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Contaminant Exposure Linked to Cellular and Endocrine Biomarkers in Southern California Bottlenose Dolphins

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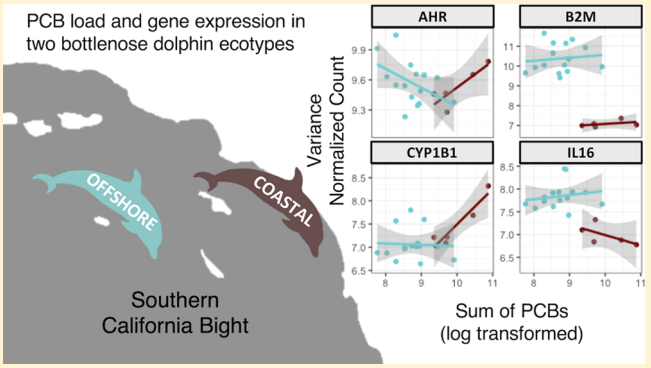
Supporting Information

ABSTRACT: Cetaceans in the Southern California Bight (SCB) are exposed to high levels of halogenated organic contaminants (HOCs), which have previously been linked to impaired reproductive health and immune responses. We used a combination of molecular tools to examine the potential physiological impacts of HOC exposure in two bottlenose dolphin (*Tursiops truncatus*) ecotypes in the SCB. We quantified 25 HOCs in the blubber of 22 biopsies collected from males between 2012 and 2016. We then analyzed genome-wide gene expression in skin using RNA-sequencing and measured blubber testosterone to compare HOC exposure with cellular and endocrine biomarkers. We found high levels of HOCs in both ecotypes with significantly higher total polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), tris(4-chlorophenyl)methanol (TCPMOH), and chlordane-related compounds in the coastal ecotype versus the offshore ecotype. We found evidence of PBDE bioaccumulation in both ecotypes, however, the pattern of bioaccumulation or endocrine disruption for other HOCs was different between the ecotypes, suggesting potential endocrine disruption in the coastal ecotype. We also observed correlations between HOCs and gene coexpression networks enriched for xenobiotic metabolism, hormone metabolism, and immune response that could indicate cellular effects from HOC exposure. By integrating measurements of HOC load with both transcriptome profiling and endocrine biomarkers, our approach provides insight into HOC exposure and potential impacts on wild cetacean health in southern California.



INTRODUCTION

Marine mammals in the Southern California Bight (SCB) possess some of the highest concentrations of halogenated organic contaminants (HOCs) in the world, most notably legacy contaminants like DDT, PCBs, and PBDE.[1−3] While certain legacy contaminants have declined in the SCB since their production has been discontinued,[1] top predators are still exposed to a large number of HOCs throughout their lifespan[4,5] and legacy compounds remain a threat to long-term population health.[6] Marine mammals are also known sentinels of ocean and human health and can serve as a model with which to investigate health risks due to exposure to marine contaminants in humans and other top predators.[7,8] Exposure to HOCs has been linked to compromised reproductive health in *Delphinus delphis* in the region[5] yet relatively few studies have investigated physiological responses to HOC exposure in free-ranging individuals despite long-standing concerns of the prevalence of HOCs in the SCB.[1,9]

HOCs are often nonlethal at low doses in wildlife but a large accumulated chemical burden can reduce fitness and increase vulnerability to other stressors, such as extreme climatic events and infectious diseases.[10] Putative effects of sublethal exposure to HOCs in marine mammals are similar to those observed in humans, i.e., immunosuppression, increased physiological stress, and impaired reproduction.[11−16] These sublethal effects are thought to be caused in part by the disruption of endocrine hormones,[17−19] including testosterone, a hormone important for sexual and reproductive development.

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One way to assess how HOC loads are impacting wild cetaceans is by quantifying hormone levels in blubber, the thick layer of adipose tissue directly under the skin. Blubber hormone analysis offers an opportunity to evaluate endocrinology in live, free-ranging cetaceans using biopsies composed of skin and blubber collected in the field. Because HOCs accumulate with age in male cetaceans[13,20] and blubber testosterone also increases with maturity,[21] a positive relationship between blubber testosterone with HOC load could provide a measure of bioaccumulation. Blubber analyses can also be used to assess evidence of endocrine disruption[5] and blubber testosterone has already been used to demonstrate potential endocrine disruption within mature *D. delphis*.[5] In tandem with contaminant analysis, blubber analyses can help link contaminant exposure with maturity status or reproductive health in free-ranging marine mammals.

Cetaceans may experience other physiological responses to HOC exposure at a cellular level. RNA-sequencing (RNAseq) is one approach that offers a snapshot of an animals' cellular response to an environmental stressor, including anthropogenic contaminants, by measuring genome wide gene expression (i.e., the whole transcriptome). By evaluating how targeted gene expression changes with HOC load, it is possible to test hypotheses regarding the mechanistic relationship between HOC exposure and physiological effects.[22−24] Genome-wide gene expression provides an unbiased approach for inferring cellular function; it can not only test hypotheses regarding expression of targeted genes but it can also be used to generate new hypotheses for future studies, even in the absence of existing physiological information.[25,26]

Here we integrated three molecular tools to examine how HOC load is linked to cellular and endocrine biomarkers in wild marine mammals in the SCB. We assessed HOC load in the blubber of 22 *T. truncatus* individuals belonging to two different ecotypes with different proximity to urban environments, i.e., a coastal and offshore ecotype. To correlate HOC load to cellular and endocrine activity in skin and blubber respectively, we then conducted a transcriptome analysis using RNAseq and measured blubber hormone levels. To our knowledge, this is the first study to integrate measurements of HOC load with both transcriptome profiling and endocrine biomarkers using biopsies collected from wild cetaceans. This novel approach provides crucial insight into HOC exposure and potential impacts on wild cetacean health in southern California.

MATERIALS AND METHODS

Sample Description and Collection. All samples were collected under NOAA permit #14097−06 and approved by the NOAA SWPI IACUC committee (SWPI2013-06 and SWPI2015-03A). Biopsies composed of skin and blubber were collected from 22 male *Tursiops truncatus* individuals between 2012 and 2016 via crossbow from two ecotypes residing within the Southern California Bight. All samples were collected in fall or winter (between October and February) with one individual sampled in June. Though blubber composition is known to differ between seasons,[27] we did not observe blubber differences associated with season within this sample set, with the exception of the individual samples in summer (this sample was removed from hormone analysis; see Data Analysis: HOCs and testosterone). Seven individuals were sampled from the coastal ecotype and 15 from the offshore ecotype. Ecotype and sex were confirmed by genetic analysis as in Kellar et al.[28] Samples were flash frozen in liquid nitrogen upon collection. A subsample of the outer skin (i.e., the epidermis) for RNAseq analysis was subsequently immersed in RNAlater and stored at −20 °C until analysis. The remaining blubber tissue, approximately 1 cm long, was stored at −80 °C.

HOC Analysis. To quantify HOCs, we extracted an 80−200 mg cross-section of blubber using a Dionex Accelerated Solvent Extractor (Dionex ASE 350, Thermo Fisher Scientific). A procedural blank was included with each ASE extraction batch. The extracts were then evaporated to obtain the final lipid weights. The lipid was spiked with two internal standards, $^{13}C_{12}$-PCB-169 (Wellington Laboratories, Guelph, Ontario, Canada) and 4′-Fluoro-2,3′,4,6-tetrabromodiphenyl ether (Accustandard, New Haven, CT, U.S.A.). Samples were treated with gel permeation chromatography to remove lipids (J2 Scientific, Columbia, MO) and further cleaned up by silica solid phase extraction (EUSIL1M6G, UCT, Horsham, PA, U.S.A.). The final eluent was spiked with a recovery standard, $^{13}C_{12}$-PCB-189 (Wellington Laboratories, Guelph, Ontario, Canada). Final fractions were analyzed via Agilent 7890 gas chromatography/5977 mass spectrometry (GC/MS).

We quantified 24 anthropogenic HOCs that were selected based on relationship with blubber testosterone as in Trego et al.[5] These included five polybrominated diphenyl ethers (PBDE 47, 99, 100, 153, and 154), five polychlorinated biphenyls (PCBs 101, 118, 138, 153, and 180), four chlordane-related compounds (*cis*-chlordane, *trans*-chlordane, *cis*-nonachlor, and *trans*-nonachlor), three dichlorodiphenyltrichloroethane related compounds (*p,p′*-DDT, *p,p′*-DDE, and *p,p′*-DDD), two hexachlorocyclohexane-related compounds (α-BHC and β-BHC), one polybrominated biphenyl (PBB 153), heptachlor epoxide, Mirex, tris(4-chlorophenyl)methane (TCPM), and tris(4-chlorophenyl)methanol (TCPMOH). We also included one natural HOC that is prominent in the region, 6-Methoxy-2,2′,4,4′-tetrabromodiphenyl ether (here referred to as 6 MeO-BDE). All standards were obtained from Accustandard except TCPM and TCPMOH (Wellington Laboratories, Guelph, Ontario, Canada). Each compound was quantified using a calibration curve of five concentration points.

The final data were then corrected for the amount of analyte, if any, that was detected in the corresponding blank sample. We removed compounds that were not detected or where the majority of measurements were below three times the amount detected in the blank samples. Blank adjusted data were then lipid normalized by sample lipid weight. A partial degradation of TCPM to TCPMOH was observed in GC/MS. In calibration standards, the degradation was consistent, so the calibration curve was linear for both TCPM and TCPMOH (r^2 = 0.995 and r^2 = 0.998, respectively). However, there is a possibility that the quantification of TCPMOH in the actual samples may represent an overestimate or underestimate. Average recovery was at 71.9% on average. One sample was further removed from the analysis due to potential contamination.

Hormone Analysis. To quantify blubber testosterone, between 80 to 150 mg of blubber was homogenized and testosterone extracted according to methods published in Kellar et al.[21] and Trego et al.[5] One sample from the coastal population did not have enough blubber for both HOC and hormone analysis and was therefore not analyzed for testosterone. Testosterone was isolated from the homogenate using a biphasic solvent extraction and analyzed with an

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enzyme immunoassay kit (ADI-900-065, Enzo Life Sciences, Farmingdale, NY, U.S.A.). Each extraction included a set of nonspiked and spiked controls to estimate extraction efficiency. Efficiency was estimated by calculating the percent of testosterone recovered in controls spiked with a known amount of testosterone, which was 82.6% in this study. Blubber testosterone concentration was lipid corrected and adjusted according to the average extraction efficiency.

RNAseq Analysis. We used RNAseq to examine genome-wide gene expression in the outer skin from the 22 biopsy samples. Skin stored in RNAlater was extracted for RNAseq with Qiagen RNeasy Mini kits (#74104, Qiagen Inc., Valencia, CA, U.S.A.). A 15 to 20 mg cross-section of skin was homogenized in Qiazol with β-mercaptoethanol using an Omni BeadRuptor. The homogenate was then processed according to the protocols provided by the Qiagen RNeasy kit. RNA quality was analyzed using an Agilent Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.) and RNA was quantified with a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, U.S.A.). According to Romero et al.,[29] a conservative quality cutoff to reduce the impact of RNA degradation lies between RIN values of 6.4 and 7.9. All samples had RIN values over 7 with the exception of 2 individuals with RIN values of 6.8 and 6.9 and were included in this analysis due to the small sample set and reasonable bioanalyzer profiles. Though low RIN values are associated with RNA degradation,[29] we did not observe any differences in transcriptome expression patterns associated with lower RIN values.

Libraries were prepared for RNAseq analysis using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (#E7420S). Libraries from each of the 22 samples were uniquely indexed with NEBNext Multiplex Oligos for Illumina (#E7600S, New England Biolabs, Ipswich, MA, U.S.A.). Library quality was checked on an Agilent Bioanalyzer and quantified via Qubit fluorometer. Final libraries were cleaned, pooled for multiplexing, and run on an Illumina HiSeq 3000/4000 platform (4 lanes in tandem with 53 additional samples not used in the present study) at the UC Davis Genome Center.

Data Analysis. All analyses were conducted on a RedHat Linux system, an XSEDE Jetstream instance,[30] or in R version 3.4.4.[31] We examined HOC patterns in the SCB and how they relate to ecotype and testosterone level with *t* tests, linear regression, and random forest classification with HOC and testosterone concentrations. Data were log transformed for all analyses except the random forest classification. We then investigated transcriptome expression related to HOC load with DESeq2[32] and gene coexpression network analysis.[33]

HOC Profiles. We used a *t* test to evaluate differences in the log abundance of all anthropogenic compounds between ecotypes to examine differences in overall anthropogenic HOC exposure between ecotypes. We summarized HOC data by ecotype and used a random forest classification model to identify different contaminant patterns between ecotypes. To do this we built two models with randomForest[34] and rfPermute[35] R packages (ntree = 4000, nrep = 1000): one that used individual compound abundance to predict ecotype and a second using the summed abundance of each compound group, where groups represent established congeners within a recognized class and their metabolites (Figures 1 and S1 of the Supporting Information, SI). To examine how HOCs covaried among samples, we used principal components analysis (PCA) with the individual compound abundance. We examined

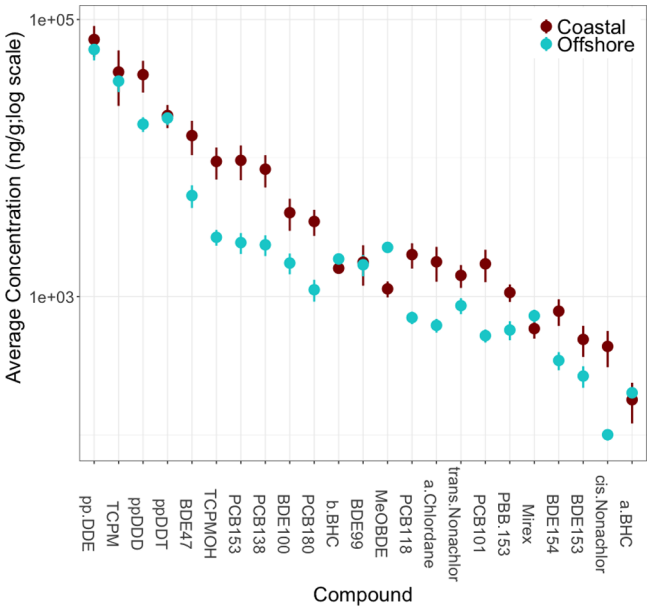

Figure 1. Average concentration of each compound by ecotype in ng/g on a log scale. The error bars represent the standard error.

significant differences between principal components 1 and 2 (PC1 & PC2) with the sum of all anthropogenic congeners as well as blubber testosterone using linear models. A biplot of the PCA is available in the SI (Figure S2).

HOCs and Testosterone. To test for evidence of HOC bioaccumulation and endocrine disruption, we examined the relationship between blubber testosterone and HOC loads. First, log testosterone levels were compared between populations with a *t* test, excluding one individual from the coastal ecotype that was sampled in June during the breeding season when testosterone spikes in males. This individual was removed from further analyses to remove confounding factors due to seasonal fluctuations in testosterone. We then compared the sum of all anthropogenic contaminant concentrations to testosterone with a linear model. To characterize relationships between contaminants and blubber testosterone levels we ran ANCOVAs with ecotype as a covariate and blubber testosterone as the response variable.

HOCs and Transcriptome Expression. We conducted a genome-guided transcriptome assembly and counted reads aligning to these reference transcripts. RNAseq data were trimmed and quality filtered with Trimmomatic (v0.36).[36] We then performed the genome-guided assembly with the Ensembl *T. truncatus* genome (Assembly turTru1, Jul 2008) using TopHat2 (v2.2.1)[37] and cufflinks (v2.2.1).[38] Transcriptome completeness was assessed with benchmarking universal single-copy orthologs (BUSCO, v3).[39,40] The assembled transcriptome was then annotated using dammit[41] on an XSEDE Jetstream instance.[30,42] We then converted protein IDs from dammit annotation to gene name when possible. All trimmed reads were then mapped to the reference transcriptome with Salmon (v0.9.1).[43] Aligned transcripts were counted by gene name with tximport (1.6.0).[44]

First, we examined relationships between HOCs and expression levels for specific genes for which we had a priori predictions regarding their relationships to contaminant exposure. We used ANCOVA to investigate whether expression of the aryl hydrocarbon receptor (AhR; a receptor known to bind with HOCs) correlated with HOC load in both

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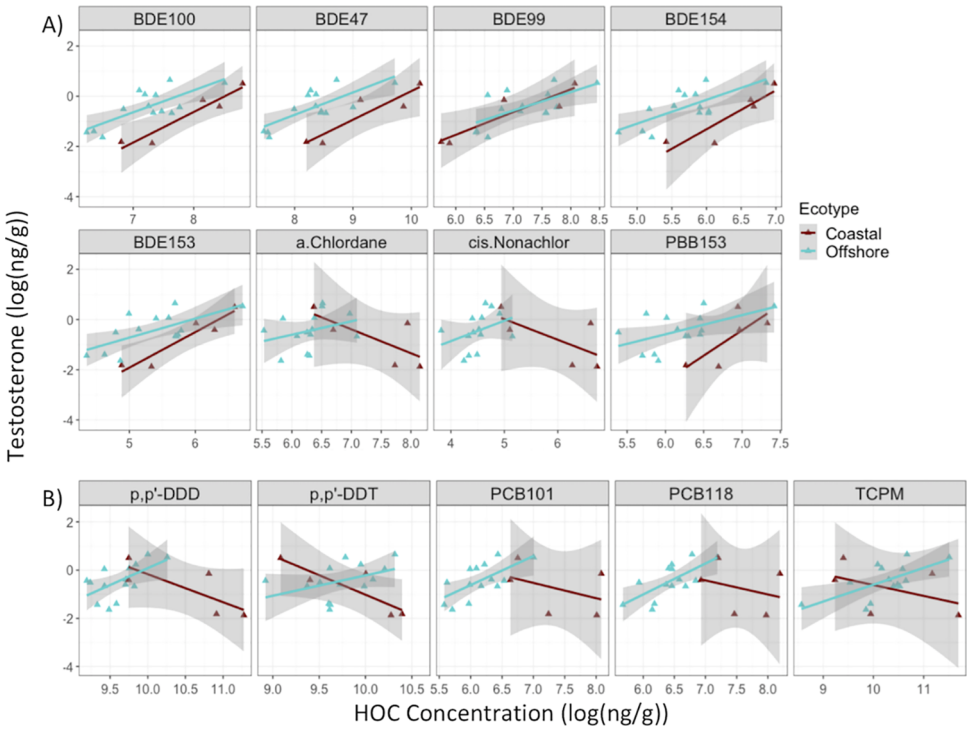

Figure 2. Log transformed HOC concentrations as they relate to log transformed blubber testosterone level in coastal and offshore ecotypes. A) Compounds with significant relationships between testosterone and HOC concentration. B) Compounds with a significant interaction between testosterone and HOC concentration.

ecotypes. Here variance stabilized AhR counts was the response variable and the predictor variables included total anthropogenic contaminant load (log transformed to meet assumptions of normality) and ecotype as a covariate. Also, given the known estrogenicity of many HOCs, we examined the relationships between DDT-related compounds (including TCPMs), PCBs, and PBDEs with estrogen related genes in our data set: estrogen receptor 2 (ESR2), estrogen related receptor alpha (ESRRA), and estrogen related receptor beta (ESRRB). Finally, we further investigated relationships between PCBs and PBDEs, known thyroid disruptors, with thyroid related genes: thyroid receptor alpha (THRA), thyroid receptor beta (THRB).

We then used transcriptomic data to evaluate potential molecular responses to HOC load in both ecotypes in a hypothesis-independent manner. Differential expression was evaluated with DEseq2 (v1.18.1).[32] DESeq2 models evaluated known sources of variation for transcriptomic data to determine the impact of including these as covariates. First, we looked at differential expression between ecotypes, with and without accounting for sea surface temperature. We then examined transcriptomic variation associated with the sum of total anthropogenic HOC, the sum of chlordane related compounds, PCBs, PBDEs, DDTs, and TCPMs (TCPM and TCPMOH). To measure the importance of accounting for covariates, we examined differential expression of HOCs without accounting for external variance and after accounting for ecotype, sea surface temperature, or both. More information on the number of genes that were significant under each model is provided in the SI along with lists of significant genes (Tables S2 and S3). Here, because sea surface temperature and ecotype accounted for some variation in our sample set, we are reporting the most conservative models: differential expression between ecotypes after accounting for variance from sea surface temperature and each HOC model accounting for ecotype and sea surface temperature. We then normalized the data using a variance stabilizing transformation in the DESeq2 package and filtered out genes with zero counts in more than 7 individuals.

We used weighted gene coexpression analysis (WGCNA)[33] to examine how HOCs correlate with coexpressed gene networks (i.e., modules of genes with highly correlated patterns of expression). We then tested for correlations between the gene module eigenvectors and ecotype, blubber testosterone, and HOC compounds. We used blockwise modules with a minimum module size of 30 and a merge cut height of 0.35. This analysis excluded the one breeding season individual referenced above to eliminate potential confounding factors due to breeding activity. To associate biological functions with gene networks, modules were tested for enrichment of gene ontology (GO) terms using TopGO.[45] We investigated enrichment of all three types of GO terms: biological functions, cellular components, and molecular function. Here we report on the top 3 biological processes and molecular functions of all gene modules. The top 5 GO terms associated with all three GO categories are reported in the SI (Table S4).

RESULTS

HOC Profiles. We quantified 25 HOCs in coastal and offshore *T. truncatus*. DDT-related compounds, including DDT and its metabolites as well as TCPM and TCPMOH, were the highest in abundance in both ecotypes (Figure 1, Table S1). Heptachlor epoxide was not at detectable levels in our samples and trans-chlordane was not detectable at sufficient levels above background noise present in the blanks and were therefore removed from all analyses. The coastal ecotype had consistently higher loads of individual HOC

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compounds and a higher average total anthropogenic HOC load than the offshore ecotype (237 μg/g, 158 μg/g respectively), though a difference in average total HOC load between ecotypes was not significant (t = 1.75, p = 0.11). When examining differences in abundance of compound groups with a random forest classification tree, PCBs, PBDEs, and chlordane-related compounds were significantly higher in coastal individuals compared to offshore individuals with an out-of-bag (OOB) error rate of 19.05%. The abundance of several individual congeners was significantly different between ecotypes, including five PCB congeners, TCPMOH, *cis*-nonachlor, and 6 MeO-BDE (OOB = 19.05%). Of these, all of the anthropogenic compounds were significantly higher in the coastal ecotype whereas the one natural compound, 6 MeO-BDE, was higher in the offshore individuals (Figure 1).

PCA identified several anthropogenic HOCs that covaried in their abundance and identified some stratification of the samples included in our analysis (Figure S2). Principal component 1 (PC1) aligned with several anthropogenic contaminant congeners and explained a majority (55.56%) of the variation in the data set. There was a strong significant relationship between PC1 and the sum of all anthropogenic contaminants (r^2 = 0.87, p < 0.001). The one natural HOC, 6 MeO-BDE, did not correlate strongly with any of the anthropogenic HOCs. Principal component 2 (PC2) further stratified our sample set and explained 18.33% of the variance. PC2 was significantly correlated with average blubber testosterone (r^2 = 0.46, p < 0.001).

HOCs and Testosterone. Average blubber testosterone for coastal and offshore ecotypes was 0.68 ng/g (se = 0.23) and 0.8 ng/g (se = 0.14), respectively. Total sum of anthropogenic contaminants was not significantly correlated with blubber testosterone across all samples but there was a significant positive relationship between total anthropogenic HOC load and testosterone within the offshore ecotype (r^2 = 0.46, p = 0.004).

For individual compounds, all PBDEs had significant positive relationships with testosterone for both ecotypes (Figure 2: p < 0.001). We found lower testosterone in the coastal ecotype when considering PBDE load for all congeners except BDE47 and BDE99 (p < 0.05). The compound groups with statistically significant relationships between HOC concentration and testosterone also included p,p'-DDT, p,p'-DDD, α-Chlordane, *cis*-Nonachlor, and PBB153. Significant differences in HOC abundance between ecotypes, after accounting for testosterone as a covariate, were observed for PCB101, PCB118, p,p'-DDT, p,p'-DDD, TCPM, TCPMOH, α-Chlordane, *cis*-Nonachlor, and PBB153. Compounds showing significant interactions between their abundances and ecotype (i.e., different relationships between HOC abundance and testosterone for each ecotype) included PCB101, PCB118, p,p'-DDT, p,p'-DDD, TCPM, TCPMOH, and β-BHC.

HOCs and Transcriptome Expression. The transcriptome analysis included a total of 17,117 genes in 20 samples. We found marginal differences in the relationship between AhR expression and anthropogenic HOC load and ecotype (p = 0.09), where AhR expression appeared to increase in the coastal ecotype and decrease in the offshore ecotype (Figure 3). Both ecotype and the interaction terms were identified as significant (p values = 0.02) but log transformed HOC load was only marginally significant (p = 0.09). ESRRA expression was significantly related to ecotype (p = 0.015) with a

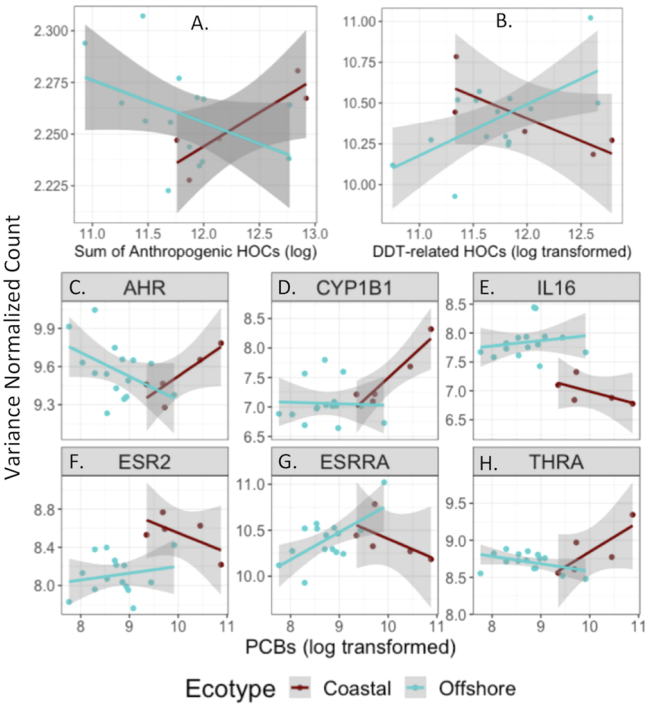

Figure 3. Aryl hydrocarbon receptor (AhR) expression associated with the sum of anthropogenic HOCs (A), estrogen related receptor alpha (ESRRA) expression related with DDT-related compounds (B). The sum of PCBs (log transformed ng/g) related to AhR (C), cytochrome P450 1B1 (CYP1B1; D), interleukin 16 (IL16; E), estrogen receptor 2 (ESR2; F), ESRRA (G), and thyroid receptor alpha (THRA; H) expression.

significant interaction between ecotype and the sum of DDT related compounds (p = 0.004; Figure 3). THRA expression was significantly related to PCBs (p < 0.001) and ecotype (p = 0.01) with a significant interaction (p = 0.002).

We found 506 genes differentially expressed between costal and offshore ecotypes. The gene with the lowest false discovery rate adjusted p-value between ecotypes was β-2-microglobulin (B2M), where expression was lower in the coastal ecotype. The coastal ecotype also had significantly higher expression of cytochrome P450 1B1 (CYP1B1) and lower expression of interleukin 16 (IL16; Figure 3). HOC loads were significantly related to expression levels of a smaller number of genes after correcting for sea surface temperature and ecotype compared to those between ecotypes (Table S2). PCBs and PBDEs were significantly related to expression levels of the highest numbers of genes (24 and 10, respectively). Fewer differentially expressed genes were significantly related to total anthropogenic HOC load as well as chlordane, DDTs, and TCPM related compounds (6, 7, 4, and 2 differentially expressed genes, respectively). We also identified several coexpressed gene networks that were significantly correlated with ecotype, blubber testosterone, and HOC concentration (Figure 4, Table 1). GO enrichment within these modules included terms associated with xenobiotic metabolism, immune response, hormone metabolism, DNA repair, and metal binding.

DISCUSSION

This research provides novel insight into contaminant exposure in two bottlenose dolphin ecotypes in the SCB and highlights potential relationships between HOC exposure and molecular biomarkers. We found evidence of differential

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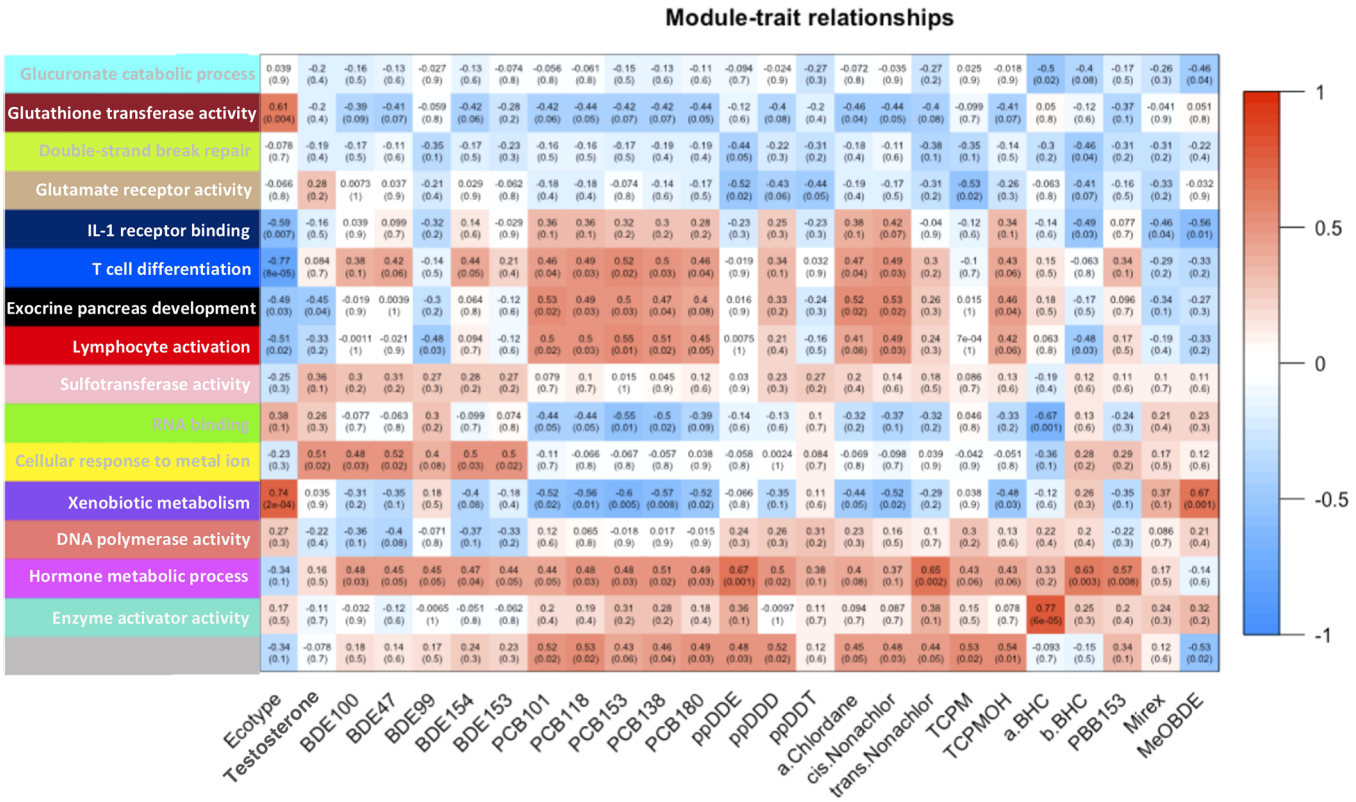

Figure 4. A heatmap of gene modules as they relate to ecotype (where coastal = 1, offshore = 2), blubber testosterone (ng/g), and HOC concentration (ng/g). Each comparison includes a correlation coefficient (top value), representing the relationship between each gene module with each factor, and a *p*-value (in parentheses). Here, a positive correlation is marked in red, and negative correlations are shown in blue. We identified the top gene ontology (GO) terms associated with each gene module and included one of these for each gene module to represent potential enrichment of these gene functions.

exposure to many anthropogenic HOCs between the two ecotypes. The coastal ecotype harbored higher body burdens of some legacy compounds, including PCBs, compared to the offshore ecotype, whereas the offshore individuals had the highest levels of the one natural HOC included in this analysis (6 MeO-BDE). By comparing HOC concentrations with blubber testosterone, we found that PBDEs were positively correlated with the testosterone levels in both ecotypes but the direction of the correlations between testosterone and other HOCs were different between the ecotypes. Gene coexpression analysis further identified gene networks related to HOC exposure and implicates HOC impacts on multiple physiological pathways in wild cetaceans in the SCB.

HOC Profiles. Legacy compounds, despite being banned 46 years ago, continue to account for a large portion of HOC load in marine mammals of the SCB, including DDT and other DDT related compounds (e.g., metabolic byproducts *p,p′*-DDE and *p,p′*-DDD as well as TCPM, a component of DDTs technical mixture,[2] and TCPMOH). This is likely due to the extremely high levels of deposition by Montrose chemical company that prompted the creation of the Palos Verdes Shelf Superfund Site off of the coast of Los Angeles County, California.[9] The lack of a significant difference in abundance of DDT-related compounds between ecotypes also indicates DDT and other related compounds are likely prevalent in other marine mammals and potentially other top predators throughout the SCB.

We did detect some differences between ecotypes in other legacy HOCs. All PCBs congeners, PBDEs, chlordane-related compounds, and TCPMOH were higher in coastal individuals, which is consistent with evidence of higher exposure of anthropogenic HOCs with closer proximity to urban areas.[46,47] Conversely, we found higher levels of one natural HOC, 6 MeO-BDE, in the offshore ecotype. This finding also aligns with previous nontargeted HOC analysis that reported higher relative abundance of natural compounds in offshore *T. truncatus*.[4] Our findings provide evidence that exposure to many toxic anthropogenic compounds or compound classes, including PCBs, PBDEs, chlordane-related compounds, and TCPMOH, are higher in the coastal ecotype that resides closer to the urbanized coastline.

HOCs and Testosterone. We observed several positive relationships between testosterone and several HOC compounds, especially in the offshore ecotype (Figure 2). In our previous research of *D. delphis*,[5] HOC concentrations were corrected for age because we used stranded animals where known age information was available. After the age correction, we observed significant negative linear regression between sum of concentrations of the anthropogenic HOCs and testosterone. In addition, within mature individuals, a negative linear relationship was found between individual anthropogenic HOCs and blubber testosterone. However, the trend was not fully reproducible in this study because we could not obtain age or maturity status (i.e., this information is not available for biopsies collected from live, wild animals). However, a negative trend was observed within the coastal ecotype only for *α*-Chlordane, *cis*-Nonachlor, *p,p′*-DDD, *p,p′*-DDT, PCB101,

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Table 1. Top Three Gene Ontology (GO) Terms Associated with Biological Processes and Molecular Function for the Gene Modules[a]

Biological Processes		Molecular Function	
GO Term	p-value	GO Term	p-value
Black Module: Ecotype, Testosterone, PCBs, a-Chlordnae, cis-Nonachlor, TCPMOH			
platelet degranulation	0.00022	siRNA binding	0.00026
exocrine pancreas development	0.00052	phosphatidylinositol phosphate binding	0.00109
lactation	0.0006	carbonate dehydratase activity	0.00168
Blue Module: Ecotype, BDE 154, PCBs, a-Chlordane, cis-Nonachlor			
negative regulation of protein localizat...	1.50E-06	calcium-dependent phospholipid binding	0.0017
negative regulation of protein localizat...	8.60E-06	calcium ion binding	0.0032
negative regulation of protein localizat...	0.0001	oxidoreductase activity, acting on the a...	0.0044
Midnightblue Module: Ecotype, b-BHC, Mirex, MeO-BDE			
cellular response to arsenic-containing ...	0.00034	interleukin-1, Type I receptor binding	0.003
response to arsenic-containing substance	0.00052	interleukin-1, Type II receptor binding	0.003
chylomicron assembly	0.00315	interleukin-1 receptor antagonist activi...	0.003
Brown Module: Ecotype, PCB118, PCB180, c-Chlordane, cis-Nonachlor			
neuron-neuron synaptic transmission	0.00059	spectrin binding	0.0035
actin crosslink formation	0.00059	glutathione transferase activity	0.004
vascular endothelial cell proliferation	0.00076	glutathione peroxidase activity	0.004
Tan Module: p,p'-DDE, p'p-DDT, TCPM			
glutamate receptor signaling pathway	0.0003	glutamate receptor activity	4.10E-05
negative regulation of Ras protein signa...	0.00042	ligand-gated ion channel activity	0.00041
RNA splicing	0.0007	ligand-gated channel activity	0.00041
Green Module: PCB101, PCB118, PCB153, PCB138, a-BHC			
transposition, RNA-mediated	4.00E-15	single-stranded RNA binding	9.50E-12
transposition	6.40E-15	RNA binding	5.90E-06
ribonucleoside monophosphate biosyntheti...	7.50E-05	troponin I binding	0.00014
Greenyellow Module: p,p'-DDE, b-BHC			
intestine smooth muscle contraction	0.00029	nucleoside kinase activity	0.0015
double-strand break repair	0.00067	cyclase activity	0.0065
RNA secondary structure unwinding	0.00083	transferase activity, transferring amino...	0.0065
Magenta Module: PBDEs, PCBs, p,p'-DDE, p,p'-DDD, trans-Nonachlor, b-BHC, PBB153			
cellular hormone metabolic process	3.20E-05	filamin binding	0.0047
hormone metabolic process	0.00061	oxidoreductase activity, acting on the C...	0.0052
negative regulation of mammary gland epi...	0.00081	anion transmembrane transporter activity	0.0074
Pink Module			
transposition, RNA-mediated	1.10E-05	single-stranded RNA binding	5.60E-05
transposition	1.30E-05	sulfotransferase activity	0.00013
extracellular matrix organization	0.00039	peptide binding	0.00019
Purple Module: Ecotype, PCBs, a-Chlordane, cis-Nonachlor, TCPMOH, MeO-BDE			
xenobiotic metabolic process	5.50E-05	single-stranded DNA 3'-5' exodeoxyribonu...	0.00051
cellular response to xenobiotic stimulus	8.60E-05	3'-5'-exodeoxyribonuclease activity	0.00102
intraciliary anterograde transport	0.00015	single-stranded DNA exodeoxyribonuclease...	0.00168
Red Module: Ecotype, BDE99, PCBs, cis-Nonachlor, b-BHC			
ventricular system development	0.0018	coenzyme binding	5.20E-05
negative regulation of long-term synapti...	0.0024	ion binding	0.00022
regulation of Ras protein signal transdu...	0.0026	cofactor binding	0.00056
Salmon Module			
RNA-dependent DNA biosynthetic process	0.0007	RNA-directed DNA polymerase activity	0.00097
anterograde synaptic vesicle transport	0.0016	DNA polymerase activity	0.00132
synaptic vesicle cytoskeletal transport	0.0016	catalytic activity, acting on DNA	0.00238
Turquoise Module: a-BHC			
regulation of phospholipid metabolic pro...	0.00025	enzyme activator activity	0.0071
ribosome biogenesis	0.00095	wide pore channel activity	0.0081
negative regulation of axon extension	0.00277	alpha-mannosidase activity	0.0088
Cyan Module: a-BHC, MeO-BDE			
negative regulation of BMP signaling pat...	0.0037	sugar:proton symporter activity	0.00017
glucuronate catabolic process	0.0043	cation:sugar symporter activity	0.00017
isopentenyl diphosphate biosynthetic pro...	0.0043	solute:proton symporter activity	0.00093
Yellow Module: Testosterone, BDE100, BDE47, BDE154, BDE153			
cellular response to metal ion	0.00011	heme binding	0.0041
cellular response to inorganic substance	0.00012	transferase activity, transferring phosp...	0.0047
membrane depolarization	0.00189	organic cyclic compound binding	0.0057

[a]For a more extensive list that includes the cellular components, see a complete table in supplemental Table S4.

PCB118, and TCPM, while the trend was opposite within the offshore ecotype.

Differences in the HOC-testosterone relationships observed between ecotypes, where the relationship is largely positive in the offshore ecotype and negative in the coastal ecotype, could be explained by the age of sampled individuals. Blubber testosterone levels can vary with a variety of factors, including between individuals, seasons, and different reproductive stages.[5,21,48] If a larger number of young individuals were sampled from the offshore ecotype than the costal ecotype, while they are still be actively accumulating HOCs as they mature, then we would be more likely to detect an increase in

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HOC load during a time where their testosterone levels are also actively increasing. Alternatively, if all samples are taken from only mature individuals from the coastal ecotype, primarily after puberty and following a longer period of accumulation of HOCs, then we would be more likely to detect signals of endocrine disruption, if apparent, without maturity as an added covariate, as found in Trego et al.[5] We previously observed highly variable HOC loads in two *D. delphis* juveniles (including one with higher levels of certain compounds than all of the mature individuals) indicating there could be high variability in HOC load during certain periods of maturity,[5] though this is typically not observed in other cetaceans where HOC patterns typically increase steadily in males throughout their lifespan.[13,20] Another possible explanation for the differential HOC-testosterone relationship within the coastal ecotype could be different feeding habits associated with age that results in different patterns of exposure to these compounds. For example, the three individuals with the highest DDT, TCPM, PCBs, and chlordane loads may have spent more time feeding in areas with higher concentrations of these HOCs.

It is notable that three coastal individuals were outliers exhibiting exceptionally high levels of *p,p′*-DDD, *α*-Chlordane, *cis*-Nonachlor, and PCB101. This suggests another potential explanation for the difference between ecotypes. These individuals represent the highest levels of legacy HOCs in the coastal ecotype and have relatively low levels of testosterone (Figure 2). These legacy HOCs are known to be androgen disruptors.[17,19,49−52] Compounds and metabolites related to these HOCs have been linked to decreases in testosterone in SCB *D. delphis*.[5] Our data suggest that individuals in the coastal ecotype may be at higher risk for endocrine disruption than the offshore ecotype, but collection of additional samples through space and time would help confirm associations between exposure and physiological effects.

HOCs and Transcriptome Expression. The transcriptome analysis provides evidence of differences in cellular pathways or impacts between the two ecotypes. Our results suggest upregulated AhR gene expression with HOC load in the coastal ecotype, similar to previous research on targeted genes.[22,53] An increase in AhR expression provides evidence for initiation of xenobiotic metabolism, or metabolism of external organic compounds, by anthropogenic HOCs. This was not apparent in the offshore ecotype where AhR expression is more aligned with AhR antagonism or down-regulation of AhR. Gene coexpression analysis also identified one gene module enriched for xenobiotic metabolism that was characterized by aryl-hydrocarbon receptor repressor (AHRR) expression and correlated with PCBs, chlordane-related compounds, and TCPM, suggesting xenobiotic metabolism may be responding to these compound classes (Table 1, Figure 4). Because AhR is primarily induced by dioxin-like compounds[54] and we quantified a dioxin-like PCB (PCB 118), the pattern we observed could be a result of exposure to other covarying dioxin-like compounds, including dioxin-like PCBs and PCB metabolites. Alternatively, AhR is also associated with other functions beyond HOC response, including hormone metabolism,[55−57] and could be indicative of different endocrine processes driving AhR expression within the two ecotypes.

In the coastal ecotype, expression of CYP1B1 was also positively correlated with PCB body burdens and with AhR expression (Figure 3). CYP1B1 is part of the cytochrome P450 family involved in organic compound metabolism, is transcriptionally activated by AHR, and has been linked to PCB load in Baikal seals.[58] CYP1B1 was proposed as a better marker for PCB exposure than CYP1A1 considering that it is also responsive to non-AhR initiated pathways,[59] including non-HOC-related estrogen metabolism.[57] Expression patterns of CYP1B1 are consistent with activation of xenobiotic compound metabolism by PCBs within the coastal ecotype. Differences between the ecotypes could be explained by threshold effects, where an increase in CYP1B1 expression is not apparent below a certain PCB concentration, as was observed in ringed seals.[53] CYP1B1 has also exhibited nonlinear relationships with PCB load in cows.[60] It is possible that within coastal animals the burdens of PCBs or other HOCs are above the threshold that is required to initiate a functional genomic response.

Transcriptome analysis further identified potential effects of increased HOC exposure, including altered immune response. Increasing loads of PCBs, PBDEs, chlordane-related compounds, and TCPMOH, and associated up-regulation of CYP1B1 in the coastal ecotype, corresponded with a decrease in expression of immune genes, including B2M and IL16. B2M is a component of major histocompatibility complex class I (MHC I), which is necessary for development of CD8 T cells. Several gene modules that correlated with burdens of PCBs, PBDEs, TCPMOH, and chlordane-related compounds were enriched with immune-related GO terms (Table 1, Figure 4), including T cell differentiation and lymphocyte activation (i.e., B or T cell activation). PCBs and PBDEs are known to cause immunosuppression in marine mammals and have been associated with declines in phagocytosis and impaired adaptive immunity as well as altered T cell and B cell activity.[14,53,61−63] Chlordane has also been associated with alternation of the adaptive immune system, but the effect appears ambiguous since activation or suppression seems dose dependent.[64,65] In addition to HOC exposure, another contributing factor to immune differences between ecotypes could include differential exposure to pathogens. This is consistent with previous research that detected increased incidence of skin lesions in offshore individuals.[66] Another explanation is that differentiation in expression of immune system genes is a result of evolved differences (either because of selection or neutral genetic drift) between discrete populations.[67,68] Though there are several possible factors influencing immune gene expression between these ecotypes, immunosuppression in the coastal ecotype due to higher exposure to certain anthropogenic compounds is one potential contributing factor.

In addition to possible immune disruption, our data further support evidence for disruption of endocrine pathways following exposure to anthropogenic HOCs. One gene module of coexpressed genes that correlated with the majority of HOCs that we measured was enriched for functions including hormone metabolism (Figure 4), which is suggestive of interactions between HOCs and endocrine regulation. Specifically, we found DDT-related compounds and PCBs were negatively related to estrogen-related gene expression. PCBs are known to bind with estrogen receptors,[69] and showed a negative relationship with ESR2 expression, though ESR2 expression was slightly higher in coastal individuals where PCBs were higher. ESRRA gene expression declined with increasing DDT-related compound and PCB load in the coastal ecotype with the opposite pattern in the offshore

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individuals. DDT, PCBs, PBDEs, and many other HOCs are known to bind with estrogen receptors, thereby reducing circulating estradiol.[69−73] Little is known about the relationship between HOCs and ESRRA expression, though ESRRA and estrogen receptor 1 can both mediate the expression of similar suites of genes (ESR1 transcripts were not present in high enough abundance to be included in this analysis).[74] Furthermore, PCBs were also linked with an increase in expression of THRA, a thyroid hormone receptor, similar to previous research linking an increase in thyroid receptor expression with a decline in circulating thyroid hormones.[12,75,76] The relationships between HOC load and estrogen and thyroid receptor expression in the coastal ecotype are consistent with previous research demonstrating HOC-induced endocrine disruption. This suggests that coastal individuals are at higher risk for declines in normal estrogen and thyroid hormone levels compared to offshore animals and has implications for reproductive and nutritional health of individuals that live in close proximity to human-impacted environments.

This is one of few studies to examine correlations between exposure to HOCs and cellular and endocrine biomarkers in wild cetaceans. Using multiple indicators of biological responses, we found that exposures to legacy contaminants, including TCPM/TCPMOH (which are not typically monitored), were biologically meaningful. This confirms that these compounds continue to pose a significant health concern for free-ranging cetaceans in the region. Incorporating full transcriptome analysis helped identify putative mechanistic links between contaminant exposure and physiological effects in free-ranging cetaceans of the SCB. Our data suggest high exposure to anthropogenic contaminants could be a concern for both ecotypes but that the coastal ecotype was at greater risk for exposure to known, toxic legacy contaminants, likely due to their greater proximity to human-impacted habitat.

Our research also highlights the need to develop and deploy minimally invasive tools and techniques for estimating age and maturity in free-ranging dolphins. New epigenetic markers, which could be collected as part of an RNA-seq workflow, should be developed to provide an estimate of age.[77] Without this information, it is difficult to distinguish clear patterns of endocrine disruption from other explanations, precluding analysis of the impacts of these legacy compounds on cetaceans in the SCB. Our findings are consistent with previously documented effects of HOCs in other mammals, including interactions with immune and endocrine pathways. By integrating these three technologies, it is possible to investigate evidence of endocrine disruption and to identify potential mechanisms of action in wild cetaceans with minimally invasive methods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b06487.

Additional supplemental tables and figures (PDF)

Gene table containing all of the normalized counts from DeSeq2 (XLSX)

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Notes

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