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PCB Related Effects Thresholds As Derived through Gene Transcript Profiles in Locally Contaminated Ringed Seals (*Pusa hispida*)

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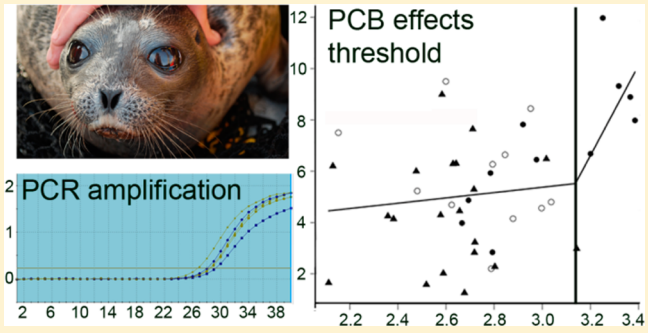
Supporting Information

ABSTRACT: Causal evidence linking toxic injury to polychlorinated biphenyl (PCB) exposure is typically confounded by the complexity of real-world contaminant mixtures to which aquatic wildlife are exposed. A local PCB "hotspot" on the Labrador coast provided a rare opportunity to evaluate the effects of PCBs on the health of a marine mammal as this chemical dominated their persistent organic pollutant (POP) burdens. The release of approximately 260 kg of PCBs by a military radar facility over a 30 year period (1970−2000) contaminated some local marine biota, including the ringed seal (*Pusa hispida*). The abundance profiles of eight health-related gene transcripts were evaluated in liver samples collected from 43 ringed seals in the affected area. The mRNA transcript levels of five gene targets, including aryl hydrocarbon receptor (*Ahr*), interleukin-1 β (*Il1b*), estrogen receptor α (*Esr1*), insulin like growth factor receptor 1 (*Igf1*), and glucocorticoid receptor α (*Nr3c1*) correlated with increasing levels of blubber PCBs. PCB threshold values calculated using best-fit hockey-stick regression models for these five genes averaged 1,680 ± 206 ng/g lw, with the lowest, most conservative, being 1,370 ng/g lw for *Il1b*. Approximately 14% of the seals in the region exceeded this threshold. The dominance of PCBs in the seals studied enabled an assessment of the effects of this chemical on gene transcripts involved in regulating the health of a highly mobile predator, something that is rarely possible in the world of complex mixtures.



INTRODUCTION

While marine mammals occupying high trophic levels in arctic food webs have been found to be contaminated with moderately high concentrations of persistent organic pollutants (POPs),[1,2] it remains unclear whether current levels represent a risk to their health. Polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides (OCPs) in the Arctic have been largely attributed to long-range transport through atmospheric processes, although some point sources do exist (e.g., military radar stations).[3,4]

The highly complex mixtures of POPs to which marine mammals are exposed in Arctic and sub-Arctic regions render it difficult to ascribe cause-and-effect relationships to any single contaminant. However, PCBs are widely considered a priority contaminant at the top of food webs with impacts on the biology of a variety of northern wildlife species. For example, endocrine disruption, reproductive impairment, and immunotoxicity in polar bears (*Ursus maritimus*) have been associated with PCBs.[5−9] An association between thyroid hormone disruption and some PCB congeners was found in beluga whales (*Delphinapterus leucas*) from Svalbard, Norway.[10] Phase I enzyme and glutathione-S-transferase (GST) activities, which biotransform group III and IV PCBs, were positively associated with PCBs in ringed seals (*Pusa hispida*) from the Baltic Sea.[11] Furthermore, PCBs were considered to be the cause of uterine occlusions which resulted in reproductive failure in grey seals (*Halichoerus grypus*) in the Baltic Sea.[12,13] More recently, changes in hepatic and circulatory vitamin A levels and hepatic *Ahr* and *Cyp1a1* mRNA levels in beluga whales from the western Canadian Arctic have been associated with PCBs.[14,15]

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In southern, more contaminated areas, chemical detoxification enzymes and altered endocrine and immune functions in several marine mammal populations have also been associated with PCBs.[16−20] In all these cases, however, correlations between health effects and PCB concentrations are based on the premise that PCBs are either the putative contaminant driving the relationship, or represent a proxy for the other cocorrelating POPs.

A PCB point source associated with a military radar station in Saglek Bay, Labrador, Canada, has contaminated the adjacent marine food web.[21−23] This radar facility has been in operation since the late 1950s; however, it was not until 1996 that extensive contamination was discovered in three areas (Site Summit, Antenna Hill, and beach area) at the site, and that PCBs were found to have entered the marine environment.[21] Very high PCB concentrations were measured in the local marine sediments, the benthic food web, and ringed seals.[21−23] Results of an ecological risk assessment indicated that shorthorn sculpin (*Myoxocephalus scorpius*) and black guillemot (*Cepphus grylle*) nestlings from the area were at increased risk of impaired reproduction or death.[24] Until now, no studies have assessed health risks to ringed seals, despite the findings that up to 60% of ringed seals sampled in the region were exposed to the local PCB source and that average PCB concentrations in adult male ringed seals exceeded a 1,300 ng/g (lipid wt; blubber) threshold for endocrine and immunotoxic effects derived for harbor seals (*Phoca vitulina*[25]).

The combination of a long-range POP "background" and a local PCB "hotspot" on the Labrador coast provides an invaluable opportunity to evaluate the effects of PCBs on the health of a free-ranging marine mammal. The present study investigates the relationship between ringed seal blubber PCB burden and hepatic mRNA abundance profiles of gene transcripts encoding proteins that play an important role in animal health with respect to chemical detoxification, the immune and endocrine systems, and the regulation of growth, development, and metabolism. Further, this transcriptomic approach provides quantitative information at the molecular level, which could serve as an early detection indicator for higher-level health effects.[19]

MATERIALS AND METHODS

Sample Collection. All tissue samples from adult (≥6 years) and subadult (<6 years) ringed seals (n = 43) were obtained from Inuit hunters in four marine inlets (Nachvak Fjord, Saglek Fjord, Okak Bay, and Anaktalak Bay) along the northern Labrador coast during the fall season (September and October) from 2009 to 2011 (see Figure 1 in ref. 23). Males/females ratio was 10/11 and 11/11 for subadult and adult seals, respectively. Sex, weight, length, girth, and blubber thickness (at the sternum) were recorded for each ringed seal. Ages were determined at Matson's Laboratory, U.S.A., by longitudinal thin sectioning a lower canine tooth and counting annual growth layers in the cementum using a compound microscope and transmitted light. Samples used for stable isotopes (muscle) and organochlorines (blubber) were stored at −20 °C prior to the analyses. Carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope signatures were evaluated because altered mRNA transcript levels have been associated with changes in feeding ecology in another marine mammal species, the arctic beluga whale.[15] Liver samples (∼1 g) collected for the measurement of mRNA abundance profiles were preserved directly in the field in RNA*later* tissue preservation solution as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, U.S.A.) and stored at −20 °C until isolation of total RNA. All tissue samples were obtained within 1 h of harvesting.

Contaminant Analysis. Ringed seal blubber samples were analyzed by the Great Lakes Institute for Environmental Research's organic analytical laboratory (Windsor, ON) (Canadian Association for Environmental Analytical Laboratories Accreditation and ISO17025 certified) for concentrations of 62 PCB congeners and organochlorine pesticides (OCPs): α-, β-, γ-hexachlorocyclohexane, α- and γ-chlordane, *cis*-nonachlor, *trans*-nonachlor, oxychlordane, heptachlor epoxide, dichlorodiphenyldichloroethane [p,p'-DDD], dichlorodiphenyldichloroethylene [p,p'-DDE], p,p'-DDT, dieldrin, and hexachlorobenzene (HCB). The detailed methodology for extraction, cleanup, and quantification of target analytes has been reported elsewhere.[23,26,27] Briefly, homogenized wet tissue (0.5−1 g), anhydrous sodium sulfate, and surrogate standard were ground with motor and pestle and then extracted following a microextraction technique.[26] Samples were analyzed for individual PCB congeners and OCPs by gas chromatography electron capture detection (GC-ECD). Percent lipid was determined using gravimetric lipid determination by weight of extract method with dichloromethane. For each batch of six samples, an in-house reference homogenate tissue, method blank, and the external PCB-34 recovery standard were analyzed for 62 PCB congeners. All PCB congeners and OCPs that were detected in 90% of the samples were included in the data analysis, in samples where an individual congener was not detected it was replaced with a random number between the detection limit (0.011 to 0.150 ng/g) and zero. Recoveries of individual PCB congeners in the homogenate reference tissue with each sample batch run were within 2 standard deviations from the mean laboratory database value derived from laboratory control charts. Recovery efficiencies for the PCB34 standard were 99 ± 0.95% (mean ± standard error). Procedural method blanks (n = 11) were below detection for all PCB congeners and OCPs. All study samples were recovery corrected for PCB congener and OCP concentrations.

Hereafter, ∑PCBs refers to the sum of the 62 PCB congeners, ∑HCH refers to the sum of α-, β-, γ-hexachlorocyclohexane, ∑chlordanes refers to the sum of α- and γ-chlordane, *cis*-nonachlor, *trans*-nonachlor, oxychlordane, heptachlor epoxide, and ∑DDT refers to the sum of p,p'-DDD, p,p'-DDE, and p,p'-DDT. ∑PCBs was used in the data analysis since both coplanar and noncoplanar PCBs have been shown to elicit toxic effects. Further, less than half (42%) of the dioxin-like PCBs were analyzed in the present study using GC-ECD.

Hepatic RNA Isolation and cDNA Synthesis. Detailed procedures on total RNA extraction and cDNA synthesis are described elsewhere.[15,19,20] Briefly, each sample was homogenized in a 1.5 mL microcentrifuge tube using a Retsch MM301 mixer mill (Thermo Fisher Scientific, Ottawa, ON, Canada) following the addition of 700 μL TRIzol reagent (Thermo Fisher Scientific) and a 3 mm diameter tungsten-carbide bead. Samples were homogenized in two 3 min intervals at a frequency of 20 Hz with a cooling period of 2−3 min on ice and 180° rotation of the mixing chambers between intervals. Isolated hepatic total RNA was resuspended in 40 μL of diethyl pyrocarbonate-treated distilled deionized water and stored at −80 °C. RNA purity and concentration were assessed by spectrophotometry at A_{260} and A_{280} and 1 μg of each sample was subsequently used to prepare cDNA with the High

Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, U.S.A.). Each cDNA sample was diluted 40-fold with PCR-grade water prior to gene-specific analysis.

Quantitative Real Time Polymerase Chain Reaction (qPCR) Assay. Eight genes were selected to provide an overview of the biological consequences of exposure to PCBs, based in large measure on past mechanistic studies of this chemical class. These include aryl hydrocarbon receptor (*Ahr*), thyroid hormone receptor α (*Thra*), estrogen receptor α (*Esr1*), thyroid stimulating hormone β (*Tshb*), retinoic acid receptor α (*Rara*), interleukin-1 β (*Il1b*), insulin like growth factor receptor 1 (*Igf1*), and glucocorticoid receptor α (*Nr3c1*). These protein-encoding genes play critical roles in detoxification pathways, immune and endocrine systems, and the regulation of growth, development, and metabolism. Three additional transcripts were chosen as normalizers for correction of input variation and assessment of sample quality: ribosomal protein L8 (*Rpl8*), β-like 2 actin (*Actbl2*), and eukaryotic translation elongation factor-1 α (*Eef1a1*).

Expressed gene sequences were isolated from ringed seal by PCR-directed cloning using degenerate primers designed against aligned gene sequences from different mammal species as described elsewhere[15,20] and deposited in NCBI GenBank (Supporting Information, SI, Tables S1 and S2). The obtained species-specific cDNA sequences were used to evaluate previously established marine mammal qPCR primer sets for efficacy toward ringed seal as well as perform species-specific de novo design with Primer Premier 5 (PREMIER Biosoft International, Palo Alto CA, U.S.A.). All primers were obtained from Integrated DNA Technologies Inc. (Coralville, IA, U.S.A.). Gene-specific qPCR was assessed in liver using a three-tier quality control process as outlined in detail elsewhere.[28] All 11 primer pairs satisfied the criteria for use of the comparative $\Delta\Delta C_t$ quantification method.[29] Ringed seal qPCR primer pair sequences used in the present study are presented in SI Table S1.

qPCR assays using SYBR Green I-based detection were conducted on a MX3005P Real-Time PCR System (Agilent Technologies Canada Inc., Mississauga, ON, Canada), as described previously.[30] For all expressed gene sequences investigated, quadruplicate amplification reactions (15 μL) were performed for each liver sample. The amplification thermocycle program for each transcript consisted of an initial enzyme activation step at 95 °C (9 min) followed by 40 cycles of 95 °C denaturation (15 s), 60 °C (or 57 °C for *Rara*, *Tshb*, *Il1b*) annealing (30 s), and 72 °C elongation (45 s). A subsequent thermodenaturation profile was included from 55 to 95 °C to evaluate qPCR amplification quality. Inter-run variation was assessed by the use of a standard control amplification reaction that included a pooled ringed seal cDNA sample. Specificity of target amplification was assessed by including a no DNA template control. Replicate cycle threshold (C_t) data for each sample were averaged and transformed to fold change values using the comparative $\Delta\Delta C_t$ method.[29] Data normalization, which accounted for variation in cDNA input, used a geometric mean calculated from the three normalizer genes (*Rpl8*, *Actbl2*, *Eef1a1*). These three genes displayed strong intercorrelation in abundance across the qPCR data set with a Cronbach's alpha of 0.852 and demonstrated strong normalizer scores in RefFinder (http://www.leonxie.com/referencegene.php?type=reference).

Stable Isotope Analyses. Muscle tissue (0.5−1 g) was freeze-dried and homogenized. Lipid was extracted from all samples using a chloroform/methanol extraction and then dried for analysis. Carbon and nitrogen isotopic analyses were performed using Continuous Flow Ion Ratio Mass Spectrometer (CFIR-MS) (Finnigan MAT Delta^{plus}, Thermo Finnigan, San Jose, CA, U.S.A.). Detailed methodology on the procedure has been reported elsewhere.[23] Stable isotope abundances are expressed in delta (δ) values as the deviation from standards in parts per thousand (‰) using the following equation:

$$\delta_{sample}‰ = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where R is the ratio of heavy to light isotope ($^{15}N/^{14}N$ or $^{13}C/^{12}C$) in the sample and standard. The standards used for carbon and nitrogen analyses were Pee Dee Belemnite limestone formation and atmospheric nitrogen, respectively. Precision based on two standards (bovine muscle (NIST 8414) and tilapia fish muscle internal laboratory standard; n = 65 for each) were <0.16‰ and <0.08‰ for $\delta^{15}N$ and $\delta^{13}C$, respectively. Accuracy of isotope analysis, based on the NIST standards sucrose (NIST 8542) and ammonia sulfate (NIST 8547) analyzed during the present study (n = 3 for each) were within <0.1‰ of certified $\delta^{15}N$ and $\delta^{13}C$ values.

Data Analysis. Unless otherwise stated, univariate statistical analyses were performed in SPSS Version 20.0 (IBM Software, Armonk, NY, U.S.A.). Data were log-transformed when necessary to meet the normality assumptions for parametric analyses. Linear and nonlinear "hockey-stick" regression analyses were used to determine relationships between blubber PCB concentrations and hepatic mRNA abundance. Hockey-stick regression analyses were carried out using R version 2.9.0 (http://cran.r-project.org/bin/windows/base/old/2.9.0/). The "hockey-stick" regression assumes a constant background mRNA abundance up to a threshold tissue concentration of PCBs, above which mRNA abundance increases in concert with PCB concentrations reflective of a threshold response.[31] The two segments meet at the change-point (or "threshold") value. The derived equation is expressed as

$$\text{mRNA abundance} = a \quad \text{when} [\text{PCB}] < [\text{PCB}]_T$$

and mRNA abundance = $a + b$ ($[\text{PCB}] - [\text{PCB}]_T$) when ($[\text{PCB}] > [\text{PCB}]_T$), where the estimated parameters are a = background mRNA abundance of seals not eliciting a response; b = the slope of the relationship between mRNA abundance and tissue PCB concentration [PCB] above $[\text{PCB}]_T$, the threshold PCB concentration. The model generates best estimates for a, b, and $[\text{PCB}]_T$.

The best variable or combination of variables (biological: age, sex, year, weight, length, girth, blubber thickness, $\delta^{15}N$ and $\delta^{13}C$; contaminant: ∑PCBs, ∑DDTs, ∑chlordanes, ∑HCH, HCB, dieldrin) to describe the mRNA abundance profile of each gene was selected using the lowest Akaike information criteria (AIC) (SYSTAT Version 13, Systat Software Inc., San Jose, CA, U.S.A.). The Akaike differences (Δi) and normalized Akaike weights (w_i) were calculated to select the best variable or variables. The models with a ΔAIC of zero and up to two were considered to have the most support.[32]

Significant relationships were further evaluated using Principal Component Analysis (PCA) (Pirouette, Infometrix, Bothell, WA, U.S.A.). Gene transcript fold change values were autoscaled (scaled to variable mean and standard deviation) before PCA. The PCA scores from axis one and two were regressed by each of the biological or contaminant variables.

Table 1. Blubber Contaminant Concentrations (ng/g Lipid Weight) for Subadult (<6 yr; Male and Female Combined), Adult Male (≥6 yr), and Adult Female (≥6 yr) Ringed Seals Collected from Northern Labrador, Canada (Mean ± Standard Error and Range)

	subadults (A)	adult female (B) (B)	adult male (C)	Anova	Tukey's
n	21	11	11	N/A	N/A
∑PCBs[b]	477 ± 62 (130−1390)	630 ± 296 (142−1090)	1290 ± 231 (464−2430)	$p < 0.001$[a]	(AC: $p < 0.001$; BC: $p = 0.005$)
∑DDTs	235 ± 24 (36−444)	196 ± 174 (31−682)	555 ± 96 (189−1,110)	$p < 0.001$[a]	$p < 0.001$ (AC; BC)
∑chlordanes	23 ± 2 (9−42)	28 ± 14 (8−50)	54 ± 13 (11−161)	$p = 0.003$[a]	(AC: $p = 0.002$; BC: $p = 0.003$)
dieldrin	27 ± 6 (12−70)	30 ± 11 (16−52)	36 ± 6 (14−59)	$p = 0.557$	
∑HCHs	52 ± 4 (18−99)	38 ± 13 (21−56)	52 ± 5 (36−85)	$p = 0.080$	
HCB	13 ± 6 (3−133)	7 ± 1 (3−16)	5 ± 1 (2−10)	$p = 0.550$	

[a] $p < 0.05$ among subadults (A), adult females (B), and adult males (C). [b] ∑PCBs include the following: 19, 18/17, 24/27, 16/32, 26, 28/31, 33/20, 22, 45, 46, 52, 49, 47/48, 44, 42, 64/41/71, 40, 74, 70/76, 66/95, 91, 60/56, 92/84/101, 99, 97, 87, 85, 136, 110, 151/82, 144/135, 149, 118, 134, 146, 153, 105/132, 141, 179, 137, 130/176, 138/163, 158, 178, 187/182, 183, 128/167, 185, 174, 177, 156/171/202, 157/173/200/204, 172, 180, 201, 170/190, 199, 203/196, 207, 194, and 206.

RESULTS AND DISCUSSION

Contaminant Concentrations in Ringed Seals. Concentrations of ∑PCBs, ∑DDTs, and ∑chlordanes were higher in the blubber of adult male ringed seals than in subadult and adult female ringed seals ($p \leq 0.05$; Table 1). No differences ($p > 0.05$) were found between subadult males and females for ∑PCBs and the 5 OCPs (∑DDT, ∑chlordanes, ∑HCHs, dieldrin, and HCB) measured. No differences ($p > 0.05$) were found between subadult and adult female ringed seals for ∑PCBs and the 5 OCPs. Such results are typical for pinnipeds, where females reduce their POP burden by transferring fat-soluble contaminants to offspring via placental and lactational transfer.[33,34] The ∑HCHs, HCB, and dieldrin concentrations did not vary ($p > 0.05$) between sexes or age classes. Similar results between sexes have been observed previously in ringed seals for these contaminants.[23,33]

While average ∑PCB and OCP concentrations for ringed seals at the Labrador sites fell within the range observed across the Canadian Arctic,[1,23,33,35,36] three features stood out in the present study. First, some individuals (14%) exhibited much higher PCB levels than expected for ringed seals in the Arctic, something we previously attributed to feeding in the contaminated Saglek Bay.[23] Second, PCBs accounted for 58−68% of the total POP profile in Labrador ringed seals (Table 1), which far exceeds the contribution observed in ringed seals elsewhere in the Canadian Arctic (~25−40%).[1,33,37] DDT was consistently ranked as the second most prevalent POP, with some variation (∑Chlordanes or ∑HCHs) across the three age/sex categories for the third POP (Table 1). This variation is likely due to differing biological status related to seal age and sex that influence POP uptake and metabolism. Third, some seals in the area were previously shown to have heavier PCB congener profiles than others, consistent with exposure to a local source.[23] Polybrominated diphenyl ethers in blubber and perfluoroalkyl compounds in liver have been detected in ringed seals across the Canadian Arctic at lower or similar concentrations, respectively,[38,39] to the legacy OCP concentrations in the present study. These compounds were not included in our study but would likely show similar trends to the legacy OCPs.

Labrador ringed seals offer a unique opportunity to examine PCB effects in a marine mammal species as PCBs dominate and concentrations of other POPs are extremely low compared to background levels in more industrialized regions. While none of the adult female seals exceeded proposed PCB effects thresholds for marine mammals,[25,40,41] nearly half (46%) of the adult males and 1 (5%) of the subadults (<1 yr) exceeded endocrine and immune thresholds. Overall, these results suggest that ringed seals inhabiting the northern Labrador coast are at increased risk for endocrine and immune disruption from PCBs compared to seals inhabiting other areas in the Arctic. This increased risk can be attributed to the exposure to the "local" PCB source at Saglek Bay.

PCB-Related Changes in Hepatic mRNA Abundance. We know of no other case of a marine mammal population that has been solely exposed to a PCB point source, such that this study afforded us with the opportunity to investigate the association between liver mRNA abundance profiles and PCB concentrations.

A correlation was observed between 5 of the 8 gene transcripts assessed (Aryl hydrocarbon receptor (*Ahr*), interleukin-1 β (*Il1b*), estrogen receptor α (*Esr1*), insulin like growth factor receptor 1 (*Igf1*) and glucocorticoid receptor α (*Nr3c1*)) and ∑PCB concentrations; with both linear and nonlinear regressions being significant (SI Table S3; Figure 1; Table 2; $p < 0.05$). Coefficients of determination (r^2) were highest for the nonlinear regressions compared with the linear regressions. We therefore present the nonlinear "hockey stick" regressions (Figure 1; Table 2). Modeling of data using a "hockey-stick" regression has been previously used to establish sediment quality thresholds and effects thresholds in biota.[31,42] Furthermore, this approach provided a model that incorporates a change point representative of a contaminant level (threshold) below which an effect is not expected.[31] The correlation between hepatic *Ahr*, *Il1b*, *Esr1*, *Igf1*, and *Nr3c1* mRNA abundance and ∑PCB concentrations and their associated effects thresholds for ringed seals from Labrador (Table 2) suggest that contaminant-associated responses in molecular end points can be detected at levels considerably lower (~10- to 20-fold) than those currently observed in ringed seals from the

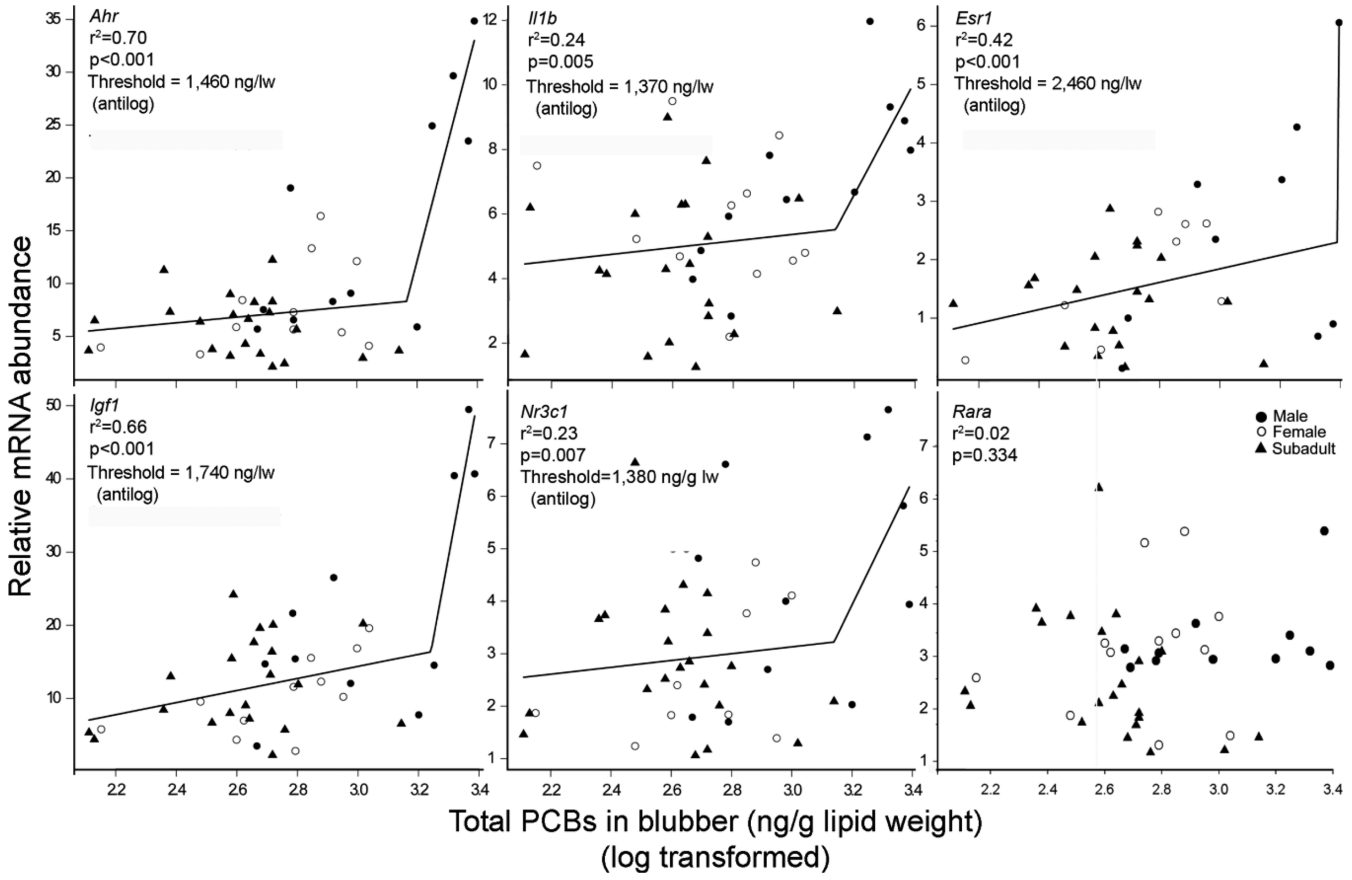

Figure 1. Hepatic mRNA transcript levels for five genes (*Ahr*, *Il1b*, *Esr1*, *Igf1*, and *Nr3c1*) correlated with ∑PCBs (ng/g lipid) in the blubber of ringed seals (n = 43). *Rara* is included as a representative of the three other transcripts examined that exhibited no association with ∑PCBs.

contaminated Baltic Sea where increased phase 1 enzyme activity and endocrine effects have recently been reported[11,43] and where a history of reproductive and developmental anomalies exists.[13]

For ringed seals, ∑PCB threshold values estimated for the five genes averaged 1,680 ± 206 ng/g lw, with the lowest being 1,370 ng/g lw for *Il1b* (Table 2). Three of the five genes (*Ahr*, *Il1b*, and *Nr3c1*) had a threshold similar to those derived for endocrine and immune effects in harbor seals (1,300 ng/g lw,[25]) and vitamin A disruption in beluga whales (1,600 ng/g lw,[14]), but lower than most other thresholds reported for marine mammals.[40,41] In contrast, *Esr1* (2,460 ng/g lw ∑PCBs) and *Igf1* (1,740 ng/g lw ∑PCBs) had the highest thresholds, suggesting that these parameters may be less sensitive to PCBs compared with the other molecular end points. Thus, the most conservative threshold value identified (1,370 ng/g lw ∑PCBs) may be considered as the most protective among those thresholds for ringed seals.

Adult males had the highest ∑PCB levels and exceeded our proposed threshold concentration of 1,370 ng/g lw (Table 1; Figure 1). One subadult seal had a PCB concentration (1,390 ng/g lw) that just slightly exceeded the effects threshold concentration. This is consistent with previous findings where the majority of adult males and a minority of subadult ringed seals exceeded the effects threshold (1,300 ng/g lw,[25]) for immunotoxicity and endocrine disruption in harbor seals.[23]

The ligand-induced aryl hydrocarbon receptor (AHR) mediates the metabolism of many POPs, including dioxin-like PCBs.[44] Such compounds bind to the AHR and modulate the activity of the transcriptional regulator which, in turn, induces

Table 2. Effects Threshold Estimates for the Hockey Stick Regression of the Relationship between Hepatic mRNA Gene Transcripts and Blubber PCB Concentrations in Ringed Seals (n = 43)

gene	threshold (log ng/g lw)	threshold (antilog ng/g lw)
Ahr	3.16	1460
Il1b	3.14	1370
Esr1	3.39	2460
Igf1	3.24	1740
Nr3c1	3.14	1380
average ± SE	3.23 ± 2.31	1,680 ± 206

expression of phase 1 and 2 xenobiotic detoxification enzymes, including cytochrome P450 enzymes (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*) and UDG-glucuronosyltransferases (*Ugt1a1*).[45] Consistent with the results of the present study, hepatic *Ahr* mRNA levels were correlated with non-*ortho* planar PCB concentrations in Baikal seals (*Pusa sibirica*)[46] and with total PCB concentrations in arctic beluga whales (*Delphinapterus leucas*).[15] In addition, positive relationships between blubber *Ahr* and PCB concentrations have been observed in heavily contaminated killer whales (*Orcinus orca*) from the Northeastern Pacific,[20] striped dolphins (*Stenella coeruleoalba*) from the Mediterranean,[47] and fin whales (*Balaenoptera physalus*) from the Mediterranean Sea and Gulf of California.[48]

A weight of evidence suggests that elevated PCBs and other POPs (e.g., polychlorinated dibenzo-*p*-dioxins and furans) may have contributed to marine mammal epizootics through reduced immunocompetence.[49] *Il1b* encodes a pro-inflamma-

Table 3. Akaike Information Criterion (AIC) in Combination with Backwards Stepwise Regression[a]

gene	predictors	r^2	p-value	AIC	AIC_c	ΔAIC_c	w_i
Ahr	PCBs, age, log_{10}weight	0.49	<0.001	256.5	264.9	0	0.71
	PCBs, age, log_{10}weight, HCH	0.50	<0.001	257.8	260.3	1.3	0.53
	PCBs, age, log_{10}weight, HCH, chlordanes	0.52	<0.001	258.2	262.9	1.7	0.11
Il1b	PCBs, age	0.26	0.005	178.3	179.4	0	0.48
	PCBs, age, log_{10}blubber	0.26	0.014	179.3	181.2	1.1	0.28
Esr1	PCBs, HCH	0.27	0.007	112.8	114.2	0	0.25
	PCBs, HCH, log_{10}weight	0.30	0.012	113.6	115.7	0.8	0.17
	PCBs, HCH, log_{10}weight, age	0.33	0.014	114.3	119.9	1.5	0.12
Igf1	PCBs, sex	0.24	0.007	328.3	329.5	0	0.42
	PCBs, sex, age	0.25	0.014	329.6	331.4	1.3	0.53
	PCBs, sex, age, HCH	0.28	0.017	329.9	332.4	1.5	0.47
Nr3c1	PCBs, sex, age, log_{10}weight, $\delta^{13}C$	0.35	0.009	148.9	151.5	0	0.29
	PCBs, sex, age, log_{10}weight, $\delta^{13}C$, HCH	0.37	0.015	149.3	154.0	0.42	0.24

[a]This confirms that PCB concentrations was the best variable to explain the variations of hepatic mRNA levels for the five genes which correlated with blubber PCB concentrations in ringed seals. Models with ΔAIC_c below 2 are presented. [b]AICc = second order AIC $n\log(\sigma 2) + 2K$) bias adjusted AIC for small sample size = AIC + $(2K(K + 1/(n - K - 1)$ where K is the total number of estimated regression parameters including $\sigma 2$ (no intercept) and n is sample size. [c]Δ_i = AIC differences computed as $AIC_i - AIC_{min}$. [d]$w_i = \exp(-1/2\Delta_i)/\Sigma\exp(-1/2\Delta_r)$.

tory cytokine that is involved in the pathological process of a number of diseases, including cancer.[50−52] Similar to our present observations, hepatic *Il1b* mRNA levels in ringed seals from the Baltic Sea were associated with increased hepatic PCB concentrations.[43] Expression of *Il1b* is additionally induced by dioxins via AHR.[53,54] We observed that *Il1b* and *Ahr* mRNA levels in the liver of ringed seals from the Labrador coast are positively correlated with PCB concentrations in blubber, suggesting that dioxin-like PCBs may be playing an active role in this animal population through AHR-dependent induction of both genes.

PCBs exhibit both estrogenic and antiestrogenic activity.[55] For example, PCBs can interfere with endocrine signaling by mimicking endogenous hormone action through binding to estrogen receptors and modulating their function with resultant impact on estrogen-dependent processes, such as steroid metabolism.[55] A few studies have looked for a possible link between disruption of estrogen-dependent processes and/or estrogen levels and PCBs in marine mammals. Female polar bears did not show a significant relationship between estradiol and PCB levels in plasma although a borderline negative relationship was observed between estradiol and PCB-118 in females with offspring.[56] Harbor seals fed PCB/DDE-contaminated fish from the Wadden Sea, The Netherlands, exhibited a perturbation in the estrus cycle consistent with the reduced reproductive success of seal populations from this geographic area.[57] Killer whales (*Orcinus orca*) from the Northeastern Pacific showed blubber *Esr1* mRNA levels to be positively correlated with blubber ∑PCB concentrations.[20] This latter observation is consistent with the results of the present study. Further, previous studies have reported a decrease in circulating levels of estrogen and PCBs in marine mammals from contaminated areas.[56,57]

Igf1 plays an important role in regulating cellular differentiation and proliferation, as well as a number of tissue-specific functions.[58] *Igf1* is also involved in the development of a number of diseases, including cancer, diabetes, and growth disorders.[59−61] Previous studies have suggested that exposure to environmental contaminants, such as PCBs and other aromatic hydrocarbons, may alter *Igf1* homeostasis in rats and humans.[62−64] Contaminant-related variation in *Igf1* mRNA levels has not been reported previously in marine mammals. However, *Igf1* mRNA levels were altered by prenatal PCB exposure in rats[63] and a negative correlation was observed between circulating levels of *Igf1* and PCB concentrations in human serum.[64]

The glucocorticoid receptor (NR3C1) is a steroid hormone receptor involved in regulating growth, development, metabolism, and apoptosis. Contaminant-related effects on *Nr3c1* mRNA levels have not been reported previously in marine mammals. However, there is evidence that PCBs can reduce the number of brain glucocorticoid receptors and that some PCB metabolites can bind competitively to glucocorticoid receptors.[65,66] In addition, a reduced or delayed cortisol response was observed in fish experiencing stress in heavily polluted waters,[67−69] suggesting that PCBs and other organochlorine contaminants may alter cortisol secretion. Further, grey seal (*Halichoerus grypus*) and ringed seal populations in the heavily polluted Baltic Sea suffered from a disease syndrome which is thought to have been caused by increased glucocorticoid hormones concentrations.[12]

The remaining 3 gene transcripts (Thyroid hormone receptor α (*Thra*), Retinoic acid receptor α (*Rara*), Thyroid-stimulating hormone β (*Tshb*)) in ringed seals exhibited no relationship with PCBs. These transcripts are involved in hormone signaling pathways, regulation of growth and metabolism, and development.[18,19,43] These results differ from those of more contaminated marine mammals, which may reflect the lower PCB dose to which our Labrador ringed seals were exposed. For example, *Thra* mRNA transcripts increased with PCB concentrations in harbor seals[19] and killer whales from the Northeastern Pacific Ocean,[20] and the liver of ringed seals from the Baltic Sea.[43] These marine mammals have concentrations 6 to 300-fold greater than ringed seals in the present study. Higher *Rara* mRNA transcripts with increasing PCB concentrations were observed in the liver of ringed seals from the Baltic Sea[43] and harbor seals from the Pacific Ocean.[18] Lastly, higher *Tshb* mRNA transcripts were reported with increasing PCB concentrations in the liver of ringed seals from the Baltic Sea.[43]

While univariate statistical approaches in the present study provided evidence of PCB-related increases in five hepatic gene transcripts (*Ahr*, *Il1b*, *Esr1*, *Igf1*, and *Nr3c1*), best-fit models using AIC (Table 3) confirmed that PCBs explained the

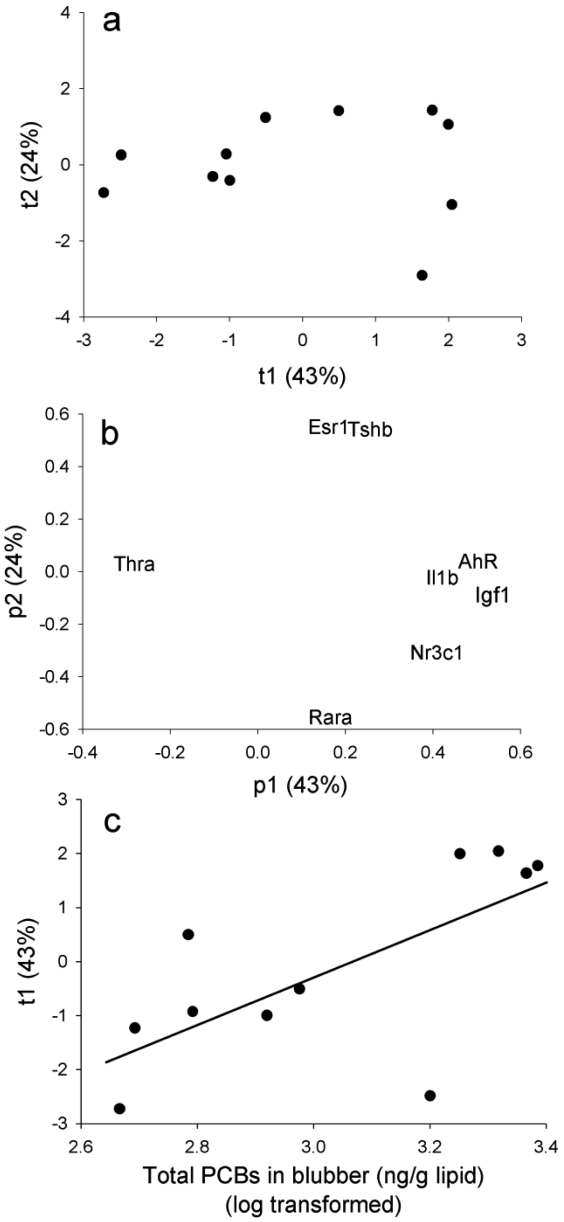

Figure 2. (a) Principal component analysis (PCA) of hepatic mRNA transcript levels of adult male Labrador ringed seals (n = 11). Each circle on the scores plot represents an adult male seal. (b) Eight mRNA gene transcripts were included in the PCA; a divergence between three gene transcripts (*Ahr*, *Il1b*, and *Igf1*) which correlated with ∑PCBs in the blubber and the other 5 gene transcripts was observed along the variable loadings of the first principal component (p1) (c) Sample scores of the first principal component (t1) was correlated with ∑PCBs.

variation in the five end points. In addition, age, sex, and weight (a proxy for age) contributed to the final model for 3, 2, and 2 of the 5 significant gene targets, respectively (Table 3). These statistical results build on the observations above, which showed that the adult males were most contaminated and were driving the up-regulation of the five PCB-correlated molecular end points (Figure 1).

Age and Sex-Class vs Vulnerability to PCB Effects. The females in the present study were far less contaminated than the adult males, which can likely be attributed to transfer of PCBs through the placenta and nursing to their young.[33,34] In this way, our findings that the females and subadults exhibited no relationship ($p > 0.05$) with PCBs, while the adult males did for 3 of the 5 significant gene transcripts (*Ahr*: $r^2 = 0.76$; $p = 0.007$; *Il1b*: $r^2 = 0.62$; $p = 0.004$; *Igf1*: $r^2 = 0.45$; $p = 0.025$) illustrates the way in which the contaminant burdens of our study animals straddle our derived effects threshold. The lack of relationship between *Esr1* and *Nr3c1* and ∑PCBs in adult males may be due to the small sample size (n = 11) and increased variation for these two transcripts. The previously demonstrated endocrine and immune threshold of 1,300 ng/g lw[25] is remarkably similar to the inflection point of our most conservative effects threshold (1,370 ng/g lw), providing strong support for an effects threshold in phocid seals for PCBs in this range. In transcriptomic studies of more contaminated marine mammal populations, PCBs drive gene expression response with secondary, limited contributions from age and sex,[20,43] further substantiating our threshold derivation for relatively low levels of PCBs.

PCA was used to further explore the factors underlying these patterns in adult males (Figure 2). Forty-three percent of variance in mRNA levels was explained by the first PCA factor (Figure 2a). The PCA variables plot of mRNA transcripts revealed a divergence of the three mRNA gene transcripts (*Ahr*, *Il1b*, and *Igf1*) which correlated with ∑PCBs and the other 5 gene transcripts (Figure 2b). ∑PCBs were correlated (r^2 = 0.42; $p = 0.04$) with the sample scores of the first principal component (t1) (Figure 2c). None of the other biological variables correlated with t1 or t2 ($p > 0.05$). Despite the lack of relationship between *Nr3c1* and ∑PCBs for adult males, *Nr3c1* was positioned relatively close to these three transcripts, suggesting that it too may be affected by ∑PCBs. These observations corroborate with our univariate findings for adult males with mRNA transcript levels for three of the eight genes being driven by ∑PCBs. Collectively, our findings suggest that while some Labrador ringed seals have low PCB levels which do not elicit detectable effects, others, notably the adult males are affected by elevated PCBs.

This present study provides a unique opportunity to evaluate the effects of PCBs on the health of a wild marine mammal, as this chemical class dominated tissue residues over other POPs. Despite declining PCB concentrations and associated effects in bottom-feeding fish (shorthorn sculpin) and seabirds (black guillemots) at Saglek Bay following remedial action,[70] we show here protracted effects in a long-lived high trophic level pinniped.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 provides qPCR primer pair sequences for genes used in ringed seal (*Pusa hispida*) liver. Table S2 provides primers and isolated ringed seal expressed gene sequences not submitted to NCBI GenBank due to the minimum length requirement of GenBank. Table S3 shows the linear regression relationships between five mRNA transcripts (*Ahr*, *Il1β*, *Esr1*, *Igf1*, and *Nr3c1*) and ∑PCBs. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Notes

The authors declare no competing financial interest.

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