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PCB-Associated Changes in mRNA Expression in Killer Whales (*Orcinus orca*) from the NE Pacific Ocean

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Supporting Information

ABSTRACT: Killer whales in the NE Pacific Ocean are among the world's most PCB-contaminated marine mammals, raising concerns about implications for their health. Sixteen health-related killer whale mRNA transcripts were analyzed in blubber biopsies collected from 35 free-ranging killer whales in British Columbia using real-time quantitative polymerase chain reaction. We observed PCB-related increases in the expression of five gene targets, including the aryl hydrocarbon receptor (AhR; $r^2 = 0.83$; $p < 0.001$), thyroid hormone α receptor (TRα; $r^2 = 0.64$; $p < 0.001$), estrogen α receptor (ERα; $r^2 = 0.70$; $p < 0.001$), interleukin 10 (IL-10; $r^2 = 0.74$ and 0.68, males and females, respectively; $p < 0.001$), and metallothionein 1 (MT1; $r^2 = 0.58$; $p < 0.001$). Best-fit models indicated that population (dietary preference), age, and sex were not confounding factors, except for IL-10, where males differed from females. While the population-level consequences are unclear, the PCB-associated alterations in mRNA abundance of such pivotal end points provide compelling evidence of adverse physiological effects of persistent environmental contaminants in these endangered killer whales.

INTRODUCTION

Killer whales (*Orcinus orca*) in the Northeastern Pacific Ocean are among the world's most polychlorinated biphenyl (PCB)-contaminated marine mammals, reflecting in part their long lifespan, high trophic level, and limited ability to metabolize recalcitrant contaminants.[1,2] Along with reduced abundance of their preferred prey (Chinook salmon), and noise and disturbance, high levels of endocrine-disrupting contaminants are touted as conservation threats to the resident killer whale communities.[3] The fish-eating Northern and Southern resident populations are listed under Canada's Species at Risk Act (SARA) as 'threatened' and 'endangered', respectively, and the marine mammal-eating transients have been listed as threatened (http://www.sararegistry.gc.ca/default_e.cfm). The transboundary Southern residents are also listed in the USA as 'endangered' under the Endangered Species Act (http://www.nwr.noaa.gov/Marine-Mammals/Whales-Dolphins-Porpoise/Killer-Whales/ESA-Status/Orca-Recovery-Plan.cfm). While logistical, legal, and ethical constraints limit toxicological research in free-ranging killer whales, evidence from other marine mammals[4,5] would suggest that PCB-related effects including immunotoxicity, endocrine disruption, and reproductive impairment represent a distinct concern for killer whales.[3] PCB concentrations measured in biopsies collected from killer whales[1,6] exceed the effects threshold established for harbor seals (17 mg/kg PCBs in blubber) by several times.[7,8]

Given the inherent challenges associated with studying wild populations of marine mammals and/or endangered species, new molecular approaches offer the promise of insight into toxicological injury. Such approaches are of particularly utility as they require minimal amounts of tissue and provide quantitative information on specific physiological systems. Changes in the expression profiles of mRNA transcripts encoding detoxification proteins represent a key component of the biological response of an animal to chemical contaminant exposure and in the maintenance of good health. Thus, ecotoxicological screening methodologies based upon genomics provide for a potentially powerful means to evaluate the effects of anthropogenic contaminants in wildlife species.

Some studies have used DNA arrays to assess the expression of genes related to stress response and immune function in marine

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mammals.[9−11] While DNA arrays are useful in evaluating large numbers of gene transcripts at one time, no killer whale-specific DNA arrays have been developed, and independent validation (using e.g. quantitative real-time polymerase chain reaction (QPCR)) would be required to apply or adapt data generated from DNA arrays from other marine mammal species to killer whale tissues.[12] An alternative approach, and one that displays greater quantitative power, is the development of a suite of high-value QPCR-based tools that are specific to killer whales and which help to quantify the levels of mRNA transcripts which relate to mechanisms of toxic injury. Changes in mRNA transcript abundance using QPCR have been linked to exposure to persistent environmental contaminants.[13−17] For example, harbor seals in the NE Pacific Ocean have exhibited PCB-related alterations to thyroid and retinoid receptor expression.[18,19]

In the present study, we obtained biopsy samples from 35 killer whales for which detailed Supporting Information is available (i.e., age, sex, and feeding preferences). These samples were used to isolate cDNA and develop species-specific QPCR assays to determine abundance profiles for mRNA transcripts encoding protein products representative of endocrine and immune functions and chemical detoxification. This enabled assessment of the relationship between mRNA abundance profiles in killer whales and the concentrations of PCBs, the primary contaminant of concern in this species.

EXPERIMENTAL SECTION

Tissue Sample Collection. Skin and blubber samples were obtained by biopsy dart from killer whales frequenting the coastal waters of central and northern British Columbia, Canada, as previously described.[1] Biopsies were taken from the region immediately posterior to, and below, the dorsal fin using a lightweight pneumatic dart system.[20] Visual confirmation of identity, age, and sex were based on photographic catalogues of resident and transient killer whales (http://www.pac.dfo-mpo.gc.ca/science/species-especes/cetacean-cetaces). After extraction from the dart, biopsies were immediately wrapped in hexane-rinsed aluminum foil, encased in 5 cc cryovials (Eppendorf, Westbury, NY, USA), and immersed in liquid nitrogen. Samples were collected in 2003, 2004, and 2007, although the 2007 samples (n = 9) were subsampled in the field along the full length of the tissue biopsy and a ∼0.5 cm diameter portion was immersed in 5 cc cryovials containing 4.5 mL of RNA*later* solution (Applied Biosystems, Foster City, CA, USA). These samples were stored immediately on ice and, after 24 h, transferred to −20 °C prior to isolation of total RNA. The remaining tissue sample was immediately placed in liquid nitrogen in the field, as described above.

Biopsies that were stored in liquid nitrogen were subsampled in the laboratory following previously established protocols.[18,19] Skin (4 mm in depth) was removed, and the blubber biopsy was divided along its longitudinal length into pieces of approximately 20−100 mg. A 100 mg piece was used for PCB analysis following storage at −80 °C in hexane-rinsed foil inside a cryovial. A 20 mg piece of blubber (from the outer layer; see below) and a 20 mg piece of skin were used for total RNA isolation. These were soaked in 4 mL of precooled RNA*later* ICE (Applied Biosystems) as per the manufacturer's instructions and stored at −20 °C prior to total RNA isolation.

Total RNA Isolation and cDNA Preparation. Blubber biopsies varied in length, and because mRNA levels may display stratification within blubber tissue,[19] 10 mm (∼20 mg) of the outer blubber layer adjacent to the skin layer was consistently used for total RNA isolation. Total RNA was isolated from each tissue sample using Trizol reagent as previously described.[19] All samples were homogenized in 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 (Invitrogen, Burlington, ON, Canada) and a 3 mm diameter tungsten-carbide bead. Samples were homogenized in three six-minute intervals at a frequency of 20 Hz. An additional six minutes of homogenization was performed if significant amounts of nondisrupted tissue remained. Samples were cooled on ice between homogenization intervals.

After isolation, RNA pellets were resuspended in 40 μL of diethyl pyrocarbonate (DEPC)-treated RNase-free water (TEKnova, Hollister, CA, USA) and stored at −80 °C. Spectrophotometry was used to determine RNA concentration, and 2 μg of total RNA were used to prepare cDNA. Total cDNA was produced using Superscript II reverse transcriptase, as described by the manufacturer (Invitrogen). Each cDNA reaction was diluted 30-fold using DEPC-treated RNase-free water prior to analysis by QPCR.

QPCR Assay. Thirteen gene transcripts were selected for their potential to provide insight into general animal health or response to contaminant exposure. These included estrogen receptor α (ERα), thyroid receptor α and β (TRα/TRβ), aryl hydrocarbon receptor (AhR), interleukin 10 (IL-10), metallothionein 1 (MT1), heat shock protein 70 (HSP70), glucocorticoid receptor (GR), adiponectin (ADP), leptin (LEP), insulin-like growth factor 1 (ILGF-1), peroxisome proliferator activated receptor γ (PPARγ), retinoid X receptor α (RXRα), and vitamin D receptor (VDR) (Table S1, online Supporting Information). Three additional mRNA were selected as potential invariant normalizers for correction of experimental variance: ribosomal protein L8 (rpL8), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytoplasmic β actin (CBA).

cDNA sequences for the desired mRNA targets were isolated using degenerate primers designed against aligned multiple cDNA sequences from different mammalian species obtained from the NCBI Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/). Sequence identity was confirmed by BLASTn alignment. NCBI GenBank accession numbers of each isolated killer whale cDNA sequence are reported in Supporting Information Table S1. Species-specific QPCR primer pairs were subsequently designed and assessed in a three-tier quality control process using amplification reactions containing a mixed cDNA template representing a combination of skin and blubber samples as described previously.[13] QPCR primer pair specificity, sensitivity, and compliance with the requirements for the mRNA comparative ($\Delta\Delta$CT) quantification method were established. QPCR assays were conducted as described previously [19] with modifications described in the Supporting Information. Briefly, each killer whale blubber cDNA was assessed for each gene target in quadruplicate on a Realplex4 Eppendorf system (Eppendorf). The average cycle threshold (C_t) values were obtained for each mRNA and normalized using rpL8 C_t values, as this transcript was the only normalizer candidate that proved invariant between sample groups and was representative of cDNA input. Data were expressed as relative fold differences in mRNA abundance determined between individuals.

PCB Analysis. Blubber samples were analyzed for PCBs at the Institute of Ocean Sciences (Fisheries and Oceans Canada, Sidney, BC, Canada) according to established laboratory procedures.[21]

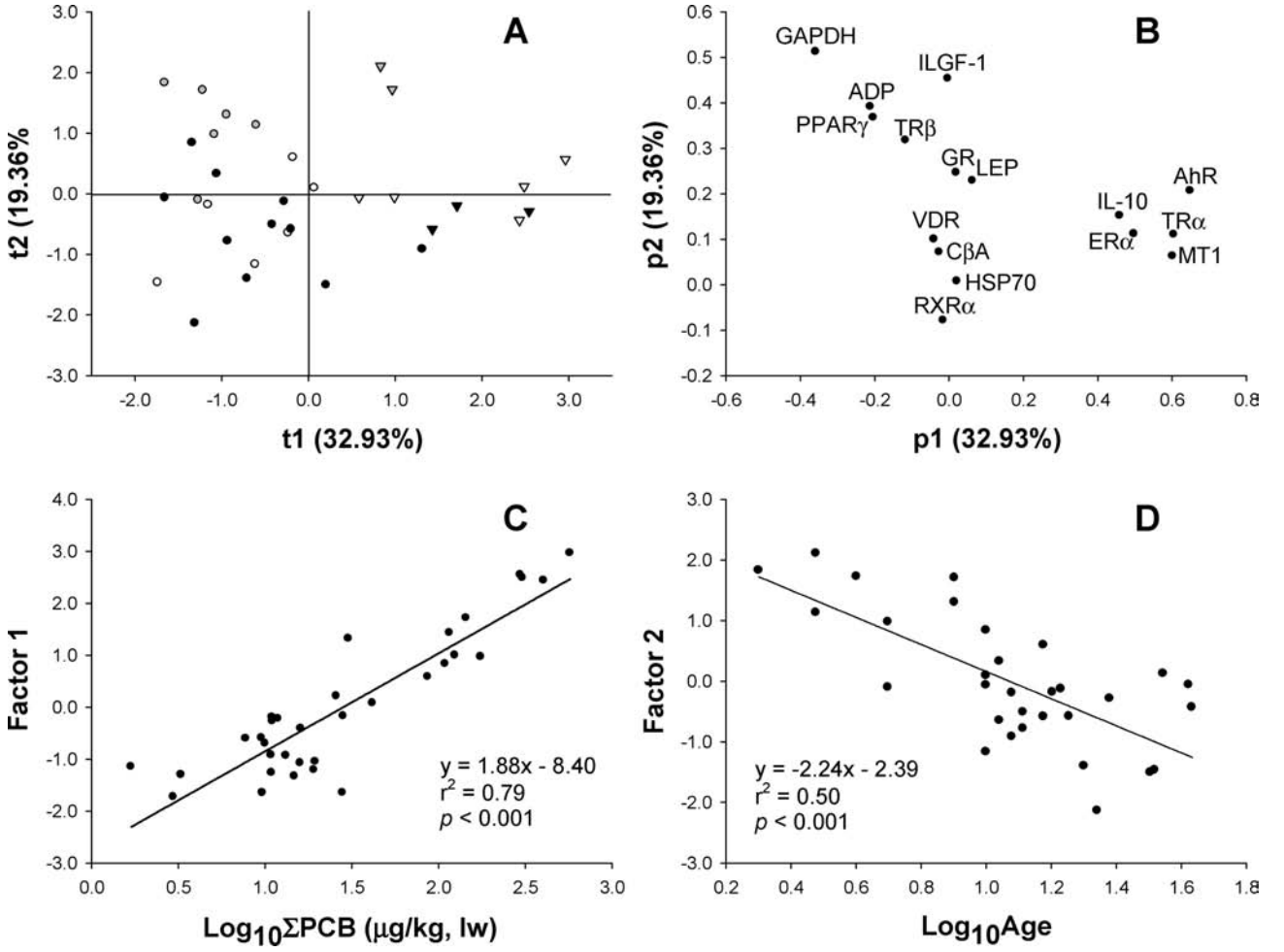

Figure 1. (A) Principal Components Analysis (PCA) of mRNA transcript abundance in blubber of transient killer whales (triangles) and northern resident killer whales (circles). Adult females are indicated by open symbols, adult males by closed symbols, and juveniles by gray symbols. (B) The 16 mRNA transcripts involved in the PCA are shown (see Table S1 for full names). (C) Factor 1 in the PCA was tightly correlated with ΣPCB concentration, while (D) factor 2 negatively correlated with animal age.

Following sample cleanup procedures, PCB congeners were quantified by high-resolution gas chromatography equipped with high-resolution mass spectrometry detection (HRGC-HRMS) as previously described.[21] Percent lipid was determined gravimetrically, and ΣPCB concentrations were lipid-normalized and $\log_{10}$ transformed. We sought 206 PCB congeners and detected 136 congeners above individual congener detection limits (DL) in greater than 70% of samples and 35 congeners above DLs in less than 70% of killer whale samples. Congener-specific concentrations for PCBs measured in each individual killer whale are provided in Supporting Information Table 2.

Data Analysis. For the purpose of this paper, we present only transcript results for blubber, as the results in blubber and skin were tightly correlated for all end points ($p < 0.05$; data not shown). The abundance of mRNA levels (reported as relative fold difference) and ΣPCB concentrations were examined for normality using the Shapiro-Wilk test and for homogeneity of variance using Levine's test. Results were considered significant at $p < 0.05$. Herein we report a full data set of PCB and mRNA data for 35 killer whales, after one 2004 killer whale outlier (>average ± two times standard deviation) was removed on the basis of mRNA results. Degradation of RNA in this sample during field collection may have led to poor subsequent QPCR quantification.

Three statistical methods were used to assess the association between mRNA levels and PCB concentrations in 35 killer whale blubber biopsies. First, an exploratory analysis of mRNA abundance patterns using a principal component analysis (PCA). PCA (using SYN-TAX Ordination 2000 (Budapest, Hungary)) was used to characterize mRNA abundance patterns among killer whales and generate insight into factors affecting them. Correlation analysis was carried out using the Pearson method for normally distributed data or the nonparametric Kendall's tau-b method when data were not normally distributed.

Second, a univariate regression analysis was conducted on the relationship between each of the mRNA end points and blubber PCB concentrations. Relative fold change of specific mRNA transcripts was regressed against logΣPCB concentrations using GLM in Systat 12 (Systat Software Inc., 2004, San Jose, CA). Analysis of variance (ANOVA) was used to test homogeneity of slopes between resident and transient populations for these regressions.

Third, analysis of covariance (ANCOVA) was carried out in Systat 12 (Systat) to determine whether any biological factors confounded the relationship between PCB concentrations and mRNA abundance end points. Interactions between PCB concentration in blubber, population, age, and sex were assessed,

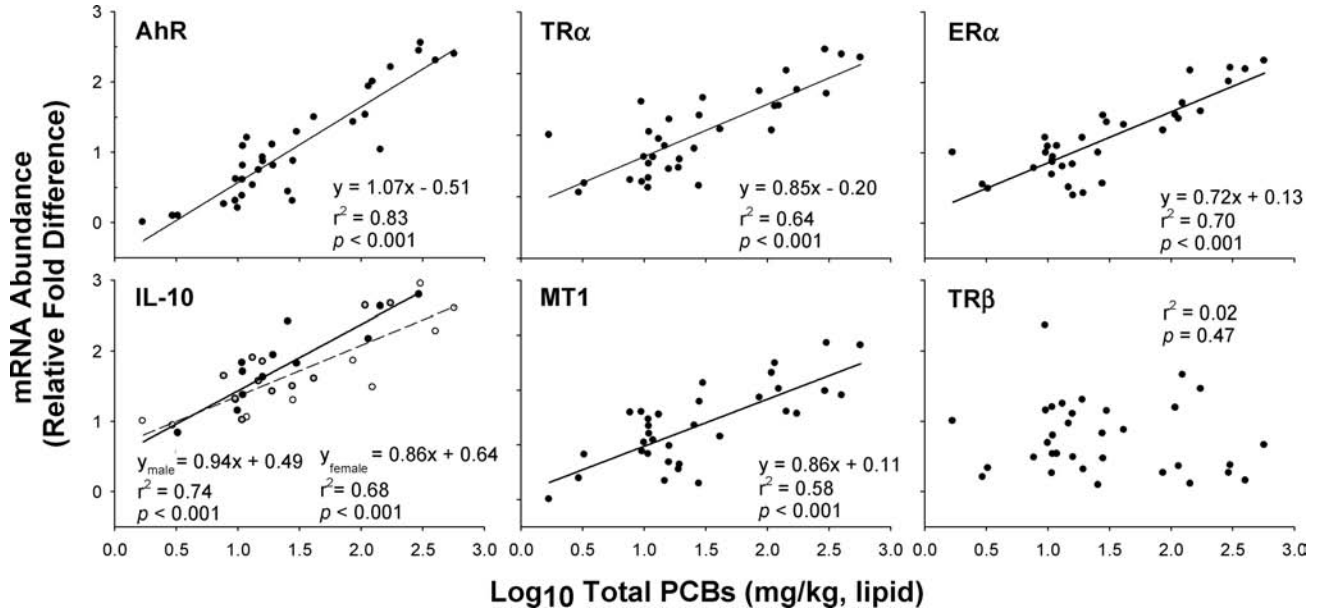

Figure 2. Transcript abundance for five mRNA (AhR, TRα, ERα, IL-10, and MT1) correlated with ΣPCB concentrations in killer whales. TRβ is included as a representative of the 11 transcripts examined that exhibited no association with ΣPCB levels. Solid lines represent relationships between mRNA abundance and ΣPCB concentrations for both sexes combined, except in the case of IL-10 where the line represents only males. For the IL-10 transcript, where sex-dependent differences exist in mRNA abundance, open symbols represent adult females, black symbols represent adult males, and gray symbols represent juvenile whales. The dotted line in the IL-10 plot represents the relationship between mRNA levels and PCBs in females.

followed by Tukey's post hoc test when normally distributed with homogeneity of variance. Krustal-Wallis and Mann−Whitney tests were used when non-normal distributions were observed. SAS Version 4.1 (SAS Institute Inc., Cary, NC) was used to calculate the Akaike Information Criterion (AIC) and its small sample size form, AICc, to determine best fit models given the data, whereby lowest AICc indicated best fit. The Akaike differences (Δ_i) and normalized Akaike weights were use to derive w_i, a "weight of evidence" to select the best variable or variables for the model. A ΔAIC of zero and up to two are considered to be the most important model variables.

RESULTS AND DISCUSSION

Biopsies were obtained from 35 killer whales (Table S2), including males and females of a range of known ages (from 2−43 years), during the years 2003 (n = 14), 2004 (n = 6), and 2007 (n = 16). These whales represent two distinct populations: the heavily PCB-contaminated marine mammal-eating transients (n = 12; mean ± SD, ΣPCB 347.0 ± 81.1 mg/kg lipid weight) and the less contaminated fish-eating northern residents (n = 24; ΣPCB = 17.9 ± 3.2 mg/kg). PCB concentrations are consistent with those previously reported for these whale populations.[1,6] While many persistent halogenated contaminants are found in marine food webs, PCBs are considered the primary toxicological concern in these killer whales and in other marine mammals in the northeastern Pacific Ocean.[3,22] Sex and $\log_{10}$Age data within each population were normally distributed, and there was homogeneity of variance for these variables between populations ($p > 0.05$).

Principal components analysis was used to characterize mRNA abundance patterns in killer whales and to explore the factors underlying these patterns (Figure 1A and 1B). Fifty-two percent of variance in mRNA levels was explained by the first and second PCA factors. Killer whale scores were differentiated on the basis of variation in the levels of 16 mRNA, with AhR, TRα, ERα, IL-10, and MT1 mRNA transcripts clustering at the right of the plot (Figure 1B). Factor 1 on the PCA correlated positively with $\log_{10}$ΣPCB concentrations ($r^2 = 0.79$; $p < 0.001$; Figure 1C) as well as the total toxic equivalency concentration (ΣTEQ) calculated for the PCBs ($r^2 = 0.46$; $p < 0.001$, data not shown). Given the overwhelming contribution of PCBs to the sum TEQ, the strong correlation between sum TEQ and sum PCB was not surprising ($r^2 = 0.61$; $p < 0.001$). Factor 2 correlated negatively with $\log_{10}$Age ($r^2 = -0.50$; $p < 0.001$; Figure 1D). Juvenile whales clustered in the upper quadrants of the PCA plot, while adults clustered toward the center and the lower quadrants of the plot (Figure 1A). Transcripts that contributed to the variation in the juvenile killer whales include GAPDH, ILGF-1, ADP, PPARγ, LEP, and TRβ. Higher mRNA transcript levels for these end points in juveniles may reflect the heightened metabolic and physiological demands associated with growth and development in younger animals.[23]

Five of the 16 mRNA transcript markers assessed in killer whales had strong positive correlations with PCB concentrations, including the AhR ($r^2 = 0.83$, $p < 0.001$), TRα ($r^2 = 0.64$, $p < 0.001$), ERα ($r^2 = 0.70$, $p < 0.001$), IL-10 ($r^2 = 0.74$, $p < 0.001$; 0.68, $p < 0.001$, males and females respectively), and MT1 ($r^2 = 0.58$, $p < 0.001$) (Figure 2 and Table S3). There were no correlations between the levels of any of the other gene transcripts evaluated and ΣPCB concentrations ($p > 0.05$) (Table S3). Results of ANOVA indicate that there was homogeneity of slopes between resident and transient populations for all 5 gene targets ($p > 0.05$). These results, coupled with the observation that the five mRNA transcripts clustered together in the PCA with killer whales containing elevated PCB levels, strongly suggests that the PCBs and related compounds influence mRNA abundance for these genes.

As ΣPCB concentrations in marine mammal-eating transient killer whales are higher than those in fish-eating northern residents,[1]

Table 1. Best Fit Regressions Using Akaike Information Criterion (AIC) Revealed That PCB Concentrations, and Not Age or Sex, Influenced Gene Expression (Relative Fold Change) of the Five Targets Which Correlated with PCB Concentrations in Killer Whales[d]

gene	model #	model equation	r^2	p value	AIC	AIC_c[a]	ΔAIC_c[b]	w_i[c]
AhR	1	y = LogPCB	0.84	<0.001	**21.25**	**22.00**	**0.00**	0.61
	2	y = LogPCB + Population + Population*LogPCB	0.86	<0.001	20.17	24.17	2.17	0.21
	3	y = LogPCB + LogAge + LogAge*LogPCB	0.82	<0.001	21.29	24.44	2.44	0.18
	4	y = LogPCB + sex + sex*LogPCB	0.84	<0.001	27.45	31.45	9.45	0.00
	5	y = LogPCB + Year + LogPCB*Year	0.74	<0.001	29.05	33.05	11.05	0.00
TRα	1	y = LogPCB	0.58	<0.001	**52.78**	**53.53**	**0.00**	0.74
	2	y = LogPCB + Population + Population*LogPCB	0.59	<0.001	55.79	57.79	4.26	0.09
	3	y = LogPCB + LogAge + LogAge*LogPCB	0.56	<0.001	54.66	56.80	3.27	0.14
	4	y = LogPCB + sex + sex*LogPCB	0.60	<0.001	58.89	62.98	9.45	0.01
	5	y = LogPCB + Year + LogPCB*Year	0.62	<0.001	57.22	61.22	7.69	0.02
ERα	1	y = LogPCB	0.66	<0.001	**29.04**	**29.79**	**0.00**	0.84
	2	y = LogPCB + Population + Population*LogPCB	0.70	<0.001	29.44	35.44	5.65	0.05
	3	y = LogPCB + LogAge + LogAge*LogPCB	0.62	<0.001	33.21	35.35	5.56	0.05
	4	y = LogPCB + sex + sex*LogPCB	0.70	<0.001	32.60	36.60	6.81	0.03
	5	y = LogPCB + Year + LogPCB*Year	0.70	<0.001	32.58	36.58	6.79	0.03
IL-10	1	y = LogPCB	0.68	<0.001	29.18	29.93	1.53	0.29
	2	y = LogPCB + Population + Population*LogPCB	0.68	<0.001	33.07	35.07	6.67	0.02
	3	y = LogPCB + LogAge + LogAge*LogPCB	0.73	<0.001	30.84	34.84	4.91	0.05
	4	y = LogPCB + sex + sex*LogPCB	0.78	<0.001	**26.28**	**28.40**	**0.00**	0.62
	5	y = LogPCB + Year + LogPCB*Year	0.71	<0.001	33.36	37.36	8.96	0.01
MT1	1	y = LogPCB	0.43	<0.001	**61.38**	**62.38**	**0.00**	0.66
	2	y = LogPCB + Population + Population*LogPCB	0.45	<0.001	64.41	66.41	4.03	0.09
	3	y = LogPCB + LogAge + LogAge*LogPCB	0.62	<0.001	62.82	64.96	2.58	0.18
	4	y = LogPCB + sex + sex*LogPCB	0.44	<0.001	68.95	72.95	10.57	0.00
	5	y = LogPCB + Year + LogPCB*Year	0.53	<0.001	62.84	66.84	4.46	0.07

[a] AIC_c = second order Akaike information criteria (AIC = nlog (σ^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 σ^2 (no intercept), and n is the sample size. [b] Δi = AIC difference computed as AIC_i - AIC_{min}. [c] $w_i = \exp(-1/2\Delta_i)/\Sigma\exp(-1/2\Delta_i)$. [d] The best fit model for each gene was selected by lowest AIC (identified by bold characters). The one exception among these targets was IL-10, which was influenced by a combination of sex and PCB concentrations.

we assessed whether population (e.g., diet or genetics) influenced relationships between mRNA abundance end points and ΣPCB concentrations. Best fit models using AICc (Table 1) revealed that population did not influence any of the relationships observed between mRNA transcript levels (relative fold difference) and ΣPCB concentrations. Age and sex also had no significant influence on these relationships (Table 1), except in the case of IL-10 where males displayed a greater relationship of IL-10 transcript level to ΣPCB concentration relative to females. For AhR, TRα, ERα, and MT1, best fit models were supported by weighted probability measurements of 61% or greater in favor of model 1 (y = LogPCB) with remaining models (Table 1) having ΔAICc values all >2 and model weights <0.4. For IL-10, the best fit model (y = LogPCB + LogAge + LogAge*LogPCB) was supported by a weighted probability of 47%, although model 1 (y = LogPCB) also had strong model weight (47%) with ΔAICc value of <2. Similar to our observations, no relationship between TRα or TRβ mRNA levels and body weight (a proxy for age) was observed in free-ranging juvenile harbor seals, although adults were not investigated.[19] In contrast to killer whales, AhR transcript abundance did correlate with age in Baikal seals,[16] whereas the role of age in a study of IL-10 mRNA levels in diseased versus healthy harbor porpoise was unclear.[14] In addition to assessing the biological factors (i.e., population, age, and sex) that could have confounded the results of this study, we assessed the effect of interannual variation on the relationship between gene expression and PCB concentrations in sampled killer whales. While gene expression for IL-10 (p = 0.033), AhR (p = 0.045), and TRα (p = 0.045) varied between sampling years, this did not confound the very strong relationships observed for the five gene targets which exhibited correlations with PCB concentrations (p < 0.05; Table 1). A larger sample size would presumably provide insight into the extent to which additional factors (e.g., food limitation) might influence gene expression in killer whales.

Evidence from carefully controlled laboratory and semifield studies demonstrates the ability of PCBs to disrupt endocrine function, immune function, and metabolic processes, leading to developmental, reproductive, and immune system impairment.[24] The PCA and subsequent univariate and multivariate evaluations presented herein identify five particular gene transcripts that have strong associations with PCBs in free-ranging killer whales.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that controls the expression of a diverse set of genes involved in xenobiotic metabolism.[25] The AhR is implicated in the biochemical, teratogenic, and lethal effects of dioxin-like compounds that include PCBs.[26,27] The contaminant-related responsiveness of AhR, and subsequent induction of cytochrome (CYP) P450 expression, has been used as a biomarker of

exposure to dioxin-like PCBs and related compounds in marine mammals and other wildlife.[25,28,29] Consistent with our results, hepatic AhR mRNA in Baikal seals (*Pusa sibirica*) correlated with increasing non-*ortho* planar PCB concentrations as well as to hepatic CYP1A mRNA and protein levels.[16] It was suggested that up-regulation of AhR may result in metabolic and physiological changes through enhancement of AhR-CYP pathways.[16] Induction of CYP1A1 through AhR action has been shown to mediate PCB-related effects on TRs [30] as well as inhibiting ERα activity [31] in rodents.

Thyroid hormones (THs) play central roles in growth, development, and metabolism in vertebrates, largely through regulation of mRNA expression via the nuclear receptors TRα and TRβ.[32] PCBs and related compounds have been associated with a disruption of TH physiology, including hormone synthesis, circulatory transport of TH, and TH metabolism in the liver and brain.[33,34] In marine mammals, altered circulating concentrations of THs have been observed in PCB-exposed individuals.[19,35−37] Our present observation of a disruption of TRα mRNA transcript but not TRβ in killer whales is consistent with results obtained in free-ranging harbor seals from British Columbia and Washington State, providing additional support for the notion that PCBs represent a credible threat to the health of marine mammals.[19] Although we could not measure TH in the blood of free-ranging killer whales, decreased circulating TH in harbor seals occurred as TRα mRNA levels in blubber increased; this was speculated to reflect a contaminant-related increase in metabolic turnover.[19] This could represent a major concern for marine mammals that rely on blubber for thermoregulation and energy storage in a cool ocean climate.

Estrogens are an important class of steroid hormones that influence essential biological functions such as growth, cellular differentiation, organ development, and reproduction. Hormonal actions are mediated through a class of nuclear ligand-inducible transcription factors including estrogen receptors (ERs).[38] PCBs have estrogenic or antiestrogenic effects on ER expression, interfere with ligand-activated ER binding to promoter-associated estrogen response elements, and disrupt hormone-dependent transcription of ER inducible genes including cytochrome P450 monooxygenases 1A1, 1A2, or 1B1.[39] Negative correlations between circulating levels of sex hormones (estrogen, testosterone, and progesterone) and PCB concentrations have been observed in marine mammals inhabiting contaminated areas.[40−43] To our knowledge, contaminant-related variation of ER mRNA levels has not been reported in any other marine mammal. PCB-exposed rodents had no differences in expression of ERα relative to controls.[44] However, there is some evidence that overexpression of ERα may alter steroid hormone concentrations and/or AhR-dependent CYP1A1 expression and consequently lead to reproductive and neurotoxic effects.[31]

IL-10 is a potent anti-inflammatory cytokine produced by lymphoid cells that functions in the termination of an immune response.[45] PCB-related effects on immune function have been reported previously, including diminished cellular and humoral responses.[8,46−48] Increases in IL-10 mRNA in diseased harbor porpoises correlated with PCB concentrations.[14] Continuous production of IL-10 can suppress cytokine responses to illness and heighten susceptibility to bacterial infections.[49] The observed relationship between increased IL-10 mRNA transcript levels and PCBs in killer whales may, therefore, reflect a chronic stimulatory effect on the immune system by these contaminants or may be due to increased incidence of inflammatory and/or disease processes in highly exposed killer whales.

Metallothioneins (MTs) comprise a family of cysteine-rich proteins that bind heavy metals (e.g., Cd, Hg, and Ag), providing protection against metal toxicity and oxidative stress.[50] While PCBs are not typically associated with MTs, the endocrine-disrupting activities of PCBs and their metabolites may interfere with metal-induced expression of MT.[51] In addition, Hg is a known ligand of MT1 [50] and, in its methylated (organic) form, biomagnifies in aquatic food webs along with PCBs.[52] Increased MT1 mRNA expression has been observed in sled-dogs fed whale blubber containing high concentrations of both Hg and organochlorines.[53] Although we did not measure Hg in the present study, positive relationships between Hg and PCB contaminant levels in aquatic wildlife (reviewed in ref 52) could underlie our observed relationship between MT1 mRNA levels and blubber concentrations of PCBs. Accordingly, our findings may indicate a physiological response to increasing Hg concentrations in killer whales. While marine mammals are thought to be able to sequester and/or detoxify ingested Hg,[54] continued delivery to aquatic ecosystems of anthropogenically released Hg will likely increase the risk of Hg-related toxicity in high trophic level wildlife including killer whales.

The remaining 11 genes evaluated in this study did not exhibit any relationships with PCB concentrations in killer whales; these included TRβ, VDR, HSP70, GR, ADP, LEP, ILGF-1, PPARγ, RXRα, GAPDH, and CBA (Table S3). While PCBs may affect VDR in mammals,[54,55] the evidence that PCBs and related compounds can affect these remaining 11 genes is scant. The indifference of these 11 gene targets to high PCB concentrations in our killer whales illustrates the apparent strength of the five remaining gene targets as biomarkers of toxicity.

PCBs are not the only contaminants of concern in killer whales in the NE Pacific Ocean. Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated biphenyls (PBBs), polychlorinated naphthalenes (PCNs), polybrominated diphenyl ethers (PBDEs), and organochlorine pesticides have been detected in biopsies obtained from free-ranging individuals.[3,57−60] While it is unlikely that PCB contamination alone explains our observations of altered mRNA abundance end points, this chemical class is present at very high concentrations in killer whales and correlates strongly with the mRNA transcript end points currently investigated. In addition, a weight of evidence from laboratory-based studies provides a plausible mechanistic basis for our observations. PCBs therefore represent the predominant toxicological concern in killer whales, but a contribution from other chemical contaminants cannot be entirely ruled out. A risk-based evaluation of persistent organic contaminants measured in harbor seals in the northeastern Pacific Ocean suggested that PCBs presented a greater health risk than all other POPs combined.[22] However, rapidly increasing PBDE concentrations in fish and marine mammals in the northern hemisphere highlight the gradual shifting profile of contaminants of concern in high trophic level biota.[3,61,62]

While toxicological research in endangered species is limited by inherent technical, legal, and ethical constraints, our biopsy-based results from free-ranging killer whales provide compelling insight into the effects of one of the leading chemicals of global environmental concern. These results provide a stark reminder of the lingering health risks associated with persistent and bioaccumulative contaminants such as the PCBs, particularly in long-lived, high trophic level wildlife.[3] Although the population-level consequences of our present observations are unclear, observations of increased mortality and reduced reproductive success in

the highly contaminated southern resident killer whales, comprising fewer than 90 individuals, are of particular concern.[63] Exposure to environmental chemical contaminants, diminished abundance of preferred prey (Chinook salmon), and disturbance by human activities bring into question the viability of small killer whale populations (http://www.dfo-mpo.gc.ca/species-especes/index-eng.htm). Reduced exposure to persistent environmental contaminants will be important to the recovery and long-term survival of killer whale populations, and longitudinal health assessments using the latest techniques represent an important means of gauging progress in this regard.

■ ASSOCIATED CONTENT

Supporting Information. The Methods section details QPCR assay development. Table S1 lists DNA primers used in cloning and QPCR analysis of killer whale target mRNA and provides GenBank Accession numbers. Table S2 summarizes the levels of PCBs measured and characteristics of the killer whales analyzed for mRNA abundance in the present study. Table S3 lists regression values (r^2) and significance (p-value) of relative fold change values regressed against $\log_{10}$PCBs for mRNA of interest in blubber. This material is available free of charge via the Internet at http://pubs.acs.org.

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