.3; 1.8-21.9) (Table 1). The profiles of reproductive status in females by PCB structural group categories demonstrated elevated levels of Persistent PCBs in samples collected on females with apparent miscarriages [Preg (poss); mean, 95% CI; 73%, 61-85], and decreased levels in whales that were between the 1st and 2nd year of lactation (43%, 30-56) (Figure 4, STable 14).

An evaluation of reproductive-age females by number of calves (modeled as a continuous variable) demonstrated a significant decreased trend for both concentration of cumulative ∑40PCBs ng/g la [coefficient, -0.179; standard error (SE), 0.034; p-value, <0.001] and percent Persistent PCBs (coefficient, -0.036; SE, 0.014; p-value, 0.029) with increasing number of calves (n=24) (STable 15). Increasing numbers of calves was also associated with a significant decreased trend by concentration of ∑4PCBs ng/g la (coefficient, -0.106; SE, 0.030; p-value, <0.001), ∑2PBDEs ng/g la (coefficient, -0.073; SE, 0.026; p-value, 0.007), and *p,p'*-DDE ng/g la (coefficient, -0.103; SE, 0.041; p-value, 0.015) in the combined dataset (n=52) (STable 15).

Accordingly, a significant decreased trend was also associated with birth order in juvenile whales (∑4PCBs: coefficient, -0.202; SE, 0.035; p-value, <0.001; ∑2PBDEs: coefficient, -0.071; SE, 0.020; p-value, 0.033; and *p,p'*-DDE: coefficient, -0.279; SE, 0.062; p-value, <0.001) in the combined dataset (n=22) (STable 16). A similar trend was marginally significant for ∑40PCBs (p=0.060), but was based on 5 samples (STable 16).

Paired high-mass fecal samples were collected from two nursing whales, J35 nursing her first calf J47 and K12 nursing her fifth calf K43. Samples of J35 were collected on lactation days 223 and 663, and samples of K12 were collected on lactation days 302 and 640. For both whales, the concentration of cumulative ∑40PCBs ng/g la decreased across lactation days; J35 decreased from 33.4 to 5.6 (an 83% difference) and the concentration in K12 decreased from 3.6 to 3.3 (an 8% difference) (STable 17). Similarly, the difference in ∑11PBDEs ng/g la across lactation days was an 84% and 18% decrease in J35 and K12, respectively, and ∑6DDTs ng/g la decreased by 80% and 19%, respectively (STable 17). Likewise, the percent of Persistent PCBs in their scat decreased across lactation days; J35 decreased from 70% to 48% and K12 from 54% to 47% (Figure 5; STable 17).

Endocrine disruption

All measured hormones were tested (T3, T4, T3/T4, GC, P4, E, and T) to investigate the relationship between toxicant (∑4PCB, ∑2PBDE, and *p,p'*-DDE) and hormone levels, however few models demonstrated a significant association. Progesterone level, adjusted for age-sex class with parity, was negatively associated with ∑2PBDE in female whales (estimate, -0.0980; 95% CI, -0.1701 to -0.0260; p-value, 0.0084) (STable 18). Estrogen, adjusted for age-sex class

with parity, was negatively associated with ∑4PCB (estimate, -0.2497; 95% CI, -0.4268 to -0.0726; p-value, 0.0063) and ∑2PBDE (estimate, -0.2410; 95% CI, -0.3657 to -0.1163; p-value, 0.0002), but not *p,p'*-DDE (estimate, -0.1555; 95% CI, -0.3318 to 0.0208), in female whales (STable 18). In consideration of multiple comparisons, application of a Bonferroni correction (dividing the critical value, 0.05, by the number of models, n=21) resulted in a new critical value of 0.00238. Using this conservative critical value, only the inverse association between estrogen and ∑2PBDE remained significant.

DISCUSSION

The overall objective of this study was the development of a non-invasive and innovative technique for the assessment of contaminant burden in SRKWs. This study reports levels of PCBs, PBDEs, and *p,p'*-DDE from 140 samples collected from 54 unique whales across a 4-year sampling period. This is a well-studied population allowing sample data to be linked to individuals (through genotyping analysis) and life-history data (including pod membership, age, birth order, etc), as well as other biologic metrics such as pregnancy status (using hormone measures from the same sample). We successfully demonstrate modulation in contaminants level and profile predicted by prey availability. Contaminant accumulation and mobilization patterns are shown to be related to age-sex class and reproductive factors such as pregnancy and lactation, consistent with previous findings using measures from blubber biopsy samples (Ylitalo et al. 2001, Krahn et al. 2009). This study supported previous findings and added a more detailed interpretation due to increased sample size and repeat sampling from individual whales. Our study provides empirical evidence on modulation of POPs levels by prey availability, and accumulation and depuration of POPs, specific to Southern Resident killer whales. The findings

from this study will be available for application in biologically appropriate models and risk assessments to promote population recovery and pollution cleanup to keep our marine environment healthy.

Effects of diminished prey abundance

Decreased prey (Chinook salmon) abundance has been associated with increased mortality (Ford et al. 2009) and decreased fecundity (Ward et al. 2009) in this population of killer whales. These effects may be compounded by increased toxicant exposure during this nutritional shortage. We found the estimated rate of change of cumulative levels of POPs excreted in SRKW scat samples significantly decreased across a gradient of increased prey availability, as measured by FR Chinook (Table 2; tPOP; estimate, -0.1065; 95% CI, -0.1958 to -0.0172). Likewise, the profile of toxicants was modulated by prey availability. When prey availability was the lowest (e.g., May and June), the more persistent, lipophilic compounds (Persistent PCBs; PCB structural groups 1, 2, and 5) were predominately excreted (Table 3; PCA Factor 1; estimate, -0.5523; 95% CI, -1.0610 to -0.0436). The Persistent PCBs are more resistant to metabolism and are more lipophilic [log octanol:water partition coefficient (log Kow): 6.7-7.1] than the Readily Metabolized PCBs (log Kow: 5.9-6.3), thus have a larger capacity for being retained and accumulating in the fat-rich tissues of the killer whales (Erickson 1997). Thus, an increase in these compounds when food is scarce (prey abundance is low) indicates the source of the exposure is likely from internal lipid stores that are metabolized for energy. When prey availability was highest (e.g., July and September), a greater percentage of the PCBs excreted were from the Readily Metabolized PCB structural groups 3 and 4. These Readily Metabolized PCB congeners likely came from a recent prey source, instead of blubber, because there is

limited bioaccumulation of these compound. This was corroborated by the increased contaminant values measured in the Spring (∑40PCBs; mean, 73.9; 95% CI, 39.6 to 137.7), when FR Chinook abundance is low, compared to Summer (∑40PCBs; mean, 32.0; 95% CI, 18.3 to 56.0) when the FR Chinook abundance is high (Table 1). The Fall samples for this study were collected before the decline in the FR Chinook run.

The Persistent PCBs, found in greater abundance during low prey availability, contain more congeners with a high toxicity potential than the group of Readily Metabolized PCBs. PCB congener toxicity has been described by their ability to induce bioactivating enzyme systems; those defined as 3-methylcholanthrene-type (3-MC-type) and mixed type mixed-function oxidases (mixed-type) are considered to have the greatest toxicity potential (McFarland and Clarke 1989). A second group of phenobarbital-type (PB-type) inducers also have a potential for toxicity, but it is comparatively less than those listed above. The congeners that comprise PCB structural groups 1, 2, and 5 include three mixed-type enzyme inducers (PCB128, 138, and 170) and four PB-type inducers (PCB153, 180, 183, and 99). The congeners that comprise PCB structural groups 3 and 4 include two mixed-type enzyme inducers (PCB105 and 118) and three PB-type inducers (PCB166, 87, and 101). No 3-MC-type inducers were analyzed in this study. This suggests the elevated levels of Persistent PCBs during low prey availability are comprised of a chemical profile with a higher toxic potential than the profile when prey is more abundant. The elevated toxic potential in addition to increased levels of toxicant described above may contribute to the increased mortality and decreased fecundity associated with decreased prey availability (Ford et al. 2009, Ward et al. 2009). That said, while the Readily Metabolized PCBs

are less abundant overall (25% of ∑40PCBs; STable 5), they also contain congeners with a high toxicity potential and should not necessarily be regarded as nontoxic.

Our analysis assumes exposure to a consistent toxicant source (chemical profile and level) throughout the season. However, the FR Chinook salmon are divided into 4 major geographical stock complexes, and 3 timing groups (early/spring run, before July 15; summer run, between July 15 and September 1; and fall run, after September 1) (DFO 1999). Our findings could be reflective of a change in toxicant profiles of FR Chinook over the course of the salmon timing groups, such as migration-related metabolism leading to a decrease in the more Readily Metabolized (i.e., less chlorinated) PCB congeners (Cullon et al. 2009). Our findings could also be influenced by a change to a prey source, such as chum salmon (*Oncorhynchus keta*) or Pacific halibut (*Hippoglossus stenolepis*), that may have a different contamination profile during the timeframe of sample collection (Hanson et al. 2010). Interpretation of the ∑4PCB and tPOP metric should be interpreted with consideration that all PCB congeners in these metrics are classified as Persistent PCBs, whereas the ∑40PCB metric includes both Persistent and Readily Metabolized PCBs. Further study evaluating the toxicant profiles on FR Chinook salmon and other potential prey sources would allow for the calculation of a Metabolic Index that would quantify relative bioaccumulation in the food chain to indicate whether the toxicants are from a prey or blubber source (Wolkers et al. 2004).

Contaminant accumulation and mobilization patterns

Individual contaminant patterns were expected to vary with respect to age and sex differences based on previous studies of accumulation patterns of POPs in wild cetacean populations (Ross

et al. 2000, Ylitalo et al. 2001, Wells et al. 2005, Hall et al. 2006, Krahn et al. 2009, Yordy et al. 2010). Particularly, an increase in contaminant level related to age with the exception of toxicant offloading due to placental and lactation transfer was anticipated. The positive association for years of accumulation of tPOPs with parous females, post-reproductive age females, and males was expected based on bioaccumulation of these compounds (Figure 3; STable 12) (Ross et al. 2000, Wells et al. 2005, Krahn et al. 2009). The suggested inverse association for juveniles was expected, based on the growth dilution of toxicants in calves (estimate, -0.069; 95% CI, -0.159 to 0.021) (Hickie et al. 2007, Krahn et al. 2009). However, the suggested inverse association in nulliparous females (estimate, -0.027; 95% CI, -0.084 to 0.030) was surprising and may be due to toxicant offloading from fetal or neonate loss that was never documented, continued growth dilution effect (whales reach full size around age 20) (Fearnbach et al. 2011), orphan-induced lactation (a phenomenon reported in *Tursiops)* (Oftedal 1997), or some other unexplained reason. The association of tPOPs and years of accumulation was tested for non-linearity to assess any apparent growth dilution effects during the post-juvenile years when growth continues, albeit at a lower rate (Christensen 1984, Fearnbach et al. 2011); a linear association was the best fit model. Years of accumulation and post-juvenile growth dilution was further evaluated with adult male and female whales stratified by those 10-20, and 20 or greater, years old. The conditional mean of adult parous females less than 20 years old with respect to years of accumulation was 0.022 (95% CI, -0.029 to 0.073), and greater than 20 years was 0.075 (95% CI, 0.018 to 0.133) (data not shown). The conditional mean of adult males less than 20 years old was 0.004 (95% CI, -0.033 to 0.041), and greater than 20 years was 0.031 (95% CI, -0.008 to 0.070) (data not shown). Although not statistically significant, this suggests the accumulation rate was lower in whales aged 10-20 than whales greater than 20 years old which supports an effect of continued yet

tapered growth dilution through the age of 20. Adult males and parous females under the age of 20 maintained a suggested positive association with years of accumulation, whereas nulliparous females, all less than 20 years old, continued to demonstrate decreased tPOPs with years of accumulation (estimate, -0.027; 95% CI, -0.078 to 0.024) (data not shown). As such, although growth dilation may contribute to this effect in nulliparous females, alternative explanations, as listed above, are likely.

A closer look at the estimated rate of accumulation in adult males, selected because they do not experience known toxicant offloading beyond metabolism and elimination, indicate the accumulation patterns may vary by class of compound. Specifically, the estimated rate of accumulation was greatest for *p,p'*-DDE (estimate, 0.023; 95% CI, -0.004 to 0.050), followed by ∑4PCBs (estimate, 0.014; 95% CI, -0.012 to 0.039), and ∑2PBDEs (estimate, 0.003; 95% CI, -0.014 to 0.020) (STable 12). This corresponds with a previously reported lack of an age related accumulation trend for PBDEs in bottlenose dolphins (*Tursiops truncates*) and killer whales (Wolkers et al. 2007, Yordy et al. 2010), but contradicts evidence of bioaccumulation of PBDEs in ringed seals (*Phoca hispida*), beluga whales (*Delphinapterus leucas*), and killer whales (Wolkers et al. 2004, Mongillo et al. 2012). Likewise, the levels of ∑2PBDEs from this study by age-sex class (female, nulliparous > post-reproductive female > juvenile > adult male >> female, parous) did not match the expected pattern as demonstrated by classes of compounds (∑4PCBs and *p,p'*-DDE) with well-established bioaccumulation patterns based on age and reproductive status (post-reproductive female > adult male > juvenile > female, nulliparous >> female, parous) (Table 1).

The estimated rate of years of accumulation of tPOPs in parous females (estimate, 0.048; 95% CI, 0.014 to 0.082) following birth of last calf, minus two years to account for lactation, was elevated compared to lifetime accumulation in males of a similar age (estimate, 0.019; 95% CI, -0.005 to 0.043), and statistically greater ($p<0.05$) than lifetime accumulation in post-reproductive females (estimate, 0.007; 95% CI 0.003 to 0.010). This finding suggests the re-accumulation of POPs between pregnancies, once lactation has ended and transfer of POPs to a calf has ceased, may occur at an increased rate. In the analysis specific to *p,p'*-DDE the rate of accumulation in parous females (estimate, 0.076; 95% CI 0.072 to 0.080) was statistically greater than both adult males (estimate, 0.023; 95% CI -0.004 to 0.050) and post-reproductive females (estimate, 0.006; 95% CI -0.003 to 0.014).

Using scat toxicant data paired with individual whale biological data, the current study provides a measurement of contaminant mobilization in SRKWs through depuration by lactation transfer from a mother to her calf. Marine mammals closely related to killer whales [Family: Delphinidae; bottlenose dolphin *(Tursiops truncates*), common dolphin (*Delphinus delphis*), and humpback dolphin (*Sousa Chnensis*)] have been documented to produce milk that is 10-30% fat (Oftedal 1997, West et al. 2007). This fat is largely derived from the mobilization of endogenous lipid reserves in the blubber during milk production, thus the lipid-rich milk also contains stored lipophilic toxins that are transferred with the lipid (Pomeroy et al. 1996, Wolkers et al. 2002, Debier et al. 2012). As such, the decreased toxicant levels in parous female whales compared to other age-sex classes reported in this study (Table 1), and the significant inverse association of ∑40PCBs ng/g la (coefficient, -0.179; p-value, <0.001), ∑4PCBs (-0.106; 0.001), ∑2PBDEs (-0.073; 0.007), and *p,p'*-DDE (-0.103; 0.015) with increasing number of calves, is

not surprising (STable 15). The demonstrated decrease in relative abundance of Persistent PCBs from the first year of lactation (mean, 95% CI; 52, 41-63) to later in lactation (1-2 years) (mean, 95% CI; 43, 30-56) indicates a greater mobilization of endogenous lipids during the first year of lactation (Figure 4; STable 14). Interestingly, nursing calves and pups of common bottlenose dolphins (*Tursiops truncatus*), harp seals (*Phoca groenlandica*), and gray seals (*Halichoerus grypus*) have been demonstrated to have a lower proportion of highly chlorinated PCBs (Persistent PCBs) compared to their mothers (Addison and Brodie 1987, Wolkers et al. 2002, Yordy et al. 2010). Possible explanations for this include selective transfer of lower chlorinated (i.e., more readily metabolized) PCBs from blubber stores for milk production, a physical barrier at the level of the placenta or mammary glands, or degradation or metabolism of certain congeners in circulation (Ross et al. 2004). West et al. (2007) demonstrated a significant increase in fat over the course of lactation in Delphinidae (West et al. 2007). As fat reserves are depleted, dietary fats may become a greater contributor of lipids for milk production, as evidenced by the suggested increase in relative abundance of Readily Metabolized PCBs during late lactation (1-2 years) (mean, 95% CI; 45, 33-57) compared to earlier in lactation (<1 year) (mean, 95% CI; 35, 25-45) (Figure 4; STable 14). In support of this the contamination patterns of J35, nursing her first calf, and K12, nursing her 5th calf, demonstrated an increase in percentage of Readily Metabolized PCBs over the course of lactation days (Figure 5). By the second year of lactation both J35 and K12 had a similar percentage of Readily Metabolized PCBs (43% and 43%, respectfully) suggesting that in both whales lipids for milk production were derived from dietary fats moreso than endogenous lipids. The steeper slope in the decrease of Persistent PCBs in J35 across lactation days may be related to this being her first calf, thus her pre-lactation level of Persistent PCBs was higher than K12 who was nursing her 5th calf. This

suggests that the blubber stores of Persistent PCBs in K12 were lower, likely due to off-loading of her toxicant burden to previous calves.

The amount of placental and lactational transfer of maternal toxicant body burden to calves in killer whales is not known, but has been estimated to be 3-5% and 70-90%, respectively, based on average values reported previously in other species of delphinids [bottlenose dolphins (*Tursiops truncatus*) and striped dolphins (*Stenella coeruleoalba*)] (Mongillo et al. 2012). It has been demonstrated that the majority of maternal contaminant burden is transferred to first-borns with diminishing transfer to subsequent calves (Wells et al. 2005). The current study demonstrates an 83% difference in ∑40PCBs levels in J35 over 440 days of nursing her first calf. and an 8% difference in ∑40PCBs levels in K12 over 338 days nursing her fifth calf. Thereby supporting that 70-90% transfer of maternal body burden may only apply to the first calf. This is corroborated by evidence of a decreasing trend in toxicant burden in reproductive-age females by number of calves (∑40PCBs: coefficient, -0.179; p-value, <0.001; tPOPs: coefficient, -0.107; p-value, 0.004) (Table 1; STable 15), and a decreasing trend in toxicant burden in juvenile whales by birth order (tPOPs: coefficient, -0.245; p-value, <0.001) (Table 1; STable 16).

Endocrine disruption

POPs are often referred to as 'endocrine disruptors' because these compounds have been demonstrated to interfere and interact with cell messaging systems (i.e., hormones) responsible for homeostasis and the regulation of developmental processes (Kavlock et al. 1996). PBDEs and PCBs share a structural similarity to thyroid hormone and have been demonstrated to disrupt thyroid hormone levels (Brouwer et al. 1998, Hallgren et al. 2001, Hallgren and Darnerud 2002,

Costa et al. 2008). Specifically, consistent evidence has shown an associated decrease in total and free thyroxin (T4), the precursor hormone of triiodothyronine (T3) (Costa et al. 2008). Disruption in thyroid hormone homeostasis has also been associated with PCB exposure; experimental studies have demonstrated decreases in circulating levels of T4 in rats (Brouwer et al. 1998, Hallgren et al. 2001, Hallgren and Darnerud 2002), although the findings have been inconsistent in epidemiologic studies (Salay and Garabrant 2009). A PCB-related increase in thyroid hormone receptor α mRNA has been specifically identified in blubber biopsy samples from killer whales (Buckman et al. 2011). As such PBDE and PCB levels in this study were expected to modify measures of thyroid hormone from the same sample. If an association was present, it may have been masked by the effects of prey modulation on thyroid and contaminant levels. Further evaluation with a larger sample size may highlight this distinction, if it exists.

Our finding of a significant inverse association between PBDEs and estrogen (estimate, -0.2410; 95% CI, -0.3657 to -0.1163) was not entirely surprising to due previous demonstrations of PBDE interacting with estrogen receptors (Costa et al. 2008). PBDE exposure has specifically been associated with decreased serum estradiol concentrations in females rats (Lilienthal et al. 2006), and their offspring (Lilienthal et al. 2006, Talsness et al. 2008). Conversely, there is more supporting evidence for an association of PCB exposure and modified estrogens levels (Haave et al. 2003, Kraugerud et al. 2010, Buckman et al. 2011), but no association was found in this study.

Future steps

Impaired reproductive success and compromised immunologic effects have been associated with POP exposures in laboratory studies (Brouwer et al. 1998) and in wildlife species (Tyler et al. 1998). A threshold for PCB-related health effects of 17,000 ng/g PCBs lipid weight in aquatic mammals (Kannan et al. 2000) has previously been applied to SRKW blubber biopsy samples (Krahn et al. 2009). Nine of the 12 blubber samples analyzed were reported to be above the threshold, the exception being three parous whales with recent off-loading of their toxicant burden to their calves (Krahn et al. 2009). Application of a similar metric to contaminant levels measured in scat samples would be a useful tool for assessing health risks using longitudinal collection of multiple scat samples per whale across seasons and years. A preliminary analysis exploring the application of this health-effect threshold to toxicants measures in scat can be found in Appendix A.

Calf mortality in the SRKW population has been described to be approximately 37–50% during the first year of life (CWR 2015). However this may underestimate calf mortality since most births occur in the winter months when this population is not monitored as closely due to their extended time spent outside of the Salish Sea, and does not account for miscarriages. Our study provides evidence of delayed reproductive success in 4 nulliparous out of 21 (19%) reproductive-age females sampled. Three out of the four nulliparous females (J31, J32, and L90) had a possible pregnancy (i.e., apparent miscarriage; defined above) over the course of this 4-year study. Of these, J32 was found dead with an almost full term fetus in December of 2014 (DFO 2014). The relative abundance of Persistent PCBs was elevated in adult females with an apparent miscarriage (mean, 95% CI; 73%, 61-85) (Figure 4; STable 14) compared to other

states of reproduction suggesting the profile of contaminants in these whales may have a higher potential for toxicity. The nulliparous females in this study demonstrated an estimated rate of change of the conditional mean of tPOP with respect to years of accumulation to be -0.027 (95% CI, -0.084 to 0.030), whereas the rate of change in males of a similar age (10-20 years) was 0.004 (95% CI, -0.033 to 0.041) (Figure 3; data not shown); this unexpected decrease in toxicant level of nulliparous females might be explained by undocumented fetal or neonate loss. Fecundity generally starts around age 10, reaching a maximum between ages 20-22 (Ward 2009). The four nulliparous females discussed above range from age 16-22. The delay in producing a successful calf in these and other female whales would impact growth potential and long-term viability of this endangered population. Further sampling with particular focus of reproductive age females, coupled with measurements of pregnancy status and toxicant level and profile, would contribute to the understanding of calf survivorship and POP levels in this population.

A risk characterization of reproductive effects associated with PCB exposure in bottlenose dolphins (*Tursiops truncatus*) has been previously performed based on measurements of PCB-associated reproductive failure in mink (*Mustela vison*) (Schwacke et al. 2002). The authors reported an expected 79% (95% CI: 44-89%) of nulliparous females, in excess of normal background incidence, to suffer from reproductive failure such as stillbirth or calf mortality in their first offspring. Similarly, DDT levels in female bottlenose dolphins have been demonstrated to be 3 times higher in those whose calves did not survive more than 6 months; PCB levels were 2.5 times higher (Reddy et al. 2001). High POP burden (PCBs, PBDEs, *p,p'*-DDE, and hexabromocyclododecane) has also been associated with a high number of ovarian

scars in female common dolphins (*Delphinus delphis*) neither pregnant nor lactating suggesting they may not be able to reproduce and therefore continue to ovulate (Murphy et al. 2010). The same study found a lower number of ovarian scars associated with POP concentration in harbor porpoise (*Phocoena phocoena*) suggesting the contaminants were inhibiting or disrupting ovulation. Information specific to PBDE levels and calf survivorship in Delphinidae is limited, although there is evidence of a decrease in fecundity and longer time to pregnancy associated with PBDEs levels in humans (Harley et al. 2010), and evidence of an associated delay in puberty onset in rats (Lilienthal et al. 2006).

A predictive calf survivorship model in relation to maternal PCB blubber levels was previously developed (Hall et al. 2006) using PCB levels reported in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Florida (Wells et al. 2005) combined with an estimated dose-response relationship (Reddy et al. 2001) and age-specific fecundity and survival data from a mark-recapture study (Wells and Scott 1990). A calculation of 0.50 probability of calf survivorship, based on the predictive calf survivorship equation, equates to an estimated maternal ∑22PCB ng/g la blubber level of 9.17 ng/g la. A preliminary analysis exploring the application of this predictive calf survivorship model to toxicants measures in scat can be found in Appendix A.

Looking ahead, refinement of the toxicant measures in scat in relation to the toxicant measures in blubber, and application of the contaminant levels in scat from SRKWs with known life history data and biologic metrics of reproductive status such as apparent miscarriage (Wasser et al. in prep), may support the development of a biologically appropriate model of calf survivorship

specific to the SRKW population. However, a better understanding of excretion rates and patterns of these toxicants is essential before applying metrics developed for contaminant measures in blubber to measures in scat samples.

CONCLUSIONS

This novel study provides reliable toxicant measures, combined with other health related analyses, in scat samples of Southern Resident killer whales. Blubber biopsy sampling has been considered the "gold standard" for measuring toxicant burden in marine mammals. Non-invasively collected scat samples increase sample size and temporal frequency, and decrease sampling bias, compared to blubber biopsy sampling. We demonstrated that, in many instances, the toxicants in scat were reflective of blubber concentrations and may reflect exposure to toxicants in circulation. This study shows contaminant levels are the highest, from endogenous lipid stores, and have the greatest potential for toxicity when the whales are nutritionally compromised; these toxicant exposures may contribute to the adverse effects of increased mortality and decreased fecundity previously related to limited prey abundance. Accumulation patterns showed an expected age-related increase with the exception of nulliparous females that may have toxicant offloading from fetal or neonate loss that was never recorded. The rate of POP re-accumulation was significantly greater in parous females, following lactation, compared to lifetime accumulation rates of adult males and post-reproductive females. Mobilization of endogenous lipid stores for milk production, and subsequent transfer of POPs to a calf, occurred in the first year of lactation particularly for first born calves with diminished transfer to subsequent calves. The off-loading of POP maternal body burden associated with a first born calf ranged from 80-84% by class of compound. PBDE and PCB levels did not demonstrate an

associated disruption of thyroid hormone as expected. If an association was present it may have been masked by the effects of prey modulation on thyroid and contaminant levels. However, PBDEs did demonstrate an inverse association with estrogen concentration. Preliminary analysis suggests potential deleterious biologic effects associated with PCB levels, however application of toxicant measures in the scat to health metrics should be interpreted with caution until further investigation on the excretion rates and patterns of these toxicants is better established. The toxicant measures provided by this study broaden the understanding of contaminant accumulation and mobilization patterns, and modulation in contaminant level and profile by predicting factors such as prey availability and reproductive status. This empirical data will be available for use in population recovery, risk assessment, and mitigation efforts, as well as adaptive management practices to provide meaningful information to improve and maintain the health of our shared marine environment.

TABLES AND FIGURES

Table 1. Profile of toxicant levels in scat samples by year, season, pod, age-sex class, reproductive status, number of calves, and birth order: total samples (combined dataset), n=140 samples on 54 unique individuals; high-mass samples, n=56 samples on 34 individuals

		Sample count	Unique whales	∑4PCBs[a] (ng/g la)			∑40PCBs (ng/g la)			∑2PBDEs[a] (ng/g la)			*p,p'*-DDE (ng/g la)		
		Total (n)/ high-mass (n)		total samples			high-mass samples			total samples			total samples		
				mean[b]	95% CI		mean[b]	95% CI		mean[b]	95% CI		mean[b]	95% CI	
Year	2010	23/17	18/14	17.0	12.0	24.0	45.2	26.3	77.9	5.7	4.5	7.3	34.9	24.1	50.7
	2011	47/15	38/15	15.6	12.0	20.1	26.6	15.6	45.4	7.1	5.9	8.5	33.1	24.9	44.1
	2012	39/11	24/10	17.1	12.8	22.7	48.9	26.8	89.5	7.1	5.8	8.6	32.6	23.8	44.8
	2013	31/13	24/10	13.6	9.9	18.5	59.5	32.9	107.6	7.0	5.6	8.7	26.6	18.9	37.3
Season[c]	Spring	33/12	23/9	20.3	14.9	27.5	73.9	39.6	137.7	8.3	6.7	10.3	41.0	29.4	57.2
	Summer	45/19	32/15	16.1	12.2	21.1	32.0	18.3	56.0	6.8	5.6	8.2	33.6	24.8	45.4
	Fall	62/25	33/18	13.3	10.4	17.0	36.3	22.6	58.1	6.1	5.1	7.2	26.5	20.1	34.8
Pod	J	67/28	19/15	17.4	12.8	23.7	48.1	27.3	84.6	8.0	6.5	9.8	24.6	17.2	35.2
	K	32/13	13/7	16.1	10.9	23.7	33.2	15.0	73.4	6.3	4.8	8.1	39.4	25.3	61.4
	L	41/15	22/12	14.1	10.4	19.1	38.0	21.2	68.1	6.1	5.0	7.5	35.8	25.3	50.5
Age-sex class	Juvenile	22/5	12/3	17.2	11.7	25.1	59.0	23.2	149.8	7.9	6.2	10.1	36.9	23.8	57.4
with parity	Female, nulliparous	15/7	4/4	15.0	8.7	26.0	45.3	20.1	102.2	9.1	6.6	1.3	22.7	11.6	44.4
	Female, parous	37/17	17/12	6.1	4.5	8.3	9.8	6.0	16.0	3.7	3.1	4.5	8.7	6.0	12.5
	Female, 40+	25/11	7/6	23.4	15.2	36.1	53.9	27.9	104.0	8.3	6.4	10.7	54.9	32.4	92.9
	Adult male	41/16	16/9	20.7	15.1	28.3	61.8	36.0	106.0	7.5	6.1	9.0	47.5	32.7	68.9
Reproductive	Preg (conf)	8/4	5/3	5.1	2.5	10.3	10.0	2.6	37.4	3.2	1.9	5.4	7.9	3.7	16.8
status[d]	Preg (poss)	14/5	6/3	5.9	2.9	12.0	13.7	2.3	81.9	4.7	2.7	7.9	7.9	3.7	16.7
(Females,	Lact (<1 yr)	4/4	4/4	4.8	2.2	10.4	12.1	3.9	37.3	2.5	1.4	4.4	6.5	3.1	13.8
age 10-40)	Lact (1-2 yrs)	3/3	3/3	2.3	1.0	5.3	6.3	1.8	21.9	1.8	1.0	3.5	3.6	1.6	8.1
	Neither	24/8	12/6	7.8	5.5	11.3	11.7	4.7	29.2	5.2	3.9	6.7	12.2	8.2	18.4

a: ∑4PCBs = PCB138, PCB153, PCB180, PCB187; ∑2PBDEs = PBDE47, PBDE100; b: All geometric means adjusted for Julian day and age-sex class when not a main effect, and repeat samples from individual whale; c: Julian day did not improve the fit of the model and was not included; d: also adjusted for number of calves, 1 whale changed categories over 4-year study; L103 (born 2003)

Table 1. (cont) Profile of toxicant levels in scat samples by year, season, pod, age-sex class, reproductive status, number of calves, and birth order: total samples (combined dataset), n=140 samples on 54 unique individuals; high-mass samples, n=56 samples on 34 individuals

		Sample count	Unique whales	∑4PCBs[a] (ng/g la)			∑40PCBs (ng/g la)			∑2PBDEs[a] (ng/g la)			*p,p'*-DDE (ng/g la)		
				total samples			high-mass samples			total samples			total samples		
		Total (n)/ high-mass (n)		mean[b]	95% CI		mean[b]	95% CI		mean[b]	mean[a]		mean[b]	95% CI	
Number of	0	15/7	4/4	15.2	9.1	25.4	46.0	26.0	81.7	9.1	6.1	13.7	23.3	11.7	46.5
calves	1	11/5	6/3	10.6	6.3	18.0	16.6	8.6	32.0	4.4	2.9	6.6	16.8	9.1	31.1
(Females,	2	8/3	4/2	6.9	3.8	12.7	21.1	9.0	49.1	4.6	2.9	7.4	7.0	3.4	14.5
age 10-40)	3	7/4	3/3	3.7	1.9	7.1	6.5	3.1	13.6	2.7	1.6	4.5	4.2	1.8	9.6
	4	4/1	2/1	4.9	2.0	11.5	5.3	1.2	22.9	4.1	2.1	8.0	11.2	3.8	32.8
	5	6/3	2/2	5.9	2.7	12.7	4.6	1.9	11.2	3.3	1.8	6.0	8.8	3.2	24.0
	6	2/1	1/1	3.4	1.0	11.2	6.6	1.5	29.4	3.6	1.4	9.0	6.1	1.4	26.7
Birth order	1	9/2	5/1	32.2	22.3	46.3	199.5	.	.	9.5	6.4	14.2	90.6	48.5	169.0
(Juveniles,	2	3/0	2/0	19.2	9.2	40.3	.	.	.	10.8	6.9	16.9	37.8	12.9	110.3
age <10)	3	3/1	1/1	15.1	8.4	27.3	65.9	.	.	7.4	3.6	15.2	35.1	12.3	100.1
	4	7/2	4/1	7.6	4.5	12.6	24.2	.	.	6.0	4.0	8.9	11.6	5.4	24.9

a: ∑4PCBs = PCB138, PCB153, PCB180, PCB187; ∑2PBDEs = PBDE47, PBDE100; b: All geometric means adjusted for Julian day and age-sex class when not a main effect, and repeat samples from individual whale

Table 2. The estimated rate of change of the conditional mean of toxicant measures [tPOP, ∑4PCBs, ∑40PCBs, ∑2PBDEs, and *p,p'*-DDE (ng/g la)] with respect to prey availability (FR Chinook) when T3 is fixed at mean value and age-sex class is fixed as adult male

Toxicant measures (ng/g la)	n samples	estimated slope	standard error	95% CI
∑4PCBs	133	-0.102	0.049	-0.199, -0.005
∑40PCBs	53	-0.163	0.106	-0.376, 0.050
∑2PBDEs	133	-0.088	0.033	-0.153, -0.022
***p,p'*-DDE**	133	-0.087	0.046	-0.158, -0.016
tPOP	133	-0.107	0.045	-0.196, -0.017

Figure 1. Estimated rate of change of conditional mean of tPOP (ng/g la) with respect to prey availability (FR Chinook); line represents linear trend for adult males when T3 is fixed at mean value

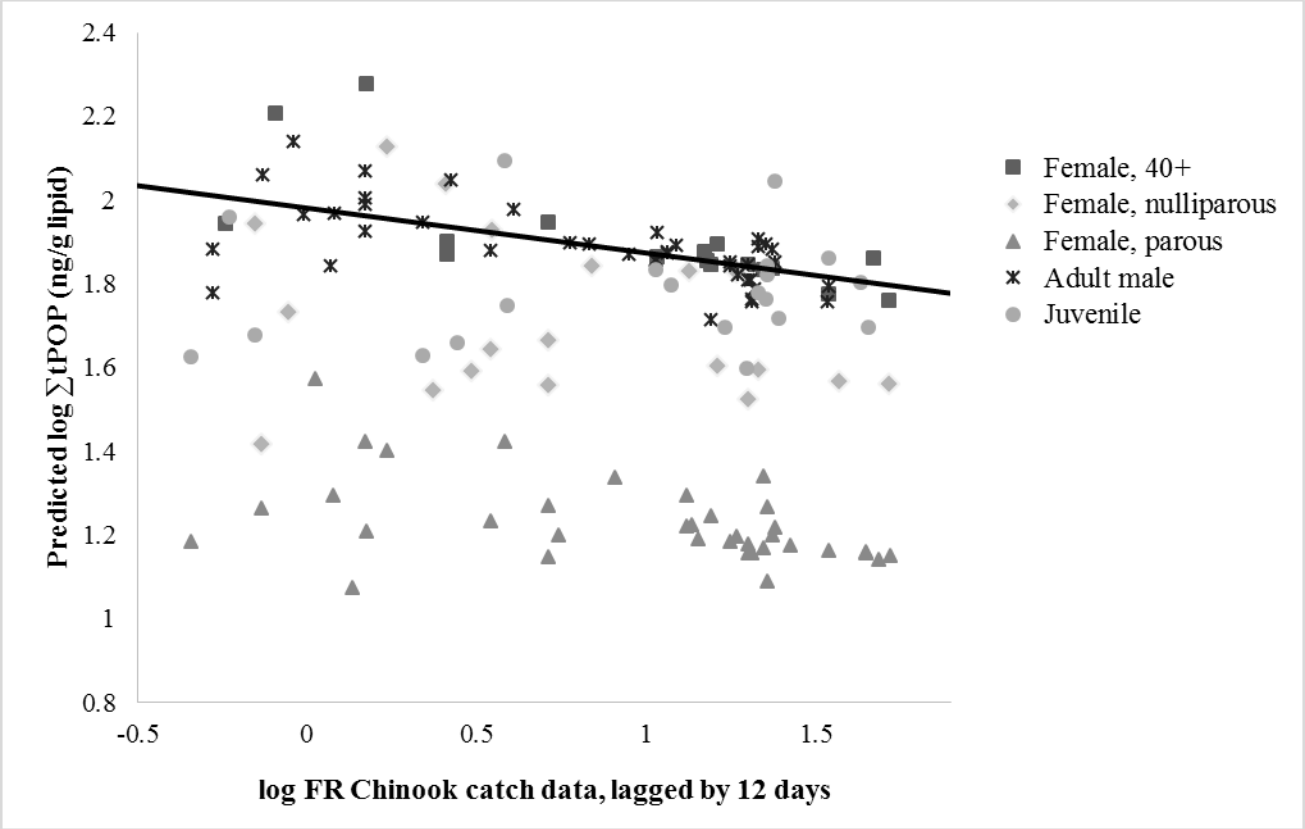


Figure 2. Rotated Factor Solution from Principal Components Analysis for PCB congeners measured in scat samples of Southern Resident killer whales; n=56 samples. Number of chlorines: ○ = 3, □ = 4, Δ = 5, ● = 6, ■ = 7. Solid line circle denotes Persistent PCB structural groups 1, 2, and 5. Dashed circles denotes Readily Metabolized PCB structural groups 3 and 4.

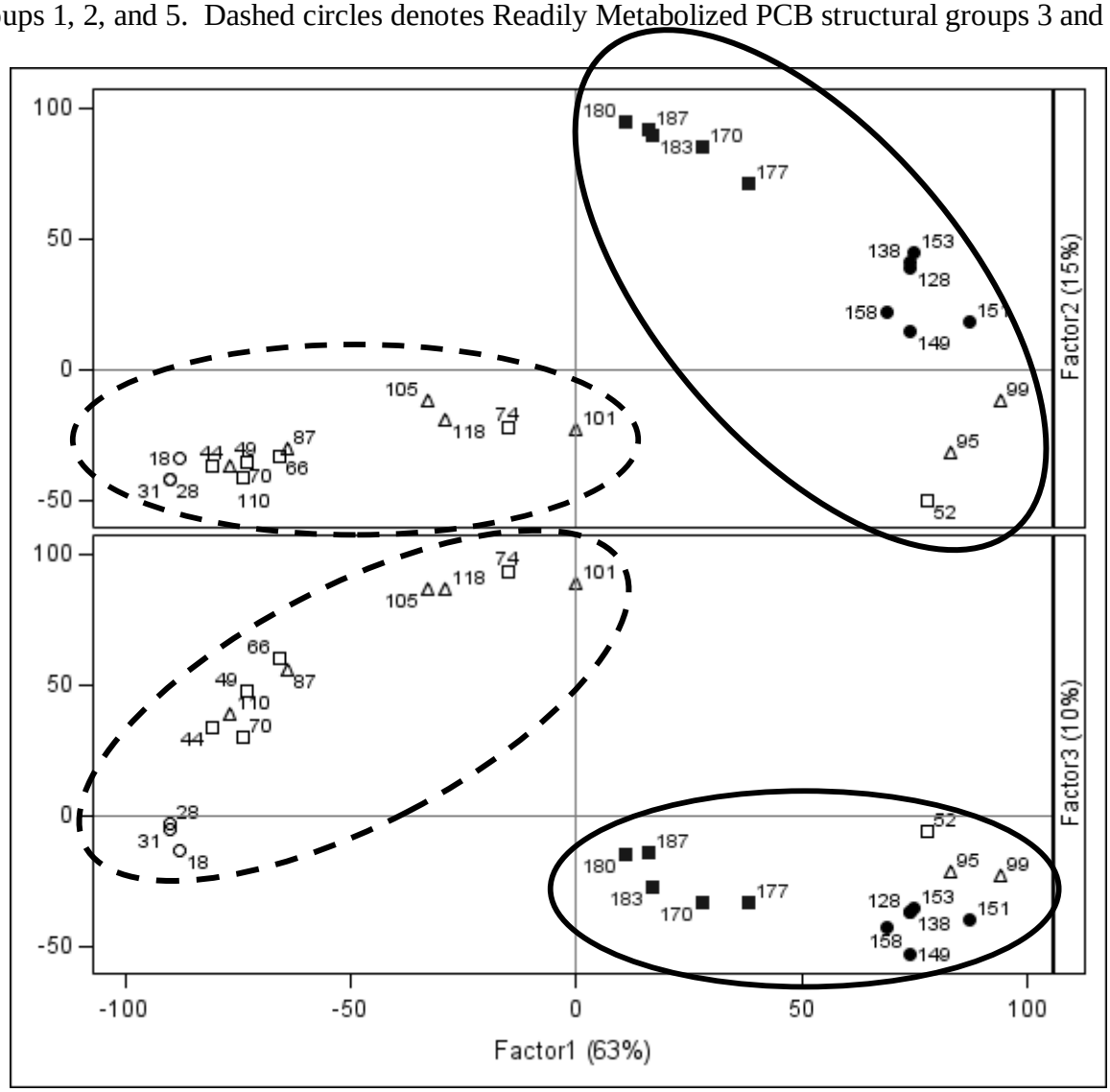


Table 3. The estimated rate of change of the conditional mean of PCA Factors 1, 2, and 3 with respect to FR Chinook for adult males when year is fixed

	estimated slope	**95% confidence interval**
Factor 1	-0.5523	-1.0610, -0.0436
Factor 2	0.2316	-0.6786, 1.0818
Factor 3	0.1074	-0.5798, 0.7946

Figure 3. Years of accumulation of tPOPs (ng/g la) measured in scat by age-sex class; n=140 samples

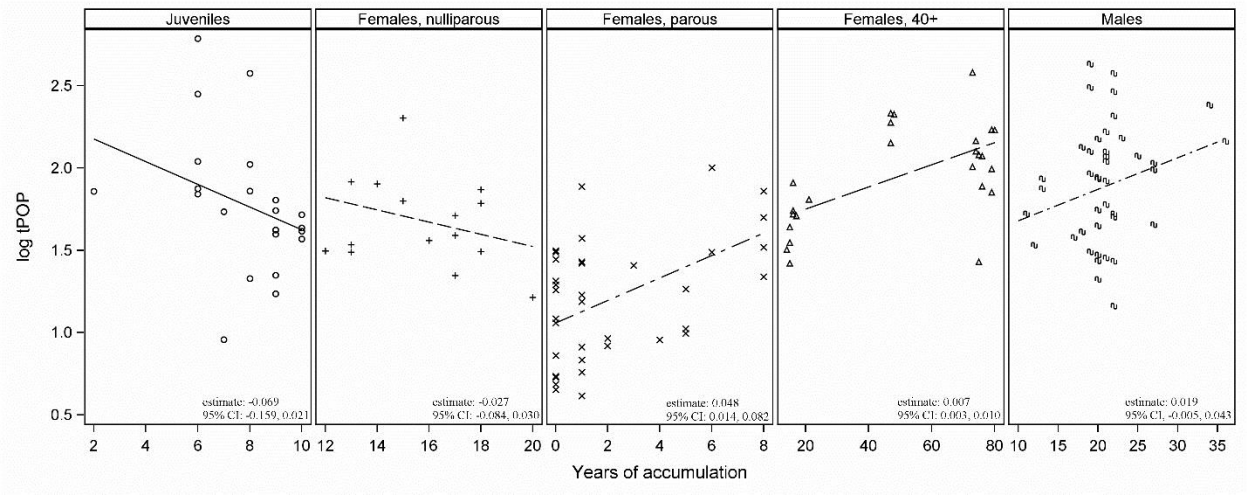


Figure 4. PCB structural group by reproductive status in reproductive-age females; n=24 total scat samples from 16 unique whales

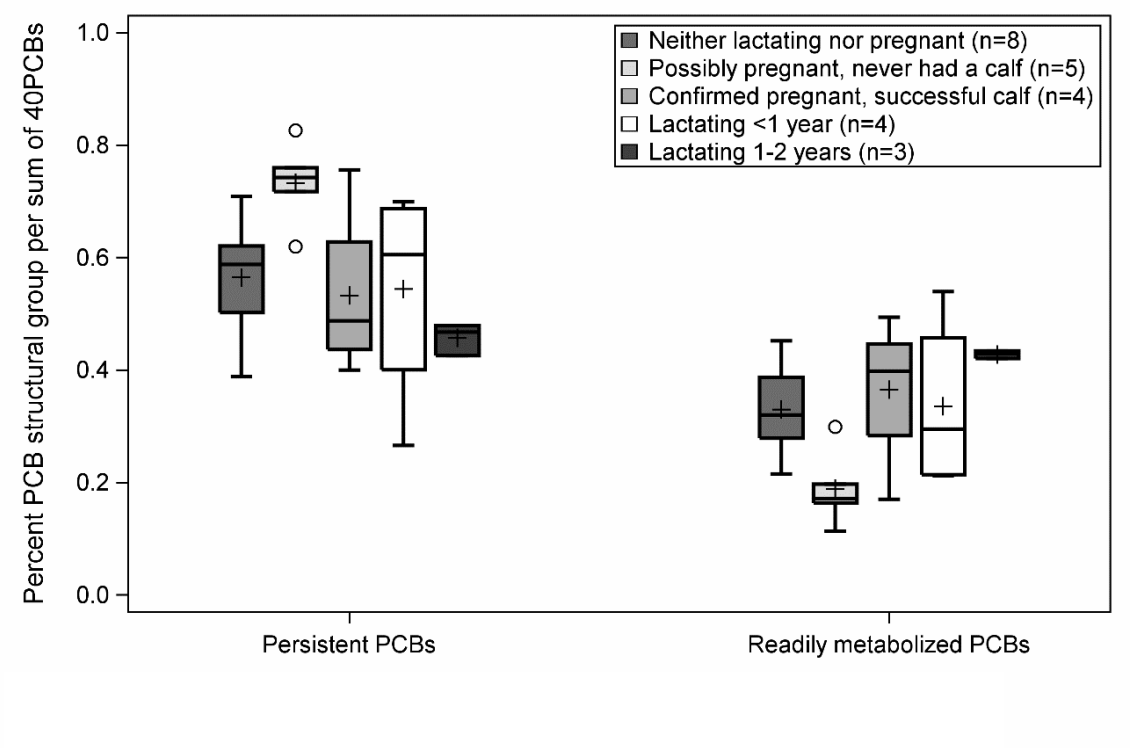
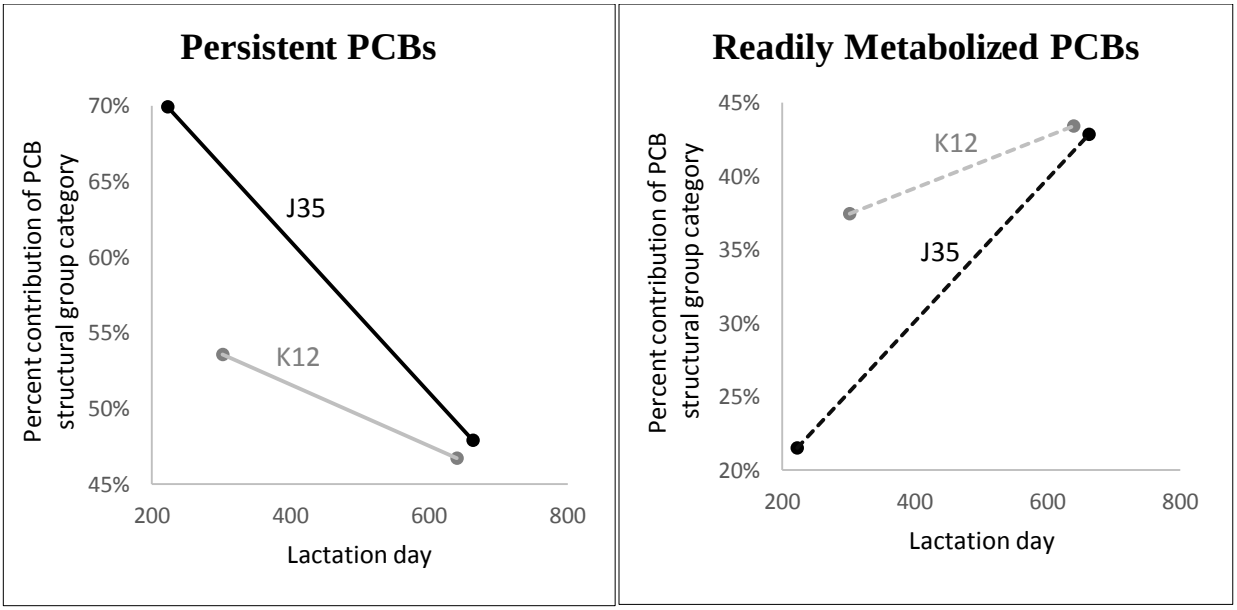
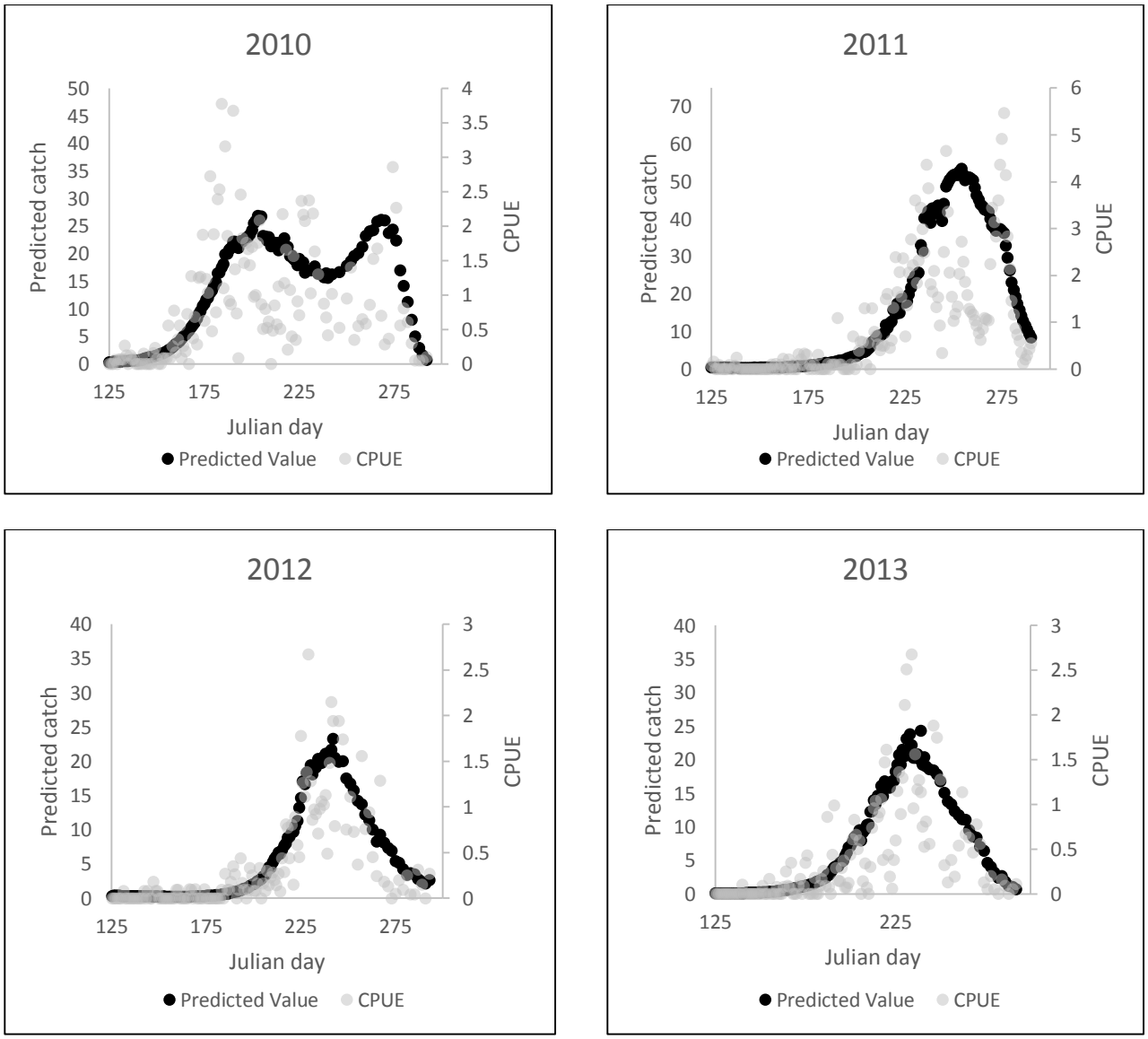


Figure 5. PCB structural group by days of lactation for repeat scat samples collected from two different lactating whales, J35 and K12



SUPPLEMENTAL TABLES AND FIGURES

SFigure 1. Albion test fishery smoothed Fraser River Chinook catch data (Predicted catch) by year, estimated using a general linear model with catch at the response variable (negative binomial), Julian day as the explanatory variable, and effort as the offset; CPUE = Catch per unit effort



STable 1. Two types of fecal samples from killer whales analyzed, high mass unprocessed samples and freeze-dried samples

	High mass unprocessed samples	**Freeze-dried samples**	**TOTAL**
Total samples	70	126	157*
Samples with genotype match**	**56**	124	**140**
Number of unique whales	34	52	54

*39 samples were analyzed by both laboratories

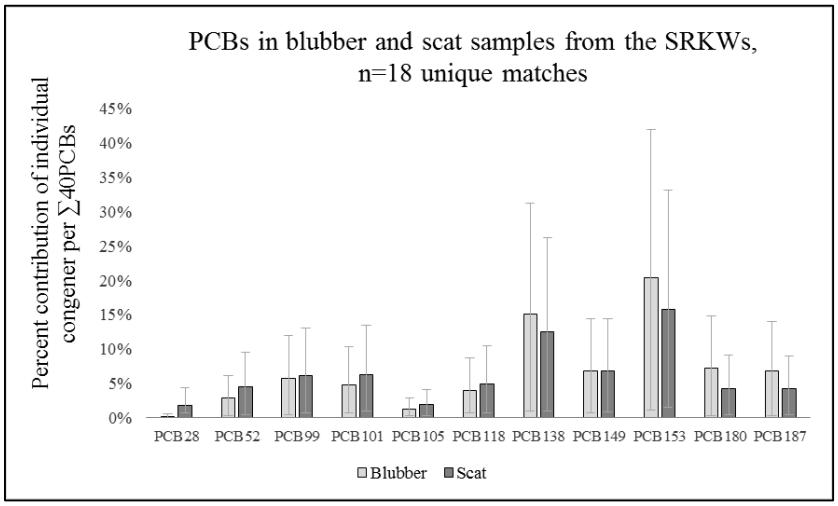
**Samples in bold were used for analyses

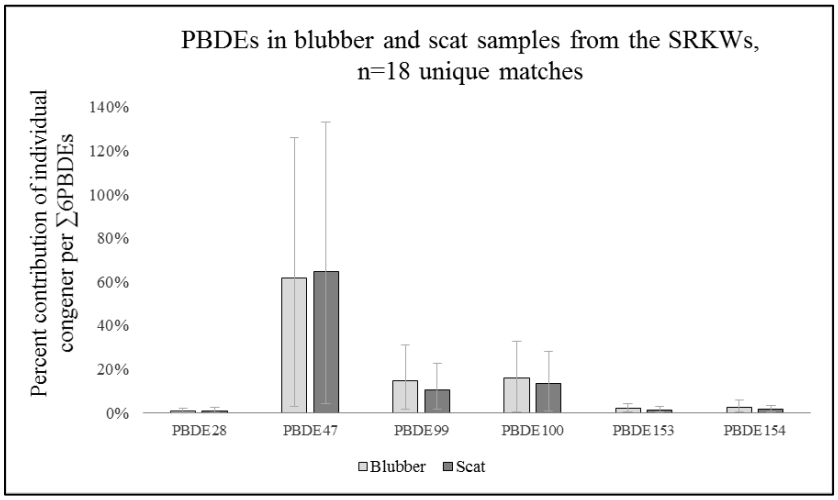
STable 2. A comparison of toxicant measures in low-mass freeze-dried scat samples, blubber biopsy samples, and high-mass unprocessed scat samples; comparisons with blubber include adult whales only

	Dried scat vs blubber,[a] n=22 unique whale matches				Dried scat vs unprocessed scat,[b] n=39 unique samples matches					Unprocessed scat vs blubber,[a] n=16 unique whale matches			
	Est	**SE**	**t-value**	**p-value**	**Est**	**SE**	**t-value**	**p-value**		**Est**	**SE**	**t-value**	**p-value**
***p,p'*-DDE**	1.018	0.190	5.37	<0.0001	0.823	0.134	6.14	<0.0001	***p,p'*-DDE**	1.018	0.190	5.37	<0.0001
∑4PCBs	0.896	0.181	4.97	<0.0001	0.760	0.140	5.44	<0.0001	**∑40PCBs**	0.886	0.203	4.36	0.0007
∑2PBDEs	0.477	0.160	2.99	0.0072	0.512	0.126	4.05	0.0002	**∑11PBDEs**	0.790	0.260	3.04	0.0089
HCB[c]	0.317	0.378	0.84	0.4114	0.610	0.448	1.36	0.1823	**HCB**	1.369	0.782	1.75	0.1018

a: Mixed effect model, adjusted for repeat sampling on individual whales (1-8 scat samples per blubber sample); b: Linear regression model;
c: hexachlorobenzene, originally considered for inclusion in the scat analyses, but not significantly correlated with blubber biopsy values (Lundin et al. in review)

SFigure 2. A comparison of toxicant measures of PCBs and PBDEs in blubber biopsy and scat samples, by congener; n=18 unique matches





STable 3. Descriptive data of killer whale fecal samples analyzed for toxicant analysis by season and year

		High-mass unprocessed samples		Total samples	
		Number of Samples (n=56)	**Number of unique whales (n=34)**	**Number of Samples (n=140)**	**Number of unique whales (n=54)**
Year	2010 (sp,s,f)[a]	17 (2,9,6)	14 (2,7,5)	23 (2,11,10)	18 (2,9,9)
	2011 (sp,s,f)	15 (2,5,8)	15 (2,5,8)	47 (9,16,22)	38 (9,15,21)
	2012 (sp,s,f)	11 (3,2,6)	10 (3,1,6)	39 (12,11,16)	24 (7,13,6)
	2013 (sp,s,f)	13 (5,3,5)	10 (5,3,4)	31 (10,7,14)	24 (8,10,6)

a: sp,s,f = spring (sp), before July 15; summer (s), between July 15th and September 1st; fall (f), after September 1st based on FR Chinook timing groups, adjusted for 12 day time lag between sampling site and Albion test fishery

STable 4. Evaluation of predicted tPOP (ng/g la) by prey availability (FR Chinook), adjusted for fecal thyroid level (T3) and age-sex class with parity; n=133

	Coefficient	**SE**	**t-value**	**p-value**
Intercept	2.7909	0.5067	5.51	<.0001
FR Chinook	-1.0835	0.43	-2.52	0.013
Juvenile	1.733	1.1318	1.53	0.1283
Female, nulliparous	0.1898	0.6049	0.31	0.7542
Female, parous	0.7895	0.6863	1.15	0.2523
Female, 40+	0.279	0.6739	0.41	0.6796
Adult male	0	.	.	.
T3	-0.3678	0.2305	-1.6	0.1132
T3 x FR Chinook	0.4446	0.1922	2.31	0.0224
T3 x Juvenile	-0.7773	0.4821	-1.61	0.1095
T3 x Female, nulliparous	-0.09063	0.2753	-0.33	0.7426
T3 x Female, parous	-0.4697	0.3124	-1.5	0.1354
T3 x Female, 40+	-0.4246	0.3113	-1.36	0.1752
T3 x Adult male	0	.	.	.

STable 5. Percent POP congener, metabolite, or group by class of compound

POP by class of compound (mean % by class)	Mean	Minimum	Maximum	Standard deviation
PCB Group1	27%	10%	36%	6%
PCB Group2	24%	10%	32%	5%
PCB Group3	13%	4%	29%	7%
PCB Group4	12%	4%	25%	6%
PCB Group5	14%	7%	22%	4%
PBDE28	1%	0%	6%	1%
PBDE47	64%	34%	79%	9%
PBDE49	6%	1%	24%	5%
PBDE66	1%	0%	5%	1%
PBDE85	0%	0%	0%	0%
PBDE99	10%	3%	16%	3%
PBDE100	13%	3%	19%	3%
PBDE153	1%	0%	11%	2%
PBDE154	2%	0%	4%	1%
PBDE155	2%	0%	10%	2%
PBDE183	0%	0%	0%	0%
o,p'-DDD	1%	0%	6%	1%
o,p'-DDE	1%	0%	4%	1%
o,p'-DDT	1%	0%	3%	1%
p,p'-DDD	4%	1%	11%	2%
p,p'-DDE	93%	85%	100%	4%
p,p'-DDT	1%	0%	3%	1%

NOTE: PCBs with a total percent contribution of <0.5% were not included: PCB17, 33, 82, 156, 191, 194, 195, 205, 206, 208, and 209

STable 6. Rotated Factor Solution from Principal Components Analysis for PCB congeners organized by PCB structural group; n=56 samples

PCB structural group category	PCB structural group	PCBs	Factor1	Factor2	Factor3
		Variance accounted for	63.1	14.6	10.0
Persistent PCBs	1	PCB153	75	45	-35
	1	PCB158	69	22	-43
	1	PCB180	11	95	-15
	1	PCB183	17	90	-27
	1	PCB187	16	92	-14
	2	PCB128	74	39	-37
	2	PCB138	74	41	-37
	2	PCB170	28	85	-33
	2	PCB177	38	71	-33
	2	PCB99	94	-12	-23
	5	PCB149	74	15	-53
	5	PCB151	87	18	-40
	5	PCB95	83	-32	-21
Readily Metabolized PCBs	3	PCB105	-33	-12	87
	3	PCB118	-29	-19	87
	3	PCB28	-90	-42	-3
	3	PCB31	-90	-42	-5
	3	PCB66	-66	-33	60
	3	PCB70	-74	-41	30
	3	PCB74	-15	-22	93
	4	PCB101	0	-23	89
	4	PCB110	-77	-37	39
	4	PCB18	-88	-34	-13
	4	PCB44	-81	-37	34
	4	PCB49	-73	-35	48
	4	PCB52	78	-50	-6
	4	PCB87	-64	-30	56

NOTE: Structural groups: Group 1 comprised congeners without any vicinal H atoms; Group 2 comprised congeners with vicinal H atoms in ortho and meta positions and ≥2 ortho-chlorine atoms; Group 3 comprised congeners with vicinal H atoms in ortho and meta positions and 1 ortho-chlorine atom; Group 4 comprised congeners with vicinal H atoms in meta and para position and ≤2 ortho-chlorine atoms; Group 5 comprised congeners with vicinal H atoms in meta and para position and ≥3 ortho-chlorine atoms.

PCB171 and 199 accounted for <70% of the variance and were excluded from the final analysis.

STable 7. Principal Components Analysis for PBDE congeners, Rotated Factor Solution

PBDEs	**Factor1**	**Factor2**
Variance accounted for	**68.2**	**20.5**
PBDE28	96	6
PBDE47	-80	-56
PBDE49	89	1
PBDE66	97	3
PBDE99	-47	81
PBDE154	46	73
PBDE155	98	1

NOTE: PBDE100 and 153 accounted for <70% of the variance and were excluded from the final analysis

STable 8. Principal Components Analysis for DDT metabolites, Rotated Factor Solution

DDTs	**Factor1**	**Factor2**	**Factor3**
Variance accounted for	**46.0**	**27.7**	**17.0**
opDDD	83	-7	-41
opDDE	90	12	12
opDDT	-5	98	3
ppDDD	63	15	-72
ppDDE	0	-12	94
ppDDT	17	92	-27

STable 9. Evaluation of PCB factor loading 1 by prey availability (FR Chinook), adjusted for age-sex class with parity and year; n=56 samples

	coefficient	standard error	t-value	p-value
Intercept	0.7194	0.2738	2.63	0.0119
FR Chinook	**-0.5523**	**0.2522**	**-2.19**	**0.0340**
Juvenile	-0.4560	0.5930	-0.77	0.4461
Female, nulliparous	-1.2002	0.4428	-2.71	0.0096
Female, parous	-1.9454	0.5248	-3.71	0.0006
Female, 40+	-0.3728	1.3418	-0.28	0.7825
Adult male	.	.	.	.
FR Chinook x Juvenile	0.4996	0.5037	0.99	0.3268
FR Chinook x Female, nulliparous	0.7244	0.4162	1.74	0.0890
FR Chinook x Female, parous	0.1812	0.4310	0.42	0.6763
FR Chinook x Female, 40+	0.5005	0.9913	0.50	0.6162
FR Chinook x Adult male	.	.	.	.
Year, 2010	0.7334	0.2507	2.93	0.0055
Year, 2011	0.3120	0.2554	1.22	0.2286
Year, 2012	0.3652	0.2181	1.67	0.1013
Year, 2013	.	.	.	.

STable 10. The estimated rate of change of the conditional mean of PCA factors for PBDE and *p,p'*-DDE with respect to prey availability (FR Chinook) for adult males when year is fixed; n=56 samples

		estimated slope	95% confidence interval
PBDE	Factor1	0.0496	-0.2131, 0.3123
	Factor2	-0.2627	-1.0609, 0.5356
***p,p'*-DDE**	Factor1	-0.1112	-0.9191, 0.6967
	Factor2	0.05367	-0.4881, 1.5614
	Factor3	0.0844	-0.8108, 0.9795

STable 11. Mixed effects regression model for tPOP with respect to years of accumulation by age-sex class.

	coefficient	standard error	t-value	p-value
Intercept	1.4979	0.2606	5.75	<.0001
Years of accumulation (Years)	0.0191	0.0122	1.57	0.1192
Juvenile	0.8137	0.4523	1.80	0.0743
Female, 40+	0.1165	0.2960	0.39	0.6945
Female, nulliparous	0.5909	0.5295	1.12	0.2665
Female, parous	-0.4044	0.2735	-1.48	0.1417
Adult male	0.0000	.	.	.
Years * Juvenile	-0.0884	0.0471	-1.88	0.0625
Years * Female, 40+	-0.0123	0.0125	-0.99	0.3258
Years * Female, nulliparous	-0.0460	0.0315	-1.46	0.1467
Years * Female, parous	0.0290	0.0210	1.38	0.17
Years * Adult male	0.0000	.	.	.

STable 12. The estimated rate of change of the conditional mean of toxicant measures [tPOP, ∑4PCBs, ∑40PCBs, ∑2PBDEs, and *p,p'*-DDE (ng/g la)] with respect to years of accumulation when age-sex class is fixed

		estimated slope	**standard error**	**95% confidence interval**	
tPOPs	Juvenile	-0.069	0.045	-0.159	0.021
n=140	Female, 40+	0.007	0.002	0.003	0.010
	Female, nulliparous	-0.027	0.029	-0.084	0.030
	Female, parous	0.048	0.017	0.014	0.082
	Adult male	0.019	0.012	-0.005	0.043
∑4PCBs	Juvenile	-0.069	0.036	-0.141	0.003
n=140	Female, 40+	0.009	0.003	0.003	0.015
	Female, nulliparous	-0.054	0.049	-0.151	0.042
	Female, parous	0.036	0.017	0.003	0.069
	Adult male	0.014	0.013	-0.012	0.039
∑40PCBs	Juvenile	0.026	0.151	-0.276	0.329
n=56	Female, 40+	0.009	0.005	-0.002	0.019
	Female, nulliparous	-0.034	0.088	-0.212	0.143
	Female, parous	0.081	0.046	-0.013	0.174
	Adult male	-0.016	0.032	-0.080	0.048
∑2PBDEs	Juvenile	-0.026	0.021	-0.068	0.015
n=140	Female, 40+	0.004	0.003	-0.002	0.010
	Female, nulliparous	-0.045	0.026	-0.097	0.007
	Female, parous	0.023	0.011	0.002	0.045
	Adult male	0.003	0.009	-0.014	0.020
***p,p'*-DDE**	Juvenile	-0.073	0.041	-0.155	0.008
n=140	Female, 40+	0.006	0.004	-0.003	0.014
	Female, nulliparous	-0.029	0.052	-0.132	0.074
	Female, parous	0.076	0.002	0.072	0.080
	Adult male	0.023	0.013	-0.004	0.050

STable 13. Mean percent contribution of PCB structural group category per ∑40PCBs and marginal geometric mean of ∑40PCBs (ng/g la) by age-sex class with parity; n=56 total samples representing 34 unique whales

	Persistent PCBs	**Readily metabolized PCBs**
	(mean, 95% confidence interval)	
Juvenile	66% (53-78)	25% (14-37)
Female, nulliparous	67% (56-78)	24% (14-34)
Female, parous	53% (46-60)	36% (30-42)
Female, 40+	69% (60-78)	22% (14-30)
Adult male	71% (64-78)	21% (14-28)

STable 14. Mean percent contribution of PCB structural group per ∑40PCBs and marginal geometric mean cumulative measure of 40 PCBs (ng/g la) by reproductive status in reproductive-age females; n=24 total samples representing 16 unique whales

	Persistent PCBs (% of ∑40PCBs)	**Readily metabolized PCBs (% of ∑40PCBs)**
	(mean, 95% confidence interval)	
Neither	56% (47-65)	33% (25-42)
Preg (poss)	73% (61-85)	19% (8-31)
Preg (conf)	54% (42-67)	36% (25-47)
Lact, <1yr	52% (41-63)	35% (25-45)
Lact, 1-2yr	43% (30-56)	45% (33-57)

STable 15. Evaluation of toxicant level by number of calves (continuous variable; range from 0-6 calves), crude mixed effects models, reproductive-age females only (age 10-40); high-mass unprocessed samples, n=24 samples on 16 unique whales; low-mass samples, n=52 samples of 21 unique whales

	high-mass samples (n=24)		low-mass samples (n=52)				
	% Persistent PCBs	**% Readily Metabolized PCBs**	**∑40PCBs**	**tPOP**	**∑4PCBs**	**∑2PBDEs**	***p,p'*-DDE**
Coefficient	-0.036	0.033	-0.179	-0.1074	-0.106	-0.073	-0.103
SE	0.014	0.012	0.034	0.0358	0.030	0.026	0.041
p-value	0.029	0.013	<0.001	0.0042	<0.001	0.007	0.015

STable 16. Evaluation of toxicant level by birth order (continuous variable; 1st - 4th calf), crude mixed effects models, juvenile whales only (age <10 years); high-mass unprocessed samples, n=5 samples on 3 unique whales; low-mass samples, n=22 samples of 12 unique whales

	high-mass samples (n=5)	low-mass samples (n=22)			
	∑40PCBs	**tPOP**	**∑4PCBs**	**∑2PBDEs**	***p,p'*-DDE**
Coefficient	-0.3494	-0.2454	-0.2050	-0.0707	-0.2743
SE	0.1182	0.0394	0.0256	0.0200	0.0497
p-value	0.0598	<0.0001	<0.0001	0.033	<0.0001

STable 17. Evaluation of percent PCB structural group by days of lactation for paired samples collected from two different lactating whales, J35 and K12

Whale	**Lactation days**	**% Persistent PCBs**	**% Readily Metabolized PCBs**	**∑40PCBs (ng/g la)**	**∑11PBDEs (ng/g la)**	**∑6DDTs (ng/g la)**
J35	223	70%	22%	33.4	7.6	11.4
J35	663	48%	43%	5.6	1.2	2.3
K12	302	54%	37%	3.6	0.7	3.0
K12	640	47%	43%	3.3	0.6	2.4

Whale	**Lactation days**	**Sum of PCB Group 1 (ng/g la)**	**Sum of PCB Group 2 (ng/g la)**	**Sum of PCB Group 3 (ng/g la)**	**Sum of PCB Group 4 (ng/g la)**	**Sum of PCB Group 5 (ng/g la)**
J35	223	10.9	8.2	3.9	2.9	4.2
J35	663	1.1	1.0	1.3	1.0	0.6
K12	302	0.85	0.71	0.68	0.62	0.36
K12	640	0.68	0.53	0.74	0.63	0.33

STable 18. Linear mixed effect model for relationship between hormone concentrations with age-sex class with parity and FR Chinook, as indicated, as fixed effects

			All samples		Female		Male	
Hormone	**Covariate**	**Toxicant**	**est. slope[a]**	**SE[b]**	**est. slope**	**SE**	**est. slope**	**SE**
T3	age-sex class	∑4PCBs	-0.0015	0.1932				
	with parity	∑2PBDEs	0.0897	0.1214				
	FR Chinook	*p,p'*-DDE	0.0092	0.1870				
T4	age-sex class	∑4PCBs	0.0036	0.0650				
	with parity	∑2PBDEs	-0.0473	0.0458				
	FR Chinook	*p,p'*-DDE	0.0112	0.0660				
T3/T3	age-sex class	∑4PCBs	-0.1390	0.0855				
	with parity	∑2PBDEs	-0.0358	0.0627				
		p,p'-DDE	-0.0807	0.0787				
GC	age-sex class	∑4PCBs	-0.0452	0.0588				
	with parity	∑2PBDEs	-0.0469	0.0406				
		p,p'-DDE	-0.0115	0.0565				
Progesterone	age-sex class	∑4PCBs	-0.0142	0.7982	-0.0514	0.0584	0.2116	0.1323
	with parity	∑2PBDEs	-0.0576	0.1245	**-0.0980[b]**	**0.0362**	0.0761	0.0849
		p,p'-DDE	0.0216	0.7137	-0.0104	0.0593	0.1965	0.1484
Estrogen	age-sex class	∑4PCBs	**-0.2246[c]**	**0.0762**	**-0.2497[c]**	**0.0889**	-0.1329	0.1500
	with parity	∑2PBDEs	**-0.1674[c,d]**	**0.0525**	**-0.2410[c,d]**	**0.0626**	0.0423	0.0943
		p,p'-DDE	-0.1616	0.0840	-0.1555	0.0885	-0.1368	0.1690
Testosterone	age-sex class	∑4PCBs	0.0377	0.0435	-0.0042	0.0536	0.0900	0.0742
	with parity	∑2PBDEs	0.0171	0.0299	-0.0582	0.0376	0.0631	0.0471
		p,p'-DDE	0.0402	0.0344	0.0287	0.0381	0.0747	0.0819

a: estimated slope; b: standard error; c: $p<0.05$; d: $p<0.0024$ (critical value after Bonferroni correction)

APPENDIX A. Deleterious biologic effects associated with scat PCB levels, preliminary analyses

A better understanding of the excretion patterns of these toxicants is essential before applying metrics developed for contaminant measures in blubber to measures in scat samples. As such, this application of toxicant measures in the scat to health metrics should be interpreted with caution until further investigation on the excretion rates and patterns of these toxicants is better established. Increased numbers of matched blubber-scat samples are needed improve the estimate of excretion across a range of toxicant concentrations by age-sex class and prey abundance. Likewise, a comparison of excretion patterns to a less contaminated reference population such as the Northern Resident killer whales, is recommended.

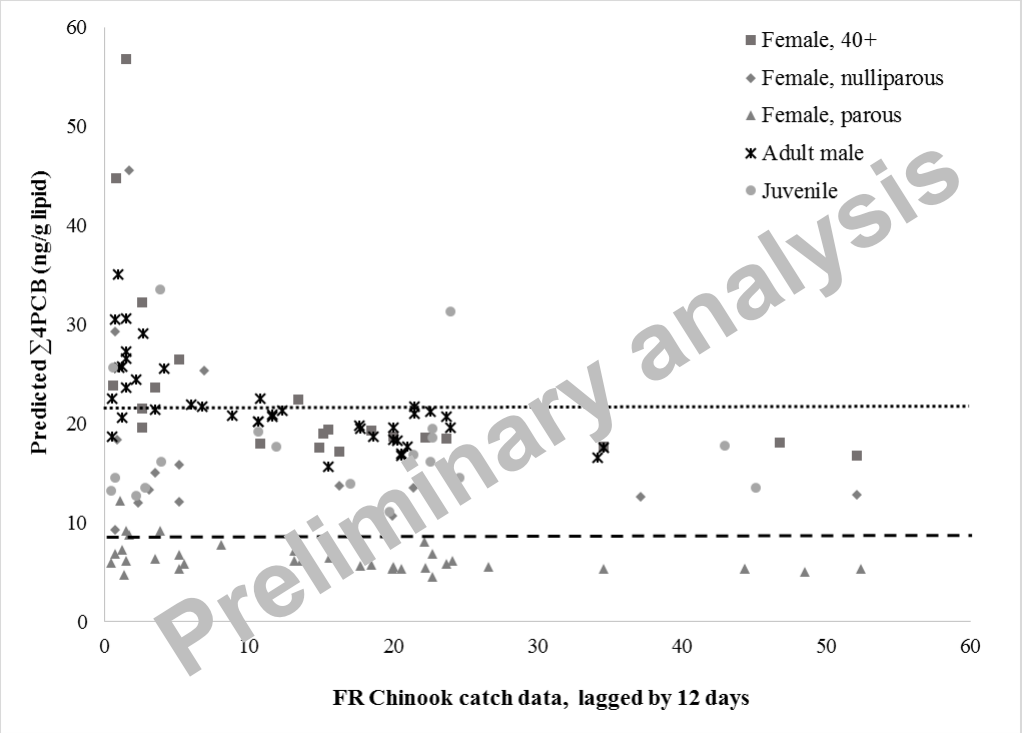
Estimated health effects threshold

A threshold for PCB-related health effects of 17,000 ng/g PCBs lipid weight in aquatic mammals has been extrapolated from experimental studies of immunologic and reproductive effects in seals, otters, and mink (Kannan et al. 2000). Conversion of this health effects threshold for PCBs in blubber to concentration of PCBs in the scat [ng/g lipid adjusted (la)] was achieved by applying the relative amount of ∑40PCBs ng/g la in the scat in relation to the amount per gram lipid in matched blubber samples (median, 0.136%), resulting in 23.1 ng/g lipid (10^{th}-90^{th} percentile of scat to blubber conversion: 5.1-54.4). This estimated median threshold value was applied to the high-mass unprocessed scat samples. Similar to previous findings by Krahn et al. (2009), almost 90% of the samples in the current study demonstrated levels above the estimated median threshold value for PCBs in scat with the exception of samples from parous females, a findings also reported by Krahn et al (2009). Four of 5 juvenile, 6 of 7 nulliparous female, 15 of

16 adult male, and 9 of 11 post-reproductive female samples in the present study were above the estimated median threshold value for PCBs in scat, and all but three (of 17) parous female samples were below the median health effects threshold, likely due to gestation and lactation transfer of toxicants to offspring.

The resulting estimated median threshold value for ∑4PCBs in scat, based on the median ∑4PCBs/∑40PCBs value of 39.1% and median scat to blubber conversion value of 0.136%, was 9.0 ng/g la (10^{th}-90^{th} percentile of ∑4PCBs/∑40PCBs conversion: 6.4-10.6), with an "upper-bound" threshold value of 21.3 ng/g la (10^{th}-90^{th} percentile of ∑4PCBs/∑40PCBs conversion: 15.0-25.0) calculated from the 90^{th} percentile blubber to scat conversion value of 0.320%. These estimated health-effects threshold values for ∑4PCBs were related to the ∑4PCBs ng/g la measure predicted from the prey availability model (Figure 6). With the exception of parous females, Figure A demonstrates the predicted ∑4PCBs ng/g la contaminant level associated with FR Chinook in all samples (n=133) is above the median health effects threshold value in scat of 9.0 ng/g la regardless of prey abundance. The upper-bound threshold value in scat of 21.3 ng/g la demonstrates the whale ∑4PCB ng/g la levels are more consistently above this estimated upper-bound contaminant level determined to elicit an effect at the physiologic level when prey abundance is low. This analysis emphasizes the importance of prey availability for the whales and also highlights how a more refined analysis could provide a metric (e.g., a minimum catch of approximately 10 fish by the Albion test fishery, adjusted for effort) describing what level of prey abundance demonstrates an increased occurrence of toxic exposures.

Figure A. Estimated health effects threshold in scat in relation to predicted ∑4PCBs ng/g la levels by FR Chinook (dashed line designates estimated median health effects threshold in scat; dotted line designates estimated upper-bound health effects threshold in scat)



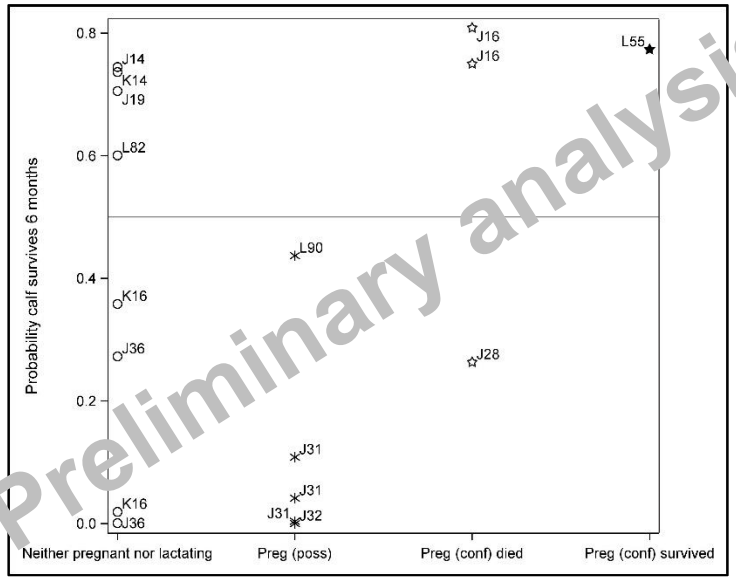
Predictive calf survivorship model

A predictive calf survivorship model in relation to maternal PCB blubber levels was previously developed (Hall et al. 2006) using PCB levels reported in bottlenose dolphins (*Tursiops truncatus*) from Sarasota Bay, Florida (Wells et al. 2005) combined with an estimated dose-response relationship (Reddy et al. 2001) and age-specific fecundity and survival data from a mark-recapture study (Wells and Scott 1990). This predictive model was developed based on ∑22PCBs (PCBs 28, 52, 95, 101, 105, 118, 128, 138, 149, 151, 153, 156, 170, 174, 180, 183, 187, 194, 195, 201, 206, and 209). A similar metric was created using the PCB dataset from the current study with the exception of PCBs 174 and 201, which were not measured. The new

metric of ∑20PCBs was incorporated into the Hall et al. reduced calf survivorship equation (Equation 2), Logit[Probability (survive first 6 months of life)] = 1.77 + (-0.193 x ∑22PCB) (Hall et al. 2006), substituting ∑20PCB for ∑22PCB. Non-lactating reproductive-age females from the high-mass sample dataset (n=17 samples from 12 unique whales) were evaluated.

The resulting probabilities of calf survivorship were less than 0.50 for all nulliparous whales (J31, J32, J36, and L90), including five samples from three whales designated as apparent miscarriages [Preg (poss)] (Figure B). One sample represented a successful pregnancy, L55, with a 0.77 probability of calf survival. Three samples were from two whales with calves that did not survive more than 6 months. The probability of calf survivorship in the sample from J28 (collected in 2012) was 0.27; the neonate from this pregnancy washed ashore Dungeness Spit in January 2013 and was genetically confirmed to be the calf of J28 (NOAA Fisheries 2013). However, J16 was sampled twice in 2010 while pregnant (probability of calf survivorship, 0.81 and 0.75) and her calf, J48, did not survive. Eight samples from six whales classified as neither pregnancy nor lactating suggest that two of six reproductive-age females in this population may have less than 0.50 probability of calf survival. Model predictions should be interpreted with caution due to the small sample size and the application of a model developed for bottlenose dolphins to killer whales. Model uncertainties outlined by Hall et al. (Hall et al. 2006) include: the model did not incorporate density dependence, multiple system effects (immune function, etc), or season variation. Also, the dose-response relationship was derived from mink (*Mustela vison*), although mink were determined to be an appropriate surrogate species because of their similar reproductive physiology to marine mammals.

Figure B. Predicted probability of calf survival in relation to maternal scat PCB concentration (equation from Hall et al. 2006), n=17 samples from 12 reproductive-age female whales that were not lactating at the time of sampling



CHAPTER 3. Polycyclic aromatic hydrocarbons in caribou, moose, and wolf scat samples from three areas of the Alberta oil sands

ABSTRACT

Impacts of toxic substances from oil production in the Alberta oil sands (AOS), such as polycyclic aromatic hydrocarbons (PAHs), have been widely debated. Studies have been largely restricted to exposures from surface mining in aquatic species. We measured PAHs in Woodland caribou (*Rangifer tarandus caribou*), moose (*Alces americanus*), and Grey wolf (*Canis lupus*) across three areas that varied in magnitude of *in situ* oil production. Our results demonstrate a distinction of PAH level and source profile (petro/pyrogenic) between study areas and species likely related to dietary exposures and bioavailability. Caribou samples indicated pyrogenic sourced PAHs in the study area previously devastated by forest fire. Moose samples from the high oil production area demonstrated PAH ratios indicative of a petrogenic source. Increased PAHs in wolf samples from the same area potentially reflect their prey preferences for moose. These findings emphasize the importance of broadening monitoring and research programs in the AOS.

INTRODUCTION

The Alberta oil sands (AOS) is the third largest proven reserve of oil in the world underlying 142,200 square kilometres (km^2) of land (Alberta 2013). The bitumen (thick, heavy crude oil), which is refined for consumer and industrial use, is mixed with sand and water deep below the earth's surface. Extraction of these oil reserves has required *in situ* drilling technologies; steam assisted gravity drainage (SAG-D) is the predominant form of extraction in the AOS, particularly south of Fort McMurray. Steam mobilizes and separates the bitumen deep below the surface (e.g., 350 meters), and only the bitumen is removed and transported. *In situ* technologies are

advertised by the Canadian Association of Petroleum Producers to have lower environmental impact than surface mining as measured by greenhouse gas emissions, land use, water use, and tailings ponds (CAPP 2014). Although this technology disturbs less land on the surface, it has been shown to have a spatial footprint equivalent to surface mining when considering increased landscape fragmentation due to seismic lines, access roads, pipelines, and well sites (Jordaan et al. 2009). The perceived environmental and public health impact of surface mining activity, north of Fort McMurray, has been the focus of controversy with demands to reduce or halt development in the AOS (Kelly et al. 2009, Timoney and Lee 2009, Gibbins 2010). However, few independent studies are available on the environmental and public health impacts of oil exploration and bitumen extraction using *in situ* methods south of Fort McMurray.

Attention surrounding the AOS stems from the perception that these reserves are integral to North American energy security coupled with local to international campaigns highlighting negative impacts on human, wildlife, and ecological health (Gibbins 2010). Of particular concern are exposures to polycyclic aromatic hydrocarbons (PAHs), a diverse groups of compounds found in bitumen, among other sources such as forest fires and diesel exhaust, known to be carcinogens and mutagens, and rank in the top 10 hazardous substances by the United States Agency for Toxic Substances and Disease Registry (Culp et al. 1998, Baird et al. 2005, Xue and Warshawsky 2005, ATSDR 2013). PAHs are resistant to degradation making it imperative for future planning initiatives to be grounded on an understanding of the impacts of PAH exposure on the maintenance of biodiversity and ecologic processes. The Woodland caribou of northern Alberta are listed as threatened (Alberta Woodland Caribou Recovery Team 2005), yet the extent and routes of PAH exposure and potential long-term impacts on this

population are poorly understood. A better understanding of PAH exposures in large terrestrial mammals is also imperative for the health of tribal and recreational hunters. Sustainable hunting communities in the AOS, particularly First Nations who have retained treaty rights to hunt and fish in their traditional homeland for subsistence (Alberta 2013), depend on the health of this ecosystem to ensure their game is pollutant free. Common big game in Alberta includes moose, caribou, and deer. Moose in particular are the most frequently consumed traditional food among First Nation groups (McLachlan 2014).

Caribou feed largely on lichen, when available (Wasser et al. 2011, Naughton 2012). Lichen can readily absorb airborne contaminants; including the lightweight and volatile 2-4 ring PAHs (Kelly and Gobas 2001, Blasco et al. 2006, Blasco et al. 2007, Belis et al. 2011), potentially exposing caribou to trace contaminants from long-range transport of the compounds. Kelly et al.(Kelly et al. 2009) calculated over 11,000 metric tons of airborne particles, consisting mostly of bitumen, as far as 50km from upgrading facilities. PAHs can persist in environmental compartments, such as lichen, due to their slow growth and longevity (Blasco et al. 2006). As such, caribou may be exposed to PAHs from land disturbance or forest fire for a prolonged period after the event. Moose feed largely on riparian vegetation, particularly Red Oster Dogwood and various willow species that depend upon moist soils that may facilitate absorption of PAHs through ground water (Naughton 2012). Although the uptake of PAH compounds varies by properties of the plant, the pollutants, and environmental conditions (Migaszewski et al. 2002, Belis et al. 2011), water and sediment is a well-established contamination source of PAHs in the AOS (Headley et al. 2001, Akre et al. 2004, Wayland et al. 2008, Hall et al. 2012, Kurek et al. 2013). PAH exposure may also occur through atmospheric transport and deposition

on the woody vegetation. Exposure to PAHs in carnivores could occur through ingestion of PAH-laden prey. An evaluation of prey hair in wolf scat samples, analyzed as part of this same ongoing work, demonstrated over 90% of the winter diet in wolves consisted of deer, moose, and caribou; 24% of wolf diet was comprised of moose, 11% of caribou, and 65% of deer after adjusting for biomass (Wasser et al. 2011). Similar findings have been reported from a separate study in Northeastern Alberta, including sites that overlap our study area (Latham et al. 2011).

Our study measured polycyclic aromatic hydrocarbons in scat (fecal) samples collected over a 2500km^2 area with varying degrees of *in situ* oil production activity, providing an unprecedented opportunity to non-invasively evaluate exposures in three terrestrial species with markedly different resource utilizations. We evaluated three wide-ranging terrestrial mammals that inhabit the AOS, Woodland caribou (*Rangifer tarandus caribou*), moose (*Alces americanus*), and Grey wolf (*Canis lupus*). This allowed us to compare exposures across species that have markedly distinct dietary differences, life histories, and associated routes of exposure for a comprehensive look at potential contamination of different environmental resources.

MATERIAL AND METHODS

Study Area

Scat samples from caribou, moose and wolf were collected over a 2500km^2 area south of Fort McMurray, spanning three caribou ranges within the East Side of the Athabasca River caribou herd that differed in oil production activity and fire histories (Figure 1). The extent of oil development and exploration was evaluated using the Alberta Energy Regulation's List of Wells in Alberta, Statistical Report 37 (AER 2014), including all reported wells starting in 1957 up

through and including 2009 in locations defined using the Alberta Township System (ATS) (ATS 2014). The Egg Pony (EP) range, located in meridian 4 including ranges 7-13 and townships 77-83, was the site of the greatest amount of oil development and exploration activity during our winter 2009 study period. The EP area had 4 active wells and 34 wells drilled and cased. The Wiau range, to the immediate south of the EP, located in meridian 4 including ranges 9-12 and townships 76-77, included oil exploration and extraction activity at a lower intensity than the EP, with no active wells and 7 wells drilled and cased. In addition, an extensive fire in 2002 (the House River fire) burned over 238,000 hectares of the range (Alberta 2013) including the entire Wiau sampling area. No other fires on record in the three study areas since 1996 were larger than 20,000 hectares. The Algar range, approximately 160 km to the north of the EP and 70 km west-southwest of Fort McMurray, along the Athabasca River, located in meridian 4 including ranges 13-17 and townships 84-87, had no active or capped wells on record although historical exploration may have occurred, as evidenced by survey lines visible on Google Earth (Google Inc; Mountain View, CA).

The study areas were selected based on established caribou herd ranges; caribou are well-known to have high site fidelity within their range (Stuart-Smith et al. 1997). Likewise, moose have distinct home ranges from 2.2-17.0 km^2, using only portions of this area within a season (Naughton 2012). Most wolf packs in northern Canada have stable home ranges and generally concentrate in areas of high prey density (Latham et al. 2011, Naughton 2012).

Sampling Design

Scat samples were located by trained detection dogs between mid-December of 2008 and mid-March of 2009, as part of the ongoing work on the impacts of AOS development on caribou, moose, and wolf (Wasser et al. 2011). The survey included 36 cells in the EP, four cells in the Wiau, and eight cells in the Algar. DNA was extracted from all scat samples and used to confirm the species, sex, and individual identities (Wasser et al. 2011). Available funding restricted the number of samples that could be analyzed in each species. We aimed to randomly select one fecal sample from 15 genetically unique individuals of each sex per species and area. However, the number of samples analyzed from each sex and species varied across study areas based on sample detections in the field. For example, only one wolf sample was collected in the Algar (not analyzed) and no male caribou or female wolf samples were collected in the Wiau.

Laboratory and Analytical Methods

PAH extraction, purification, and quantification methods were developed and performed at the University of Washington, Seattle using modified procedures from other studies (United States Environmental Protection Agency 1996, Mazéas and Budzinski 2005, Forsberg et al. 2011, Cochran et al. 2012). All samples were spiked with a surrogate standard (Acenapthylene-d10) (Accustandard; New Haven, CT USA) prior to extraction to monitor extraction recovery and for internal standard quantification using isotope dilution methods. All samples were previously lyophilized (Wasser et al. 2011). For caribou and moose samples, 1.0 gram of dry fecal material was saponified (alkaline decomposition of lipids) using 9.0mL of 1M potassium hydroxide:ethanol (80% ethanol:20% Millipore water) then extracted with 10.0mL of hexane. Due to matrix interference and poor recovery of internal standards in the wolf sample, 0.35

grams of dry fecal material was first extracted in 10.0mL of hexane, then saponified as described above. This modification served to saponify only the extract, not other components of the fecal bulk (e.g., prey hair). The final extract solution for all species received 20.0mL of magnesium sulfate and sodium chloride (4:1). The resulting hexane extract was evaporated to approximately 1.0mL, and loaded onto a 500mg Discovery-aminopropyl Solid Phase Extraction cartridge (Supelco; Bellefonte, PA USA). The target compounds were eluted using 12.0mL hexane. The final extract was evaporated to 0.5mL and stored in the dark at -20°C until analysis.

Analysis

All sample extracts and standards were analyzed using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass spectrometer (GCMS). The MS system was operated with election ionization (70 eV) and in select ion monitoring mode. The instrument was fitted with a DB-5MS column (30m length, 0.25µm film thickness, 0.25mm i.d.) (Agilent, Santa Clara, CA). The injection port was operated in splitless mode, fitted with a glass liner, and delivered 2 µL by auto-injection. The carrier gas was helium. Chromatographic separation was achieved using an initial temperature of 50°C with a 4 minute hold, ramping to 100°C at a rate of 10°C per minute, immediately followed by ramping to 145°C at a rate of 5°C per minute, to 150°C at 1°C per minute, then to 250°C at 5°C per minute, with a final ramp to 310°C at 10°C per minute with a 4 minute hold. The total run time per sample was 53 minutes.

Quantification and quality assurance

Concentrations of individual PAH compounds were based on internal standard quantification. A stock solution of 27 parent (unsubstituted) and alkylated (derivatives with one to four methyl-

group substitutions) PAHs was prepared by combining a custom mix of priority pollutant PAHs and individual PAHs [Accustandard (New Haven, CT USA), Chiron (Trondheim, Norway), and Sigma (St. Louis, MO USA)] in hexane. Target compounds included: naphthalene (NPH); 1-methylnaphthalene (MN1); 2-methylnaphthalene (MN2); 1,2-dimethylnaphthalene (DMN2); 1,6-dimethylnaphthalene (DMN); 2,3,5-trimethylnaphthalene (TriMN); 1,4,6,7-tetramethylnaphthalene (TetMN); phenanthrene (PHN); 1-methylphenanthrene (MP1); 1-methylanthracene (MA1); 2-methylphenanthrene (MP2); 3,6-dimethylphenanthrene (DMP); 1,2,5-trimethylphenanthrene (TriMP); 1,2,6,9-tetramethylphenathrene (TetMP); dibenzothiophene (DBT); fluorene (FLO), 1-methylfluorene (FL1); 1,8-dimethylfluorene (DMF); Chrysene/ben[a]anthracene (CHR/b[a]A; co-eluted); acenaphylene (ACY); acenaphthalene (ACE); anthracene (ANT); 2-methylanthracene (MA2); 9-methylanthracene (MA9); 9,10-dimethylanthracene (MA910); 2,3-dimethylanthrcene (MA23); fluoranthene (FLU); and pyrene (PYR). Each analytical batch (by species) included an eight-point standard calibration curve at the beginning and end of each run, a calibration check standard every 20 samples, a laboratory procedure blank for each extraction group, three repeat quantification samples to test instrument accuracy and precision, three repeat extraction samples to test laboratory accuracy and precision, and solvents blanks (hexane). The calibration curve concentrations ranged from 0.1 pg/µL to 0.1 ng/µL. The regression coefficient of the calibration curve slope was >0.999 for all target compounds. The calibration check standard response factors were checked for each series of samples analyzed, and were typically ±10% the expected values. PAH recoveries were calculated by comparing the relation of the surrogate standard signal intensity in the sample to that in the standards (defined to be 100%). Mean percent recovery by species were: 81% (standard deviation (sd); 13) for caribou, 84% (sd; 19) for moose,

and 73% (sd; 16) for wolf. One moose and one caribou sample were excluded from the final analysis due to a recovery greater than 3 standard deviations from the mean. Three target analytes were excluded from all analyses due to co-elution of artifact compounds in the matrix (1,2-dimethylnaphalene,1,8-dimethylfluorene, and acenaphthalene). Any measured responses in the procedure lab blanks were subtracted from the responses measured in samples from the same extraction group. Duplicate sample extractions were typically ±10% the expected values. All sample concentrations are reported as nanogram per gram dry fecal weight (ng/g dw).

Method detection limits (MDLs) were calculated as three times the standard deviation of a 5ppb spike of target analytes in replicate samples with the exception of TetMN which was calculated based on a 20ppb spike. Method detection limits were generally less than 1.0 ng/g dw, except for naphthalene (2.3 ng/g dw), phenanthrene (2.8 ng/g dw), 1,4,6,7-tetramethylnaphthalene (4.0 ng/g dw), and chrysene/benzo[a]pyrene (3.2 ng/g dw). Results reporting less than the method detection limit were replaced with the detection limit divided by the square root of two; non-detections were replaced with zero. Only target compounds that were detected in ≥25% of the samples per species were included in the final analysis.

Statistical Analysis

A number of PAH metrics were selected *a priori* to evaluate differences between study areas per species using general linear model marginal mean estimates. The *a priori* PAH metrics included total (∑)PAHs, a cumulative measure of all naphthalenes, fluorenes, phenanthrenes, and anthracenes (parent and alkylated compounds), as well as flouranthene and pyrene. The metric ∑Petro-PAHs included parent and alkylated compounds of three of the five PAHs commonly

used for identification of petrogenic source PAHs: naphthalenes, fluorenes, and phenanthrenes (Wang and Brown 2009); ∑Parent and ∑Alkyl included just the parent or alkylated compounds, respectively. The Petroleum Index evaluated the quotient of ∑Parent divided by ∑Alkyl. A greater presence of parent compounds is indicative of pyrogenic source PAHs due to cleaving off of carbon-based methyl groups during combustion. Alkylation of a parent compound is a characteristic of a petrogenic source PAH. Sex was considered as a covariate where possible and was retained if significant in the models. An effect was considered significant for $p<0.05$.

A number of PAH ratios have been successfully applied for distinguishing between petrogenic and pyrogenic PAH signatures. PAH ratios are useful for minimizing any concentration effect and are self-normalizing since variations due to instrument operating conditions, final sample volume, or matrix effects are minimized. Many PAH ratios involve pairs with the same molar mass and similar physiochemical properties for likeness when undergoing environmental fate processes. Fluoranthene and pyrene are 4-ring PAH isomers that are commonly used to distinguish between combustion (pyrogenic) and petroleum (petrogenic) sources (fluoranthene/(fluoranthene+pyrene) (F/FP)) (Soclo et al. 2000, Carls 2006, Jiang et al. 2009, Liu et al. 2009, Wang and Brown 2009, Jautzy et al. 2013). Crude oil generally contains more pyrene than fluoranthene, however fluoranthene is more stable than pyrene in high-temperatures such as combustion (Wang and Brown 2009). A ratio of F/FP <0.4 was considered indicative of petrogenic source PAHs. The F/FP ratio is considered a sensitive measure of type of combustion; a ratio of 0.4-0.5 was considered characteristic of fossil fuel combustion and >0.5 an indicator of biomass combustion (pyrogenic). An additional PAH ratio used for this study evaluated the presence of alkylation of phenanthrene, an indication of petrogenic source, in

relation to parent phenanthrene ((1-methylphenanthrene + 2-methylphenanthrene)/ phenanthrene; MP/P). A ratio >1.0 was considered an indicator of a petrogenic PAH source, and <1.0 an indicator of a mixed PAH source. This ratio has been successfully applied to characterize pyrogenic or petrogenic hydrocarbon contaminants in other studies (Notar et al. 2001, Zakaria et al. 2002, Kim et al. 2008). Proportion of samples meeting petrogenic or pyrogenic criteria for both diagnostic ratios were compared between study areas by species using a chi-square test, or Fisher's exact test when appropriate. An effect was considered significant for $p<0.05$. All statistics were performed using SAS 9.3 (Cary, NC).

RESULTS

Final analysis included 24 target analytes from 62 caribou, 69 moose, and 29 wolf fecal samples from three study areas in the AOS (Figure 1; Table 1).

Caribou. Woodland caribou samples demonstrated a statistically higher level of ∑PAHs (ng/g dw) in the fire-impacted Wiau study area (geometric marginal mean (mean), 95% confidence interval (CI); 115.4, 102.2-130.3) compared to the Algar (93.6, 84.7-103.3) and the EP study areas (97.6, 89.4-106.6) (Figure 2). Elevated mean ∑Parent levels of 28.3 ng/g dw were also demonstrated in the Wiau study area (95% CI, 24.8-32.3) compared to a mean of 23.9 ng/g dw in the Algar and 26.9 ng/g dw in EP areas (95% CIs, 21.4-26.6 and 24.4-29.5, respectfully). This increase in PAHs, particularly of parent PAHs, suggest that the source of the increase PAH levels in samples from the Wiau are from a combustion source (e.g., fire or diesel exhaust).

PAHs levels evaluated by sex were not significantly different in the EP or Algar areas. Lack of male caribou samples in the Waiu prohibited testing for sex differences.

The fluoranthene/(fluoranthene+pyrene) (F/FP) ratio in caribou samples from the Wiau area was also characteristic of a pyrogenic PAH source (Figure 3). The F/FP ratio is considered a sensitive measure of type of combustion; for this study a ratio >0.5 was considered an indicator of biomass combustion (pyrogenic). The mean ratio in the Wiau (mean, 95% CI; 0.66, 0.55-0.77) was statistically higher than the mean ratio in the EP area (0.46, 0.38-0.54) (data not shown). The cross-plot of diagnostic ratios (Figure 3) demonstrate that significantly more samples from the Wiau study area (12/14 samples; 86%) had PAH ratios characteristic of a pyrogenic source based on a F/FP ratio >0.5 and a methylphenanthrenes/phenanthrene (MP/P) ratio <1.0, indicative of a non-petrogenic source, compared to the other study areas (EP, 41% of samples; Algar, 52% of samples) ($\chi^2 = 7.61$, $p<0.022$). This pyrogenic PAH source in the Wiau caribou samples likely has a biomass combustion source origin from the House River forest fire that engulfed the area in 2002.

Moose. Moose samples did not demonstrate a difference in ∑PAHs between the three study areas indicating their level of exposure between study areas was similar. However, a statistically higher mean level of ∑Alkyl PAHs was demonstrated in the Algar study area (i.e., control site) (mean, 95% CI; 32.6, 27.1-39.3) compared to the EP (23.1, 19.7-27.1) and Wiau areas (22.5, 18.6-27.2) (Figure 2) indicating a petrogenic exposure in the Algar study area [refer to discussion

section]. This finding was dominated by alkylated naphthalenes (Table 1). Sex was not significant in any model.

The PAH ratio of F/FP demonstrated a predominant petrogenic origin of PAHs (F/FP <0.4) in the moose samples from the EP study area (i.e., area of most intensive oil production) (Figure 3). The mean ratio in the EP (mean, 95% CI; 0.23, 0.16-0.29) was statistically lower than the mean ratio in the Wiau (0.43, 0.35-0.50) and in the Algar (0.44, 0.36-0.51) study areas. Similarly the PAH ratio of MP/P, used to indicate petrogenic source when >1.0, also demonstrated a predominant petrogenic origin of PAHs in the moose samples from the EP study area. The mean MP/P ratio in the EP (mean, 95% CI; 2.22, 1.36-3.60) was statistically higher than the mean ratio in the Wiau (0.48, 0.27-0.86) and in the Algar (0.40, 0.23-0.70) study areas (data not shown). The cross-plot of PAH ratios (Figure 3) demonstrated an apparent cluster of moose samples, with 86% (24/28 samples) of samples from the EP area having PAH ratios indicative of a petrogenic origin (i.e., F/FP <0.4 and a ratio of MP/P >1.0). This proportion of petrogenic PAH samples in the EP area, based on PAH ratios, was significantly greater ($\chi^2 = 28.13$, Fisher's exact test p-value <0.001) than the proportion of petrogenic samples from the Wiau (6/20 samples, 30%) or Algar (3/21 samples, 14%) study areas.

Wolf. The Grey wolf scat samples exhibited a statistically higher mean level of ∑PAHs in the EP area (mean, 95% CI; 109.7, 84.4-142.5) compared to the Wiau area (64.4, 42.6-97.4) (Figure 2). We did not have wolf samples from the Algar study area. The elevated level of PAHs was dominated by alkylated PAHs, indicative of a petrogenic source, demonstrated by an increase in mean levels of ∑Alkyl in the EP (mean, 95% CI; 50.0, 38.4-65.1) compared to the Wiau (26.5,

17.5-40.2). A decreased Petroleum Index, ∑Parent PAH compounds divided ∑Alkyl compounds, in the EP area (mean, 95% CI; 1.75, 1.61-1.89) compared to the Wiau (2.05, 1.81-2.32) also suggest a predominance of alkylated PAHs in this area (data not shown; $p<0.05$). Lack of female wolf samples in the Wiau prohibited testing for sex differences.

The PAH ratios suggested a combination of petrogenic and pyrogenic exposure sources in the wolf samples. The ratio of F/FP was significantly lower in the EP study area (mean, 95% CI; 0.31, 0.19-0.43) compared to the Wiau area (mean, 95% CI; 0.55, 0.37-0.73) (data not shown), indicating a petrogenic PAH source. In total, 12 of the 20 (60%) wolf samples from the EP area, compared to 2 of 8 (25%) Wiau samples, had F/FP ratios <0.4, indicative of a petrogenic origin (Figure 3). However, a mean MP/P ratio of 0.41 (95% CI; 0.26-0.63) in wolf samples from the EP area indicate non-petrogenic or mixed exposure (data not shown); only three samples had a petrogenic MP/P ratio greater than 1.0.

DISCUSSION

The ability to measure PAHs in scat samples of multiple terrestrial wildlife species with distinct dietary and resource utilization differences allowed us to assess PAH contamination from petrogenic and pyrogenic sources across an expansive landscape. Our results clearly demonstrate that PAH exposures vary between study areas and species. The need to balance the maintenance of a healthy thriving ecosystem with competing economic objectives is crucial as bitumen production in the AOS is projected to more than double between 2008 and 2018 (Alberta 2013). These findings illustrate the importance of further scientific investigation and including toxicant exposures and their environmental consequences in monitoring programs of *in*

situ drilling technologies in the AOS. The noninvasive sampling method applied in this study is ideally suited for such investigations owing to the availability of wildlife scat in the environment and its relative ease of collection over large geographic areas.

Multiple lines of evidence indicate a pyrogenic PAH signature in caribou samples from the Wiau area where a 238,000 hectare fire occurred in 2002. These include an increased level of parent PAHs ($p<0.05$) and a predominance of pyrogenic diagnostic ratios (86%; $p<0.02$) compared to the other study areas. This finding is likely reflective of increased PAH levels in lichen, caribou's preferred food source, in the fire stricken area. The remaining patches of long-lived lichen would have been exposed to high level of PAHs in the atmosphere during the large-scale forest fire. By contrast, moose and wolf samples from the EP study area, where the most intensive oil extraction and exploration activity occurred, each had evidence of exposure to petrogenic PAHs compared to the other study areas. Most moose samples from the EP study area (86%; 24/28 samples) demonstrated PAH ratios suggestive of a petrogenic source; this was significantly greater than the petrogenic source PAH ratios from the other study areas ($p<0.001$). PAH exposures may have occurred through absorption of ground water by their preferred diet of riparian vegetation, deposition on woody vegetation from atmospheric transport of these compounds, or seepage through geographic strata. Wolf samples from the EP study area demonstrated statistically increased levels of total ($p<0.05$) and alkylated ($p<0.05$) PAHs, suggestive of a petrogenic source, that were not evident in samples from the other study areas. Ingestion of PAH-laden prey is the most likely source of PAH exposure in wolves. However, other routes of exposure such as social grooming or cleaning of paws after walking through contaminated snow-pack could provide additional sources of exposure. Both moose and wolf

species also showed evidence of mixed source exposures. These sources may have been better differentiated by a larger sample size.

The presumed petrogenic sources of PAHs in the AOS include crude oil exposure from natural deposits, seepage through geographic strata, potential leaks or spills, or increased exposure to natural deposits through disturbance of the landscape or the addition of steam into bitumen rich areas as part of exploration and extraction processes. Presumed pyrogenic (high-heat combustion) sources of PAHs would be historical forest fires, petroleum combustion from a related increase in road development and motorized traffic in the area, or industrial emissions. Mixed source profiles were evident in all species, likely influenced by a combination of the potential PAH sources listed above. Surprisingly, the cumulative levels of PAHs in moose were highest in samples from the Algar compared to other areas. The Algar is an area of habitat restoration with no active or capped oil wells on record, dating back to 1957 (AER 2014, COSIA 2014). These increased levels may reflect the relative proximity of the Algar study area to both the intensive surface mining activities north of Fort McMurray (70 km east-northeast of the Algar area), nearness to the Athabasca River (Figure 1) where oil seepages make their way through geographic strata into the watershed, or other unknown sources of PAH exposure. The mixed PAH source signature in the caribou samples across the EP and Algar areas could reflect a combination of smaller and/or unreported forest fires, volatilization of compounds (subsequently absorbed by the lichen) from continuously running diesel trucks (in the EP) or disturbance of the landscape, or sources outside of the study area brought in by atmospheric transport. The mixed source exposure in the wolf may be a reflection of their diverse prey, including moose

(petrogenic), caribou (pyrogenic), deer, beaver, and snow shoe hare. Other unexplained PAH sources are also possible and warrant further study.

Past studies have demonstrated bioaccumulation of persistent organic pollutants (POPs) [e.g., polychlorinated biphenyls (PCBs)] in top-level arctic carnivores, such as the wolf and other arctic wildlife (Kelly and Gobas 2001, Fisk et al. 2005, Sormo et al. 2006). PAHs are more susceptible to metabolism, the biologic half-life of benzo[a]pyrene in plasma from rats has been demonstrated to be less than 6 hours (Ramesh et al. 2001), and are less lipophilic (i.e., decreased affinity for long-term storage in fat) compared to POPs, thus do not bio-magnify as readily in the food web. Nonetheless, with declines in use of POPs, PAHs are reportedly beginning to emerge as the dominant contaminant in the arctic food web despite their more acute exposure (Laender et al. 2011). The present study measured PAHs in wolf scat samples at higher levels in areas of increased oil extraction activity. These measures are likely the result of recent PAH transfer, presumably through the consumption of PAH-laden food sources. Prey hair analyses of wolf scat from this same project, corroborated by findings from a separate study, demonstrated that 24% of the wolf winter diet consist of moose (Latham et al. 2011, Wasser et al. 2011). A recent study similarly reported elevated alkylated PAH levels in moose muscle tissue (McLachlan 2014). Since moose are also preferred game of First Nation communities proximal to the AOS, as well as common game of recreational hunters (McLachlan 2014), our reported petrogenic PAH profile in the moose samples from the area of increased oil exploration and extraction could have public health implications related to sustainable and recreational hunting in areas proximal to active oil production.

Few comparable studies exist on contamination levels and adverse effects of PAHs in wild terrestrial mammals (McLachlan 2014). Health effects of PAHs are often assessed through the concentration of 5-ring or larger compounds, such as benzo[a]pyrene, that are well studied and considered "known animal carcinogens" (ATSDR 2013); however, these compounds were not detectable in the samples from this study. Reproductive toxicities have been associated with exposures to benzo[a]pyrene in rats (Arafa et al. 2009) as well as 3-ring PAHs, such as phenanthrene, in Japanese medaka (*Oreyzias latipes*) (Horng et al. 2010), marine calanoid copepod (Bellas and Thor 2007), and enchytraeids (Sverdrup et al. 2002). Research on the health impacts of exposure to alkylated PAH compounds is limited, although a study evaluating alkyl-phenanthrene demonstrated an increase in embryo abnormalities (*Oryzias latipes*) compared to unsubstituted phenanthrene (Turcotte et al. 2011). Regardless of whether current PAH exposures in terrestrial wildlife in the AOS are at levels likely to cause carcinogenic effects or reproductive toxicities, sublethal exposures and indirect effects due to disrupted trophic interactions (Peterson et al. 2003), or toxic effects induced by ultraviolet radiation exposure (i.e., sunlight) such as the generation of reactive oxygen species that cause membrane damage (Arfsten et al. 1996, Yu 2002, Incardona et al. 2012), may still be of detrimental.

Further sampling from subsequent years would markedly improve our understanding of the extent and impact of PAH exposure among the study species. Additional sampling would be especially informative given the projected increases in petroleum exploration and development activity (Alberta 2013). It is recommended that future sampling efforts include white-tailed deer since they are the preferred wolf prey (Wasser et al. 2011) and are increasing in numbers in the AOS (Latham et al. 2011). Previous studies have demonstrated that PAH metabolites are present

in feces at higher levels than the parent compounds (Bouchard and Viau 1998, Ramesh et al. 2001, Jonsson et al. 2004, Hillenweck et al. 2008). This study focused on comparing the measurements of parent compounds within species and between ranges; thus our results are a conservative estimate of the actual PAH levels. The within species comparisons that reported findings between study areas are not due to species differences in bioavailability, metabolic capacity, or gut microflora leading to increased metabolites that would inflate the level of measured PAHs. Including a spectrum of PAH metabolites may be considered in future studies.

TABLES AND FIGURES.

Figure 1. Fecal samples collected over a 2500 km^2 area within the AOS. Sampling spanned across three caribou ranges within the East Side of the Athabasca River caribou herd, separated by dashed horizontal lines (top: Algar; middle: Egg Pony; bottom: Wiau), that differed in level of *in situ* oil production activity and fire history. Grey shaded area in main figure denotes historical wildlife. Grey solid line denotes primary and secondary access roads. Inset map denotes sampling area (**X**) within Alberta, Canada. Symbols: caribou (●), moose (■) and wolf (▲)

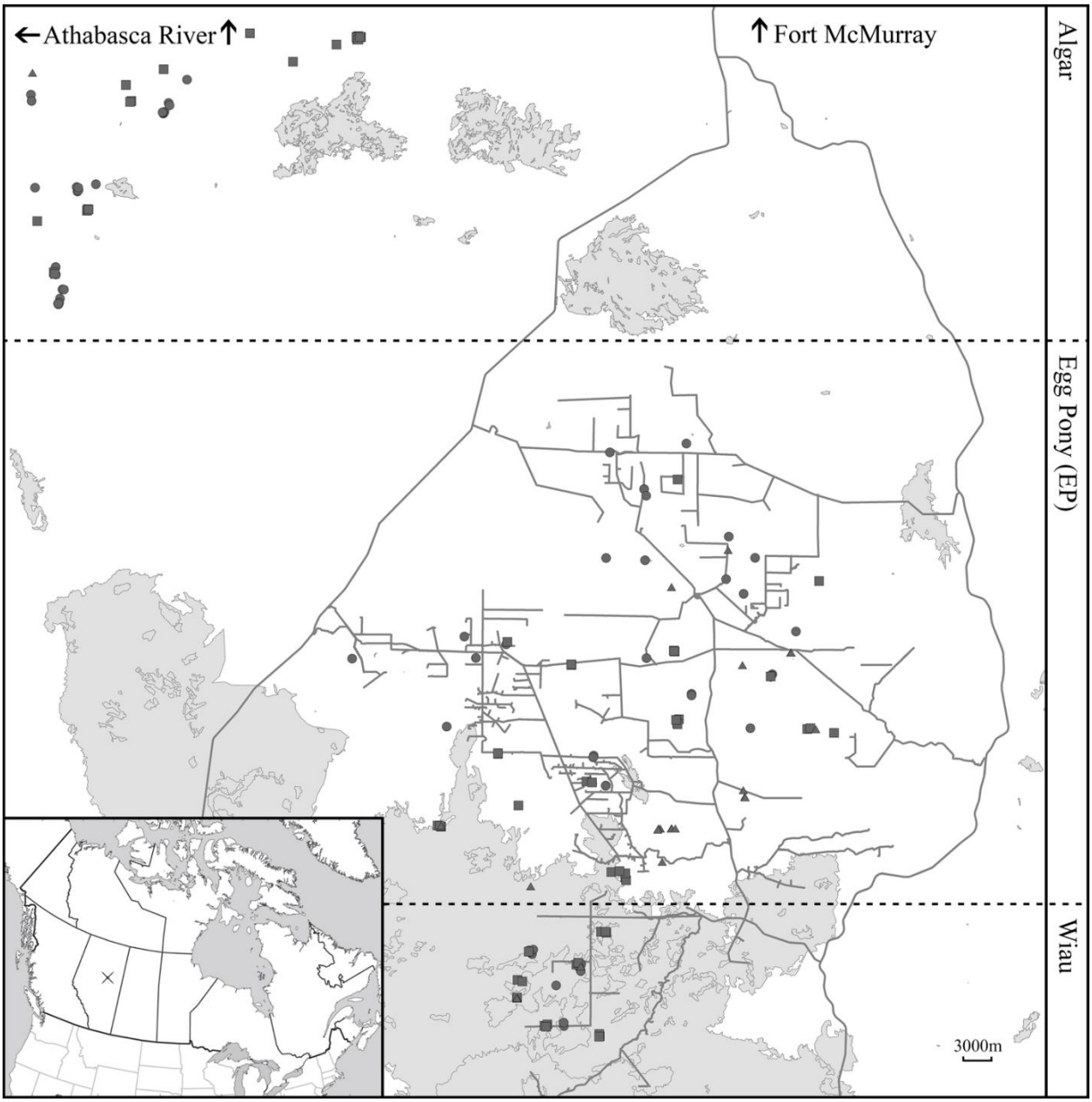
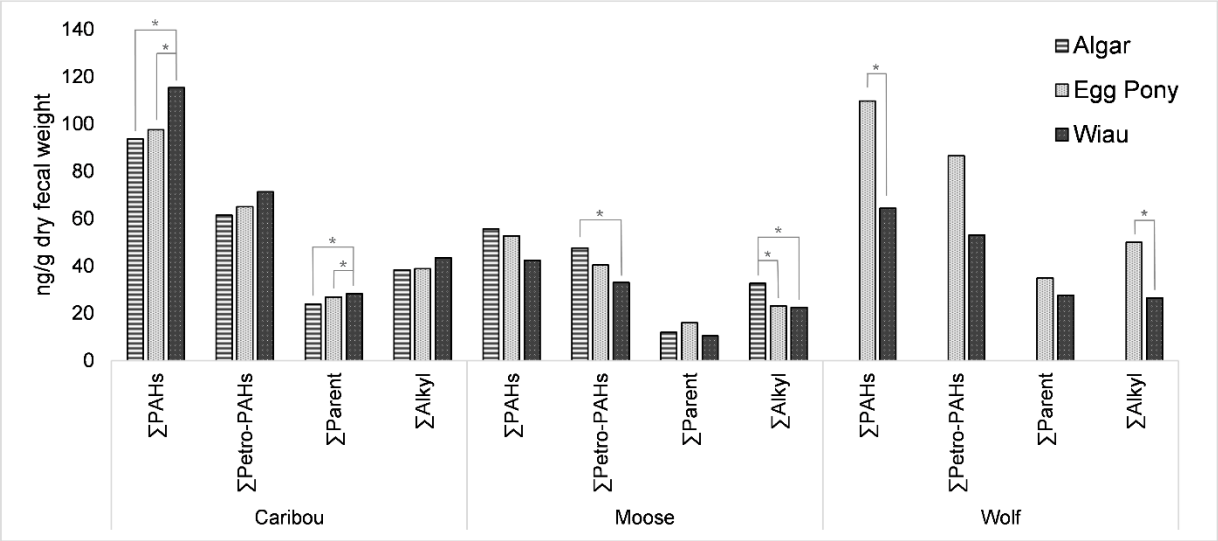


Table 1. Median and standard deviation (sd) of PAH target compounds (ng/g dry fecal weight) in fecal samples from caribou, moose, and wolf from three areas of the oil sands region of Alberta, Canada

Target compound	Caribou (n, male/ n, female)						Moose (n, male/ n, female)						Wolf (n, male/ n, female)			
	Algar (11/10)		EP (12/15)		Wiau (0/14)		Algar (7/13, 1 unk)		EP (14/14)		Wiau (6/14)		EP (10/10)		Wiau (8/0)	
	median	sd	median	sd	median	sd	median	sd	median	sd	median	sd	median	sd	median	sd
NPH	11.0	3.5	12.6	6.9	11.8	2.9	5.7	16.5	19.0	11.9	5.6	3.1	25.4	17.3	21.9	9.0
MN2	10.7	3.5	10.0	3.4	10.1	7.4	13.0	8.3	5.1	5.7	5.7	2.7	16.5	9.7	10.2	5.2
MN1	7.7	2.2	7.1	2.3	8.1	4.7	8.9	5.3	2.0	3.5	3.0	1.9	8.2	6.2	5.0	3.0
DMN	3.0	1.1	2.6	1.1	2.6	1.1	3.5	1.6	3.6	1.0	3.3	0.8	3.8	2.1	3.3	1.7
TriMN	1.9	1.1	2.0	1.3	1.8	1.8	1.2	1.1	0.6	0.9	0.5	0.7	2.1	6.0	0.1	1.2
TetMN	0.7	0.6	0.2	0.5	0.2	0.3	.	.	.	.	.	.	.	.	.	.
PHN	10.1	2.6	11.3	4.9	13.6	4.4	3.2	2.0	2.1	1.4	4.3	1.7	10.8	21.6	3.1	2.6
MP2	4.0	1.5	3.7	2.3	5.2	1.5	0.1	0.6	2.0	0.9	0.2	1.5	1.2	8.0	1.7	2.7
MP1	2.9	1.6	3.0	2.3	5.8	1.8	0.7	1.4	1.8	1.8	0.6	1.7	2.5	5.9	0.5	0.6
DMP	1.0	1.5	2.1	2.0	1.0	1.7	1.0	0.4	1.1	0.4	0.9	0.6	0.7	2.4	0.0	0.1
TriMP	.	.	.	.	.	.	.	.	.	.	.	.	0.3	3.7	0.5	0.2
TetMP	.	.	.	.	.	.	.	.	.	.	.	.	1.8	1.7	1.7	1.0
DBT	0.7	0.4	0.8	0.4	0.4	0.4	0.3	0.3	0.1	0.6	0.3	0.6	0.6	3.2	0.2	0.0
FLO	1.3	0.7	1.7	1.0	1.9	1.0	1.4	0.9	0.8	0.5	0.8	0.6	2.5	4.0	1.4	1.0
FL1	1.4	1.0	1.7	1.4	1.9	2.2	1.7	1.0	1.9	1.8	2.3	2.3	2.6	5.5	1.3	1.0
CHR/B[a]A	8.5	4.1	10.0	4.2	12.5	3.9	0.4	0.3	1.2	0.6	0.2	0.6	6.1	2.1	6.0	0.4
ACY	1.2	0.9	1.6	1.2	1.4	1.0	.	.	.	.	.	.	0.0	0.2	0.0	0.1
ANT	0.0	1.7	0.0	0.9	0.0	0.2	0.9	7.7	0.0	15.5	0.4	10.1	1.5	4.3	0.4	0.4
MA2	0.0	0.1	0.0	0.3	0.0	0.2	0.7	0.4	0.1	2.5	1.0	0.3	0.6	0.8	0.6	0.2
MA9	16.3	7.4	15.4	6.7	17.0	3.8	.	.	.	.	.	.	.	.	.	.
MA23	0.1	1.6	0.7	1.5	2.1	1.6	0.4	0.7	0.4	1.5	0.4	0.5	0.5	4.4	0.9	0.2
MA910	1.4	1.3	1.9	1.2	2.8	1.3	.	.	.	.	.	.	1.2	3.7	1.1	2.6
FLU	1.3	1.9	2.2	2.8	5.6	2.7	0.3	0.3	0.7	0.5	0.5	0.3	0.7	4.6	0.0	0.5
PYR	1.5	1.3	2.0	1.6	2.8	1.4	0.3	1.4	2.0	1.5	0.6	0.9	2.3	6.2	0.0	0.2

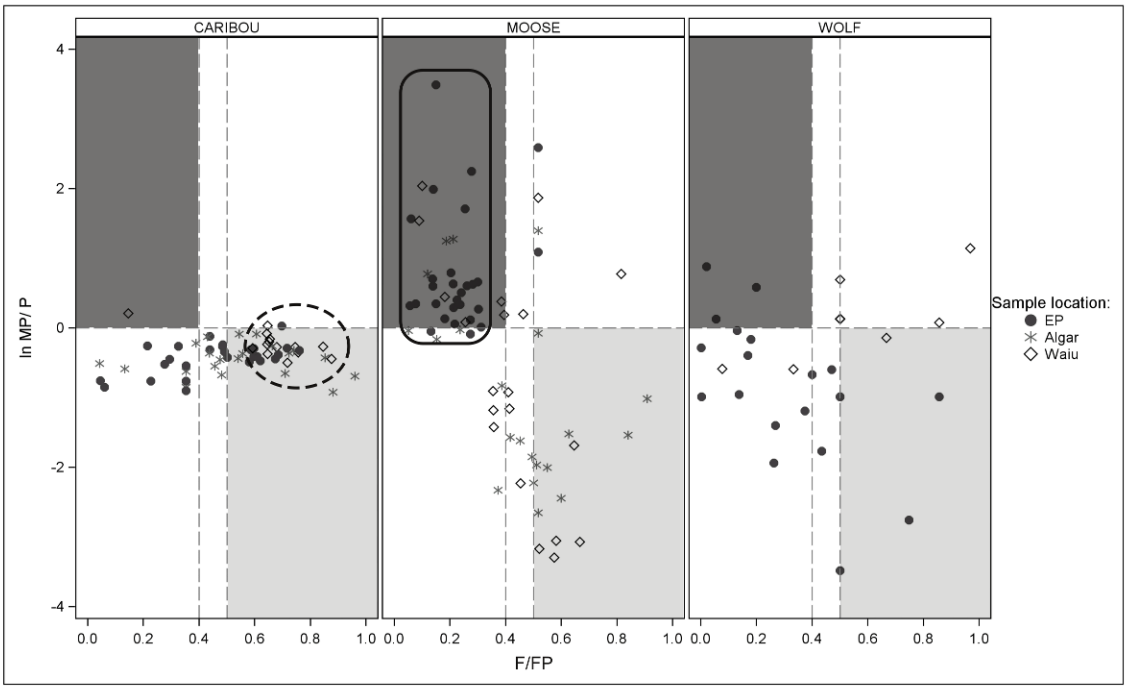
NOTE: . denotes target compound not detectable; DMN2 and FL2 not included due to co-elution of artifact compounds in matrix

Figure 2. Geometric marginal means (ng/g dry fecal weight) of PAH metrics in fecal samples from caribou, moose, and wolf from three areas of the Alberta oil sands



*p<0.05

Figure 3. PAH ratios for distinguishing pyrogenic vs petrogenic PAH source. Ratios* indicative of petrogenic sources fall above the horizontal line and to the left of the two vertical lines, samples between the two vertical lines suggest fossil fuel combustion sources, and samples below the horizontal line and to the right of the two vertical lines indicate pyrogenic (biomass combustion) or mixed sources. The left panel demonstrates ratios for caribou, the middle panel for moose, and the right panel for wolf.



*F/FP, <0.4 petrogenic, 0.4-0.5 petroleum combustion, and >0.5 pyrogenic (biomass combustion);
ln MP/P, <0 mixed source and >0 petrogenic (note the natural log scale of the MP/P metric)
NOTE: dashed circle denotes cluster of samples from the Wiau study area; solid line rectangle denotes cluster of samples from the EP study area

SUPPLEMENTAL TABLES AND FIGURES

Table S1. List of PAH target compounds and abbreviations for quantification by GCMS, retention times (RT-SIM), quantification (quant) ion and confirmation (conf) ion for GCMS analysis, Method Detection Limits (MDLs), and the percent of each compound that was detectable in samples across species.

Abbreviations	Target compounds	RT-SIM	Quant Ion	Conf Ion	MDLs (ng/ g dw)	% samples with detectable levels of target compound		
						Caribou (n=62)	Moose (n=69)	Wolf (n=29)
NPH	Naphthalene	12.56	128	127	2.27	100%	86%	97%
MN2	2-methylnaphthalene	15.11	142	141	0.71	100%	94%	97%
MN1	1-methylnaphthalene	15.47	142	141	0.25	100%	94%	100%
DMN	1,6-dimethylnapthalene	18.12	156	141	0.00	100%	99%	97%
DMN2	1,2-dimethylnaphthalene	18.86	156	141	.	0%	0%	0%
TriMN	2,3,5-trimethylnaphthalene	22.10	170	155	0.15	84%	83%	59%
TetMN	1,4,6,7-tetramethylnaphalene	26.16	184	169	4.01	65%	0%	24%
ACY	Acenaphthylene	18.76	152	153	0.14	90%	4%	38%
ACE	Acenaphthalene	19.65	154	151	.	0%	3%	28%
DBT	Dibenzothiophene	28.85	184	139	0.94	94%	71%	48%
FLO	Fluorene	22.87	166	165	0.64	89%	97%	93%
FL1	1-methylfluorene	26.74	180	165	0.30	97%	97%	93%
FL2	1,8-dimethylfluorene	29.99	194	179	.	0%	0%	0%
PHN	Phenanthrene	28.88	178	176	2.76	100%	96%	83%
ANT	Anthracene	29.19	178	176	0.15	40%	59%	83%
MP2	2-methylphenanthrene	31.85	192	191	0.66	98%	70%	62%
MA2	2-methylanthracene	32.10	192	191	0.21	50%	77%	79%
MP1	1-methylphenanthrene	32.31	192	191	0.53	98%	94%	79%
MA9	9-methylanthracene	33.13	192	191	1.33	82%	13%	0%
DMP	3,6-dimethylphenanthrene	34.21	206	191	0.48	48%	59%	48%
MA23	2,3-dimethylanthracene	35.58	206	191	0.23	68%	26%	62%
MA910	9,10-dimethylanthracene	36.59	206	191	0.43	69%	12%	31%
TriMP	1,2,5-trimethylphenanthrene	38.27	220	205	0.78	8%	1%	62%
TetMP	1,2,6,9-tetramethylphenanthrene	40.17	234	219	0.40	8%	4%	34%
FLU	Fluoranthene	35.28	202	201	0.39	89%	78%	69%
PYR	Pyrene	36.32	202	201	0.18	89%	88%	76%
CHR/ B[a]A	Chrysene/benz[a]anthracene	42.26	228	229	3.15	100%	59%	90%

Table S2. Geometric marginal means and 95% confidence intervals metrics (ng/g dry fecal weight) of PAH in fecal samples from caribou, moose, and wolf from three area of the oil sands region of Alberta, Canada

		Algar			Egg Pony			Wiau		
Caribou	∑PAHs	93.6	(84.7-103.3)	b	97.6	(89.4-106.6)	a	115.4	(102.2-130.3)	a,b
	∑Petro-PAHs	61.4	(55.4-68.0)		65.0	(59.3-71.1)		71.3	(62.8-80.9)	
	∑Parent	23.9	(21.4-26.6)	a	26.9	(24.4-29.5)	b	28.3	(24.8-32.3)	a,b
	∑Alkyl	38.4	(34.3-42.8)		38.8	(35.2-42.8)		43.5	(38.0-49.9)	
	Petroleum Index	1.62	(1.56-1.68)		1.69	(1.64-1.75)		1.65	(1.58-1.73)	
	F/FP	0.54	(0.45-0.63)		0.46	(0.38-0.54)	a	0.66	(0.55-0.77)	a
	MP/P	0.63	(0.58-0.70)	a,b	0.65	(0.60-0.71)	b	0.79	(0.71-0.89)	a
Moose	∑PAHs	55.7	(45.6-68.0)		52.7	(44.4-62.6)		42.4	(34.5-51.9)	
	∑Petro-PAHs	47.6	(38.4-59.0)	a	40.4	(33.5-48.6)		33.0	(26.5-41.1)	a
	∑Parent	11.9	(8.1-17.5)		16.0	(11.5-22.3)		10.5	(7.1-15.6)	
	∑Alkyl	32.6	(27.1-39.3)	a,b	23.1	(19.7-27.1)	b	22.5	(18.6-27.2)	a
	Petroleum Index	1.47	(1.32-1.65)	b	1.78	(1.62-1.96)	a,b	1.49	(1.33-1.67)	a
	F/FP	0.44	(0.36-0.51)	b	0.23	(0.16-0.29)	a,b	0.43	(0.35-0.50)	a
	MP/P	0.40	(0.23-0.70)	b	2.22	(1.36-3.60)	a,b	0.48	(0.27-0.86)	a
Wolf	∑PAHs				109.7	(84.4-142.5)	a	64.4	(42.6-97.4)	a
	∑Petro-PAHs				86.5	(64.6-115.8)		53.1	(33.5-84.2)	
	∑Parent				34.9	(23.7-51.2)		27.6	(15.0-50.6)	
	∑Alkyl				50.0	(38.4-65.1)	a	26.5	(17.5-40.2)	a
	Petroleum Index				1.75	(1.61-1.89)	a	2.05	(1.81-2.32)	a
	F/FP				0.31	(0.19-0.43)	a	0.55	(0.37-0.73)	a
	MP/P				0.41	(0.26-0.63)	a	1.11	(0.56-2.21)	a

NOTE: Same letters denote significance across rows, $p<0.05$

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