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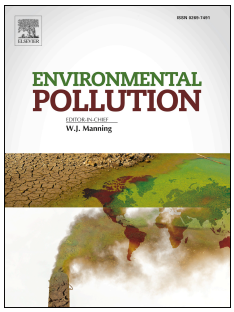

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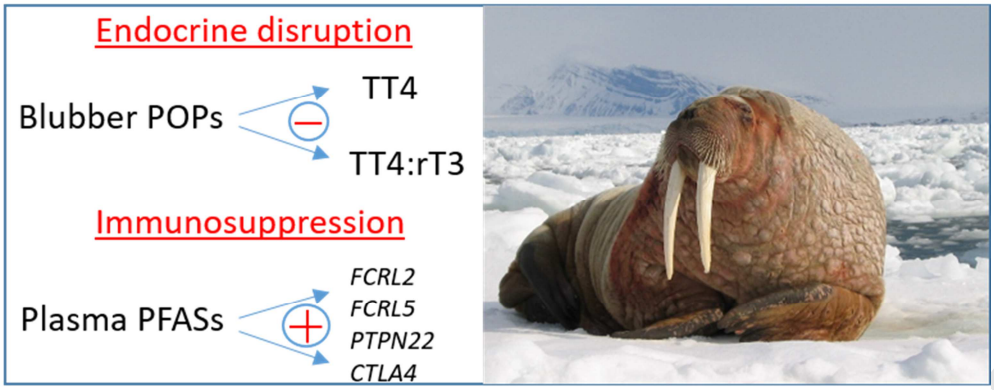

Contaminants in Atlantic walruses Part 2: Relationships with endocrine and immune systems

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Abstract

Marine mammals in the Barents Sea region have among the highest levels of contaminants recorded in the Arctic and the Atlantic walrus (*Odobenus rosmarus rosmarus*) is one of the most contaminated species within this region. We therefore investigated the relationships bewteen blubber concentrations of lipophilic persistent organic pollutants (POPs) and plasma concentrations of perfluoroalkyl substances (PFASs) and markers of endocrine and immune functions in adult male Atlantic walruses (n=38) from Svalbard, Norway. To do so, we assessed plasma concentrations of five forms of thyroid hormones and transcript levels of genes related to the endocrine and immune systems as endpoints; transcript levels of seven genes in blubber and 23 genes in blood cells were studied. Results indicated that plasma total thyroxine (TT4) concentrations decreased with increasing blubber concentrations of lipophilic POPs. Blood cell transcript levels of genes involved in the function of T and B cells (FC like receptors 2 and 5, cytotoxic T-lymphocyte associated protein 4 and protein tyrosine phosphatase non-receptor type 22) were increased with plasma PFAS concentrations. These results suggest that changes in thyroid and immune systems in adult male walruses are linked to current levels of contaminant exposure.

Capsule: The first investigation on the effects of contaminants in walruses suggest that changes in thyroid and immune systems in adult male walruses are linked to current levels of contaminant exposure.

Key words: pollutant; marine mammal; thyroid hormone; qPCR; mRNA

Introduction

Effects of environmental contaminants on endocrine and immune systems have been reported widely in humans and experimentally confirmed in various other mammals (Gore et al., 2015; Selgrade, 2007). Although several Arctic wildlife species are exposed to relatively high concentrations of environmental contaminants, there is limited knowledge of the implications for the health status of these species.

The Barents Sea is among the most polluted regions within the Arctic due to high inputs of contaminants transported by winds and ocean currents from Europe and North-America (Hansen et al., 2015; Shindell et al., 2008). Within the Barents Sea ecosystem, the Atlantic walrus (*Odobenus rosmarus rosmarus*) is one of the species that has the highest body burdens of persistent organic pollutants (POPs) (Scotter et al., 2019; Wolkers et al., 2006).Concentrations of lipophilic POPs are particularly high in this species because of the partitioning of the chemicals towards the sediments of the Barents Sea (Carrizo et al., 2017; Sobek and Gustafsson, 2014), where walruses feed on benthic mollusks (Gjertz and Wiig, 2009; Scotter et al., 2019). Some individual walruses (1-22%) also feed on higher trophic level prey such as seals (Fay, 1982; Gjertz and Wiig, 2009; Seymour et al., 2014a, b) and consequently ingest high concentrations of POPs (Muir et al., 1995; Wolkers et al., 2006). Concentrations of the main lipophilic POPs in walruses, namely polychlorinated biphenyls (PCBs) and chlordanes, are at levels slightly lower or similar to those observed in polar bears (*Ursus maritimus*) from corresponding areas in the Barents Sea and the Canadian Arctic (Muir et al., 1995; Scotter et al., 2019; Wolkers et al., 2006), whereas concentrations of perfluoroalkyl substances (PFAS) in walruses are lower than in polar bears or phocids in the Barents Sea ecosystem (Scotter et al., 2019). Previous studies on polar bears and other marine mammals such as pinnipeds and whales have associated contaminant exposure to adverse health effects such as endocrine disruption and immune suppression (reviewed by Desforges

et al., 2016; Letcher et al., 2010; Routti et al., 2018). However, to date there are no published studies investigating possible health effects of contaminants in walruses.

A large number of contaminants are known to affect mammalian thyroid systems through multiple target-points in the thyroid hormone system (Gore et al., 2015). Thyroid hormones (THs) are involved in growth, neurologic development and metabolism (McNabb, 1992; Zhu and Cheng, 2010) and altered TH levels have been associated with exposure to lipophilic POPs, their metabolites and/or PFASs in ringed seals (*Pusa hispida*), harbor seals (*Phoca vitulina*), hooded seals (*Cystophora cristata*), white whales (*Delphinapterus leucas*) and polar bears (Bourgeon et al., 2017; Gabrielsen et al., 2011; Routti et al., 2010; Tabuchi et al., 2006; Villanger et al., 2011). Both circulating TH levels as well as multiple other molecules involved in thyroid homeostasis such as receptors and enzymes involved in thyroid hormone action and metabolism, respectively, can be used as biomarkers to study effects of contaminants.

Contaminants may also target endocrine systems through lipid metabolism and stress responses (Gore et al., 2015). A nuclear receptor, peroxisome proliferator activated receptor gamma (PPARG) is the major regulator in the formation of lipid stores in adipose tissue (Cristancho and Lazar, 2011; Desvergne et al., 2006). Additionally, glucocorticoid hormones, which act through the glucocorticoid receptor (GR) encoded by gene nuclear receptor subfamily 3 group C member 1 (*NR3C1*), play an important role in basal and stress-related homeostasis and are involved in almost every regulatory network within organisms (Nicolaides et al., 2010). Recent reports indicate that a wide range of endocrine disrupting chemicals target mammalian PPARG and GR (Grimaldi et al., 2015; Routti et al., 2016), which may lead to endocrine disruption.

Contaminants may also affect the immune system directly (Desforges et al., 2016). Studies on marine mammals have related contaminant exposure to haematological alterations, lymphocyte proliferation, respiratory bursts of leukocytes, modulation of natural killer cell activity, immunoglobulin production, transcription of cytokines and morphological changes in lymphoid tissues (Desforges et al., 2016). Contaminant-induced immunosuppression has been speculated to be a contributing factor to mortality experienced by several marine mammal species infected with various pathogens (Jepson et al., 1999; Ross, 2002). Studies on captive harbour seals (*Phoca vitulina*) fed contaminated fish from the Baltic Sea showed that the seals developed significantly higher body burdens of potentially immunotoxic organochlorines and displayed impaired immune responses (de Swart et al., 1996). Monitoring contaminant related health effects is of particular importance for the Barents Sea population of walruses which is currently recovering from extensive commercial harvesting that took place over a period of 350 years, prior to protection being put in place regionally in the 1950s (Kovacs et al., 2014). Moreover, studying contaminant effects in walruses is of the highest relevance in the light of a changing climate in order to understand multiple stressors that walruses are exposed to. Because all Arctic endemic marine mammals depend on sea ice as a platform for resting and a host of other functions (Laidre et al., 2008), loss of sea ice represents a significant risk to ice-associated marine mammals such as walruses (Kovacs et al., 2011; Laidre et al., 2008). Declines in Arctic sea ice are happening particularly rapidly in the Barents Sea (Årthun et al., 2012; Laidre et al., 2015). Further, a suggested shift to feeding at higher trophic levels (Seymour et al., 2014a) may change the range and degree of exposure of walruses to a variety of pathogens and contaminants, which may influence their health and disease status (Burek et al., 2008).

The aim of the study was to investigate the effects of contaminants on the endocrine and immune systems of adult Atlantic male walruses from the Svalbard area using plasma thyroid

hormone concentrations and transcript levels of genes related to endocrine and immune systems as endpoints.

Materials and methods

Field sampling

Blubber and blood samples were collected from 38 apparently healthy adult male walruses from Svalbard, Norway, in July 2014 and August 2015. Briefly, walruses were immobilized on land with an intramuscular injection of etorphine hydrochloride, with naltrexone as a reversal agent (Ølberg et al., 2017). Tusk volume based on tusk length and girth at proximal end was used as a proxy for age (Skoglund et al., 2010). Blood from the extradural vein in the lumbar-sacral region was collected in vacutainers with heparin or ethylenediaminetetraacetic acid (EDTA) (Venoject, Terumo Corporation, Leuven, Belgium), and blubber biopsies, comprising the epidermis, dermis and the entire blubber layer were collected from the mid dorsal region using a custom-made hollow stainless steel corer (8 mm in diameter). Blood samples were kept cool and plasma and blood cells were separated by centrifugation (4000 rpm for 10 minutes) within a few hours. Blubber and plasma samples for contaminant analyses were kept at -20 °C until analyzed, while plasma for hormone and blubber and blood cells for quantitative real-time polymerase chain reaction (qRT-PCR) analyses were frozen in liquid nitrogen in the field and thereafter kept at -80 °C until analyses. Animal handling procedures were approved by the Norwegian Animal Care Authority (2013/36153-2) and the Governor of Svalbard (2014/00066-2 and 2015/00218).

Analyses of thyroid hormones in plasma

Plasma was separated from blood collected into heparinized tubes. The concentrations of total thyroxine (TT4), free (not bound to carrier proteins) T4 (FT4), total triiodothyronine

(TT3), free T3 (FT3) and reverse T3 (rT3) were measured in plasma using EIAgen enzyme-linked immunosorbent assays (ELISA; Diagnostics Biochem Canada Inc.; TT4: CAN-T4-4240, FT4: CAN-FT4-4340, TT3: CAN-T3-4220, FT3: CAN-FT3-4230, rT3: CAN-RT3-100), following the manufacturer's recommended protocols. The quality was controlled by a series of calibrations with known TH levels and two control solutions (low and high concentrations) for each hormone, all provided by the respective kits. Absorbance was measured on a microplate reader (BioTek Instruments, Inc., Vermont, USA) at 450 nm. All samples were run in duplicate and only one plate was used per hormone assay. Intra-assay variation was 6.5% for TT3 (n=3), 5.0% for TT4 (n=3), 5.1% for FT3 (n=2), 7.6% for FT4 (n=3) and 7.1% for rT3 (n=2). All samples had concentrations of TT3, TT4, FT3, FT4 and rT3 above the level of detection (LOD). Six TH ratios (TT3:FT3, TT4:FT4, TT4:TT3, FT3:rT3, TT4:rT3 and FT4:FT3) were calculated as indicators for TH bioavailability and metabolism.

Assay results were validated for walruses by performing analyses of serial dilutions and standard addition tests because the assay kits were originally developed for human subjects. Validation results are given in the supporting information (Figure S1, S2).

Transcript levels of genes in blubber samples

Adipose tissue is considered as an endocrine organ involved in coordinating for example energy metabolism and immune function (Kershaw and Flier, 2004). Transcript levels of seven target genes involved in these functions were analyzed in walrus blubber samples (Table 1). Three reference genes, beta-2-microglobulin (*B2M*), eukaryotic translation elongation factor 1 alpha (*EEF1A1*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were selected and have been used in previous studies on marine mammals, in particular for skin and blubber biopsies (Table 1) (Brown et al., 2014; Castelli et al., 2014;

158 Das et al., 2008; Mancia et al., 2012; Spinsanti et al., 2006). Blubber samples (100-150 mg)
159 were homogenized using a TissueLyser (QIAGEN, Hilden, Germany) and total RNA was
160 extracted using the Aurum Total Fatty and Fibrous Tissue kit (Bio-Rad, Hercules, CA, USA)
161 following the manufacturer's instructions and finally eluted in 40 µL of Elution Solution
162 (from the kit) and stored at -80 °C. Genomic DNA was digested by DNase-on-column
163 treatment for each sample. RNA quantity (166 ± 68 ng/µL) and purity ($A_{260/280}$ 2.02 ± 0.04;
164 $A_{260/230}$ 1.81 ± 0.30) of the isolated RNA was determined by Nano-Drop ND-100 UV–Vis
165 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). An additional
166 assessment of the integrity of the samples was done by denaturing agarose gel (1.2%)
167 electrophoresis and ethidium bromide staining. Reverse transcription reactions were
168 performed using the iScript cDNA Synthesis Kit (Bio-Rad), using 750 ng of total RNA as
169 starting amount.

170 Genes were sequenced using cDNA as a template. Primers for sequencing were designed in
171 conserved regions after the alignment of the phylogenetically closest species available in
172 GenBank and Ensembl database (e.g. *Pusa hispida*, *Phoca vitulina*, *Canis lupus* or *Felis*
173 *catus*). The selected regions were amplified by PCR reactions and run on 2% agarose gel with
174 Ethidium bromide staining. Amplification products were purified with Wizard SV Gel and
175 PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced. Sequences were
176 corrected manually using Sequencer 4.2.2 software (Gene Codes, Ann Arbor, MI, USA) and
177 the specificity of the products was checked using BLAST (http://blast.ncbi.nlm.nih.gov/
178 Blast.cgi). The base pairs sequenced in walruses and their GenBank accession numbers are
179 reported in Table S1.

180 Primers for qRT-PCR were designed on the specific walrus sequences using the Beacon
181 Designer v. 8.14 software (Premier Biosoft, Palo Alto, CA, USA). All primers were
182 purchased from Merk (Darmstadt, Germany). The efficiency of each primer pair (Table S2)

for each gene was calculated using a calibration curve with 1:5 serial dilutions of cDNA. Each primer pair presented a melting curve with a sharp peak, indicating no unspecific products or primer-dimer formation. The amplicon length was verified on 2% agarose gel with Ethidium bromide staining.

The qRT-PCR assays were carried out on 96-well reaction plates with an iCycler iQ5 (Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The reactions were performed in a total volume of 20 μL the 2x SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

The seven genes of interest, and three reference genes, were amplified for each of the 38 blubber samples. Each reaction was run in triplicate and a control with no template was included in each reaction series. One walrus sample was analyzed repeatedly to account for variation between plates. The raw cycle of quantification (Cq) values were used for downstream analyses.

Transcript levels of genes in blood cells

Transcript levels of 23 target genes related to endocrine and immune systems (Table 1) and four reference genes (*GAPDH*, actin β [*ACTB*], hypoxanthine phosphoribosyltransferase 1 [*HPRT1*] and TATA-box binding protein [*TBP*]) were determined from blood cell samples from walruses. Red blood cells and leukocytes were separated from blood collected into EDTA-tubes. Total RNA was isolated from the combined blood cells using the miRNeasy blood kit (QIAGEN, Norway), according to the manufacturer's protocol with some modifications. Briefly, blood samples (200 μL) diluted with PBS (1:1) were lysed with 700 μL of QIAzol lysis solution and homogenized with TissueLyser II (QIAGEN). Carrier RNA (MS2 RNA, Roche, Oslo, Norway) was added to the homogenized samples. Then, the manufacturer's protocol was followed for subsequent processing. The quantity (39 ± 16

ng/μL) and purity ($A_{260/280}$ 2.08 ± 0.12; $A_{260/230}$ 0.75 ± 0.35) of the isolated RNA was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific, Oslo, Norway). The RNA integrity (expressed as RNA integrity numbers (RIN)) was assessed by an Agilent 2100 Bioanalyzer using the Eukaryote total RNA 6000 Nano LabChip kit and Eukaryote total RNA Nano assay according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA, USA). RIN (from 1 to 10 - low to high RNA quality) was calculated using the 2100 Expert software (Agilent Technologies). The isolated total RNA was stored at −80 °C until analysis in elution buffers supplied with the kit.

cDNA synthesis was performed with 100 ng total RNA from samples as template, using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The amplification reactions were carried out in a thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany), with the following steps: 10 min at 25 °C, 2 h at 37 °C and 5 min at 85 °C. The quantity and quality of the cDNA was determined using a NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific). All cDNA samples were stored at −20 °C prior to gene expression analysis.

Gene-specific qPCR was carried out as previously described (Gutzkow et al., 2016) using the KAPA SYBR FAST qPCR Master Mix (2×) Universal Kit according to the manufacturer's protocol (Kapa Biosystems, Oslo, Norway) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Briefly, a 1:80 dilution of cDNA from each sample was run in duplicate for each gene of interest. All samples were analyzed on the same 384-well plate, which allowed simultaneous measurement of all samples (eight genes in each run) reducing the influence of run-to-run variation. Non-template controls (NTC) and melting curve analysis were included on each plate. Gene-specific primers were designed using Primer3Plus software (Koressaar and Remm, 2007; Untergasser et al., 2012) and in total 27 genes were targeted (Table S3). The Cq- values for 23 target genes and four reference genes were

recorded with CFX Manager Software (Bio-Rad). The PCR efficiency was determined using Lin-RegPCR software (Ruijter et al., 2009). Cq-values were used for data analyses; only *ACTB* and *HPRT1* were defined as control genes due to low target abundance of *TBP* (Cq: 34.84 ± 2.49) and abnormal efficiency of *GAPDH* (3.05).

Analyses of contaminants

Concentrations of PCBs, organochlorine pesticides (OCPs) and polybrominated diphenyl ethers (PBDEs) were determined in blubber samples, and perfluoroalkyl substances (PFAS) were determined in plasma samples from heparinized tubes; these findings were published elsewhere (Scotter et al., 2019) but the results are used herein to study associations between contaminant exposure and biological response variables in the same walruses. The analytical procedures including extraction, partitioning and clean-up, quantification, QA/QC used for the determination of 26 PCBs, *p,p*'-DDT, -DDE and -DDD, hexachlorobenzene (HCB), pentachlorobenzene (PeCB), five chlordanes, alpha-, beta- and gamma-hexachlorohexanes (HCH) and 24 PBDEs, 4:2, 6:2 and 8:2 fluorinated telomere sulfonates, $C_{4,6\text{-}10}$ perfluoroalkyl sulfonates, $C_{6\text{-}14}$ perfluoroalkyl carboxylates are similarly reported in detail in Scotter et al. (2019) and used herein. All contaminant analyses were conducted at the Norwegian Institute for Air Research, Tromsø. Analytical procedures are briefly described in the supporting information as well a full list of analysed compounds (Table S4, S5).

Data analyses

All statistical analyses were performed using R version 3.4.3 (R Core Team, 2017). Principal component analyses (PCA) were done in library *vegan* (Oksanen et al., 2017) to explore relationships between contaminants (ng/g wet weight) and plasma TH concentrations. To reduce the number of variables, only the contaminants quantified above the limit of quantification in 80% or more of the walruses were included in the statistical analyses. For

these compounds, the values below the limit of detection (LOD) were replaced by a random number between half of the LOD and the LOD (1.3% of the contaminant data). The following compounds were included in the analyses: PCB74, -99, -101, -118, -153, -170, -180, -183, -194, PeCB, α-HCH, β-HCH, γ-HCH, oxychlordane, trans-nonachlor, Mirex, *p,p'*-DDE, BDE47, BDE153, perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), perfluorononanoate (PFNA) and perfluorodecanoate (PFDA). Significance of the relationships identified by the PCA-biplot were further tested using linear models. To approximate normal distribution, contaminant concentrations were ln-transformed for linear models and further statistical analyses (Figure S3).

Bayesian analyses in the R-package *MCMC.qpcr* were used to explore the relationships between contaminants and transcript levels of genes (Matz et al., 2013). Prior to analyses, Cq-values for each technical replicate (n=2 and n=3 per walrus ID for blood cell and blubber data, respectively) were converted to molecule counts using the following equation: $E^{(Cq1-Cq)}$ in which E is gene specific amplification efficiency and $Cq1 = 79 - 21.5E$ (Matz et al., 2013). Blood samples from five individuals were not included in the statistical analyses due to low RIN values (<5) (Duale et al., 2012; Fleige et al., 2006). Generalized linear mixed models (GLMMs) with Poisson log-normal error were applied to the molecule count data using *mcmc.qpcr*-function (Matz et al., 2013). Four separate models were fitted to analyse how ln-transformed ΣPOP and ΣPFAS (ng/g ww) explained the variation in blood cell and blubber qRT-PCR data, so the responses of all genes in one matrix were analysed simultaneously. Summed concentrations of highly correlated POPs and PFASs were used as predictor variables to reduce the number of tests. Transcript levels of genes and their interactions with contaminants were included as fixed effects in the models. Walrus ID was included as a random effect in all models. Model estimates and credible intervals (Bayesian analogue of the confidence interval) were obtained using a Markov Chain Monte Carlo

(MCMC) algorithm with 12000 iterations. Models were fitted without reference genes (naïve model), and compared to models incorporating the variation of individual reference genes and/or RNA quantity, RIN (only available for blood samples) and/or geometric average of the reference genes as trackers for global effects. Models including the geometric average of the reference genes as a tracker for global effects were selected for final analyses because they showed the highest performance (i.e. narrowest credible intervals), and global patterns were present in the Cq-data. Final estimates were obtained from MCMC algorithm with 45 000 iterations (first 5000 discarded). Outliers were identified using the function *outlierSamples* in the library *MCMC.qpcr*. If outliers were present at the model fitting stage, further analyses were run with and without the outliers to explore their potential influence and results reported if the significance of the estimates differed. Model assumptions (linearity, homoscedasticity and normal distribution) were tested graphically using diagnostic plots of residuals.

Results

Contaminants

Summed concentrations of lipophilic compounds in walrus blubber samples and PFASs in plasma samples are shown in Table 1. As reported by Scotter et al. (2019) concentration range for lipophilic compounds ranged over three orders of magnitude, whereas variation in PFAS concentrations only one order of magnitude. PCBs and oxychlordane comprised 70 and 22%, respectively, of lipophilic POPs. Transformed to lipid weight (lw) concentrations, median and mean concentrations for Σ_{18}PCBs were 923 and 383 ng/g lw, whereas respective oxychlordane concentrations were 383 and 937 ng/g lw (Scotter et al. 2019). PFOS was the most dominant compound among the six PFASs detected. Mean and median values for Σ_6PFAS were 5.4 and 6.8 ng/g wet weight in walrus plasma samples.

Thyroid hormones and relationships with contaminants

All of the thyroid hormones studied (free and total forms of T4 and T3 as well as reverse T3) were found at detectable levels in walrus plasma samples (Table 2). An explorative PCA biplot suggested that the lipophilic POPs that were strongly inter-correlated (PCBs, β-HCH, chlordanes, Mirex, p,p'-DDE and PBDEs) were negatively associated with the concentrations of TT4 and TT4:rT3 (Figure 1). Linear models using ln-transformed ΣPOP as a response variable, confirmed the relationships (β=-5.9 [95% CI: -10.6, -1.2], p=0.015 for TT4 and β=-2.7 [95% CI: -5.1, -0.39], p=0.023 for TT4:rT3; Figure 2). Concentrations of FT4, TT3 as well as TT3:FT3 ratios were located close to PFOS in the PCA biplot (Figure 1), but none of these relationships were significant according to linear models ($p \geq 0.22$).

Gene transcription in blubber and relationships with contaminants All of the genes that were studied were transcribed in the blubber samples with average Cq-values per gene ranging between 15 and 30 (Figure S4) and 0.5% of the values showed low expression (Cq>35 (Duale et al., 2012)). Transcript levels of *NR3C1*, the thyroid hormone receptor alpha (*THRA*) and the retinoic X receptor alpha (*RXRA*) in blubber samples were negatively associated with plasma ΣPFAS concentrations (Table 3a). Based on the back-transformed estimates, the molecular counts of these genes decreased by ~30% within the range of the standard deviation of ΣPFAS (±3.65; Table 2). However, the inclusion of outliers did affect the significance of the results (Table 3a footnote) despite overlapping confidence intervals of the estimates. Additionally, results obtained from the naïve model were different from the model that included the variation of the reference genes (estimates for *NR3C1*, *THRA* and *RXRA*: 0.054, 0.040 and 0.048).

Gene transcription in blood and relationships with contaminants

Transcript levels of deioidinase 1 (*DIO1*), *PPARG*, fatty acid binding protein 4 (*FABP4*), phosphodiesterase 8B (*PDE8B*), solute carrier organic anion transporter family member 1c1

(*SLCO1C1*) and FC receptor like molecule 3 (*FCRL3*) showed low transcription in the blood samples with Cq-values above 35 (Duale et al., 2012) in 22-53% of the samples (Figure S4). The average Cq-values ranged from 24 to 33 for the remaining genes except *THRA* and *RXRA*, which could not be quantified in most of the samples and were thus excluded from further analyses (Figure S4). *TSHR* showed the lowest Cq-values (i.e. the highest transcript levels) among the genes analysed (Figure S4). Bayesian analyses, that incorporated geometric averages for the reference genes as trackers for global effects, indicated that the transcript levels of the four genes involved in immune function, protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) , *FCR2* and *FCR5*, increased with ΣPFAS concentrations in plasma. Based on the back-transformed estimates, the molecular counts of these genes increased by 2.6-3.8 times within the range of the standard deviation of ΣPFAS (±3.65; Table 2). Although the reference genes have not been validated for walruses, the results were not influenced by the selection of reference genes. The estimates obtained from the model that incorporated the reference genes (Table 3b) were similar to the estimates obtained from the naïve model that did not incorporate the reference genes (estimes for *CTLA4*: 0.15, *PTPN22*: 0.16, *FCR2*: 0.18 and *FCR5*: 0.21).

Discussion

Endocrine system

T4 was the dominating TH, whilst rT3 concentrations were higher than TT3 concentrations. The findings are in agreement with previous studies on circulating THs in elephant seals (*Mirounga angustirostris*) from California and white whales from Svalbard (Hansen et al., 2017; Jelincic et al., 2017). Secretion of THs, T4, and to a lesser extent T3, from the thyroid gland is regulated by the thyroid stimulating hormone (TSH) (Yen, 2001). Several enzymes regulate metabolism of THs. DIO2 catalyses the deiodination of T4 to its active form T3.

DIO3 converts T4 to inactive rT3 and DIO1 degrades inactivated THs, whereas sulfo- and glucuronyltransferases inactivate all THs (van der Spek et al., 2017). 98.5% of the total THs were bound to carrier proteins in walrus plasma samples. The free TH fraction is considered to be an indicator of TH availability, although the concept has also been debated (McNabb, 1992).

TT4 plasma concentrations and TT4:rT3 ratio were inversely related to blubber concentrations of lipophilic POPs in male walruses from Svalbard. Lower TT4 concentrations in more contaminated walruses is consistent with previous studies on pinnipeds (Brouwer et al., 1989; Kunisue et al., 2011; Routti et al., 2008b; Tabuchi et al., 2006). Contaminant-mediated thyroid disruption can occur via many different potential mechanisms. These include TH synthesis, release, transport, actions on target tissues as well as metabolism through deiodination, sulfation or glucuronidation (Gore et al., 2015; van der Spek et al., 2017; Wu et al., 2005). The negative association between TT4:rT3 ratio and POP exposure found in this study may be related to POP-mediated disruption in DIOs as DIOs catalyse the conversion of T4 to rT3 and further to 3,3'-diiodothyronine (van der Spek et al., 2017) and DIOs are targeted by halogenated contaminants (Shimizu et al., 2013).

The negative relationship between TT4 and POP concentrations in the walruses is likely related to increased glucuronication of T4, and/or enhanced uptake of T4 by liver. T4 glucuronication, which enhances hepatobiliary clearance is catalyzed by uridine diphosphate-glucuronyltransferase (UGT) 1A isozymes, whereas T3 is metabolized through other pathways (Findlay et al., 2000; van der Spek et al., 2017). UGT1A expression, which occurs mainly in mammalian (human) liver, is regulated by multiple factors including xenobiotic induced receptors: the pregnane X receptor, the constitutive androstane receptor and the aryl hydrocarbon receptor (Court et al., 2012; Findlay et al., 2000; Walter Bock and Köhle, 2005; Xie et al., 2003). Increased hepatic induction of UGTs and decreased levels of circulating T4,

but not T3, have been observed in rats following exposure to PCBs or various drugs inducing UGTs (Barter and Klaassen, 1994; Van Birgelen et al., 1994). Studies on pinnipeds have also shown contaminant-mediated induction of hepatic UGTs (Routti et al., 2008a). However, the involvement of UGT in T4 clearance in walruses is not supported by the negative relationship between TT4:rT3 ratio and POP concentrations. Studies on humans and rats have shown that UGT1A has a higher substrate preference towards rT3 rather than T4 (Findlay et al., 2000; Visser et al., 1993), and thus a contaminant-related increase in TT4:rT3 ratio and decrease in rT3 concentrations would be expected. Although UGT substrate preferences vary between mammalian species (Kakehi et al., 2015), other mechanisms may also be involved (Lecureux et al., 2009). Studies on multiple rodent species have shown that PCB-mediated decreases in circulating T4, but not T3, are explained by enhanced hepatic uptake of T4 (Kato et al., 2010; Kato et al., 2007). The enhanced hepatic uptake has been related to increased expression of hepatic T4 influx transporters (Kato et al., 2017). Contaminant-related decreases of T4 in the walruses may be associated with disruption of T4 synthesis, as thyroperoxidase, an essential enzyme in T4 synthesis, is inhibited by several environmental chemicals (Paul et al., 2014; Song et al., 2012). In addition, particularly phenolic compounds interfere with thyroid hormone transport proteins (Lans et al., 1993; Simon et al., 2011), but the consequence of this on TH levels is unknown (Miller et al., 2009).

Consequences of the lower T4 concentrations in the highly contaminated walrus males are unknown. THs are involved in metabolic processes, and therefore thyroid disruption in an Arctic species may have consequences for energy homeostasis (Jenssen et al., 2015). In the present study, only adult males were studied, but it should be kept in mind that females with developing foetuses and new-borns may be particularly susceptible to TH disruption (Braathen et al., 2004; Miller et al., 2009).

All of the analysed genes in blubber samples are involved in formation of lipid stores. Stimulation of GR (encoded by *NR3C1*) by glucocorticoids is needed in differentiation of preadipocytes into adipocytes (Cristancho and Lazar, 2011) and the amount of GR ligands is regulated by hydroxysteroid 11-beta dehydrogenase 1 (HSD11B1) which converts inert cortisone to active cortisol (Seckl and Walker, 2001). PPARG and its downstream targets fatty acid binding protein 4 (*FABP4*) and adiponectin (*ADIPOQ*), are the major regulators in terminal differentiation of adipocytes (Cristancho and Lazar, 2011; Desvergne et al., 2006). RXRA forms a heterodimer with PPARG and may also activate PPARG (Evans and Mangelsdorf, 2014). THRA, a nuclear receptor activated by T3, is involved in both lipogenesis and lipolysis in adipose tissue (Mullur et al., 2014; Zhu et al., 2010). Transcription of *THRA*, *RXRA*, *PPARG*, *ADIPOQ* and *FABP4* has been previously reported in pinnipeds (Castelli et al., 2014; Mos et al,. 2007; Tabuchi et al., 2006).

Transcript levels of *THRA* and *RXRA* in blubber were negatively related to plasma PFAS concentrations in walruses. This is in agreement with an *in vitro* study suggesting that PFASs inhibited GH3 cell (rat pituitary-derived cell line) proliferation, which is mediated by *THR* (Long et al., 2013). However, *in vitro* studies also using GH3 cells as well as *THRA* and *THRB*-mediated luciferase reporter assays suggest that the PFOS is *THR* agonist (Xin et al., 2018). The results of the relationships between transcript levels of *THRA* and *RXRA* in blubber plasma PFAS concentrations in the walruses should be interpreted with care. The results were only significant when three outliers were excluded, although the estimates were still negative and the confidence intervals overlapped. The reason why the three samples were outliers might be related to their low RNA quantity/quantity and missing data. Two of the outliers were among the quartile of the samples with the lowest RNA quantity and purity, whilst *RXRA* and *NR3C1* could not be amplified in the third outlier for unknown reasons (Table S6).

Immune system

Majority of the studied genes were transcribed in walrus blood cells and many of these regulate the function of immune cells. For example, Fc receptor like 1-5 molecules (FCRL; also known as immune receptor translocation-associated proteins, FCR homologs or cluster of differentiation [CD] 307 markers), expressed mainly on the B cell surface, up-regulate the proliferation and control function of B cells (Capone et al., 2016; Maltais et al., 2006; Matesanz-Isabel et al., 2011; Polson et al., 2006). Cytotoxic T-lymphocyte associated protein 4 (CTLA4), notch 1, protein tyrosine phosphatase non-receptor type 22 (PTPN22) and interleukin 2 receptor (IL2R) are involved in specification, maintenance and signaling of T cells (Ciofani and Zuniga-Pflucker, 2005; Jofra et al., 2017; Malek and Castro, 2010; Radtke et al., 1999; Teft et al., 2006; Waterhouse et al., 1996). Furthermore, CD40, which is a receptor expressed by B cells that is activated by the CD40 ligand, which in turn is expressed mainly by T cells, regulates both humoral and cellular immune responses (Elgueta et al., 2009). Interferon induced with helicase C domain 1 (IFIH1) is a pathogen recognition receptor, which has an essential role in the innate antiviral immune response (Malathi et al., 2007). Furthermore, PPARG, FAPB4 and ADIPOQ (the latter released from adipose tissue) control inflammatory function of marcophages (Makowski et al., 2005; Ohashi et al., 2010) whereas GR (encoded by *NR3C1*) mediates anti-inflammatory effects of corticosteroids by regulating cell adhesion (Cronstein et al. 1992). The high expression of TSHR in the peripheral immune system may be involved in an alternative regulation of metabolism by the immune system (Klein, 2014).

Transcript levels of *FCRL2*, *FCRL5*, *PTPN22* and *CTLA4* in blood cells increased with plasma PFAS concentrations in the walruses. Because *FCRL2* and *FCRL5* are highly expressed on B cells (Matesanz-Isabel et al., 2011; Polson et al., 2006), the positive relationships between *FCRL2* and *FCRL5* transcript levels and PFAS exposure in the

452 walruses may be related to the proliferation of B cells (Capone et al., 2016). Proliferation of
453 B cells has been positively associated with PFOS exposure in free-ranging bottlenose
454 dolphins (*Tursiops truncatus*), and this relationship has also been confirmed *in vitro* using
455 peripheral blood leukocytes isolated from the same species (Fair et al., 2013; Wirth et al.,
456 2014). However, studies on mice suggest suppression of B cell-mediated humoral immunity
457 following exposure to PFOS (Peden-Adams et al., 2008) and mitogen-induced B cell
458 proliferation was not modulated by PFOS (or PFOA) exposure in lymphocytes isolated from
459 ringed seal lymph nodes (Levin et al., 2016).

460 The higher transcript levels of PTPN22 and CTLA4 in walruses with higher PFAS
461 concentrations could have consequences on T cell-mediated immune defence. PTPN22 is one
462 of the key regulators of immune homeostasis by having dual roles on T cells; it inhibits
463 homeostatic proliferation, but it also promotes antigen-driven responses during acute
464 infection (Jofra et al., 2017). CTLA4 is a T cell surface molecule that inhibits T cell-mediated
465 immune defence (Teft et al., 2006; Waterhouse et al., 1996). Previous studies suggest that
466 PFOS exposure decreased T cell-mediated immune defence in human cells *in vitro* (Corsini
467 et al., 2011), whereas proliferation of T cells increased following PFOS exposure *ex vivo* and
468 *in vitro* in free-ranging bottlenose dolphins (Soloff et al., 2017). In ringed seal lymphocytes,
469 mitogen-induced T cell proliferation was not modulated by PFOS exposure (Levin et al.,
470 2016).

471 *Conclusions*

472 This study is the first to investigate relationships between biological responses and
473 contaminants in walruses. T4 concentrations were inversely related to blubber concentrations
474 of lipophilic POPs. The health impact of lower T4 concentrations in the highly contaminated
475 walruses are unknown. Relationships between PFAS exposure and transcript levels of genes

476 related to the immune system suggest that the immune responses may be compromised by
477 PFAS exposure. Future research should focus on thyroid disruption in walrus females and
478 calves. Given the role of THs in growth, neurologic development and metabolism, calves and
479 females (that allocate large amounts of energy to reproduction) are likely more vulnerable to
480 thyroid disruption than males. Future studies should also investigate potential immunotoxic
481 effects of contaminants and their relationships with diseases in Arctic marine mammals.

482

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492 **Conflicts of interest**

493 The authors declare no conflicts of interest.

494 **Table 1.** Target genes analysed in walrus blubber and blood cells.

Gene name	Symbol	Blubber	Blood cell	Involvement
Capping actin protein of muscle z-line beta subunit	*CAPZB*		x	TSH secretion[1]
Thyroid stimulating hormone receptor	*TSHR*		x	TH synthesis[2]
Phosphodiesterase 8B	*PDE8B*		x	TH synthesis[3]
Thyroid hormone receptor alpha	*THRA*	x	x	TH action[2]
Retinoid X receptor alpha	*RXRA*	x	x	THRA/PPARG heterodimer pair[4]
Solute carrier organic anion transporter family member 1c1	*SLCO1C1*		x	TH uptake by brain[5]
Deiodinase, iodothyronine type 1	*DIO1*		x	TH degradation[6]
Deiodinase, iodothyronine type 2	*DIO2*		x	TH activation (T4 --> T3)[6]
Hydroxysteroid 11-beta dehydrogenase 1	*HSD11B1*	x	x	Activation of cortisone to cortisol[7]
Nuclear receptor subfamily 3 group c member 1	*NR3C1/GR*	x	x	Cortisol action[7]
Peroxisome proliferator activated receptor gamma	*PPARG*	x	x	Formation of lipid stores; inflammatory function of marcophages[8]
Fatty acid binding protein 4	*FABP4*	x	x	Formation of lipid stores; inflammatory function of marcophages[8]
Adiponectin	*ADIPOQ*	x	x	Formation of lipid stores; inflammatory function of marcophages[8]
Fc receptor like 1,2,3 and 5	*FCRL1,2,3,5*		x	Proliferation and function of B cells[9]
Cytotoxic T-lymphocyte associated protein 4	*CTLA4*		x	Negative regulator of T cell responses[10]
Interleukin 2 receptor subunit alpha	*IL2RA*		x	T cell function[11]
Protein tyrosine phosphatase, non-receptor type 22	*PTPN22*		x	T cell function[12]
Cluster of differentiation 40	*CD40*		x	Humoral and cellular immune responses[13]
Notch 1	*Notch 1*		x	T cell development[14]
Interferon induced with helicase c domain 1	*IFIH1*		x	Innate antiviral immune response[15]
Beta-2-microglobulin	*B2M*	x		Reference gene
Eukaryotic translation elongation factor 1 alpha	*EEF1A1*	x		Reference gene
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	x	x	Reference gene
Actin β	*ACTB*		x	Reference gene
Hypoxanthine phosphoribosyltransferase 1	HPRT1		x	Reference gene
TATA-box binding protein	*TBP*		x	Reference gene

495 [1] (Panicker et al., 2010; Soto-Pedre et al., 2017); [2] (Yen, 2001); [3] (Arnaud-Lopez et al., 2008); [4] (Mangelsdorf
496 and Evans, 1995); [5] (Jansen et al., 2005); [6] (van der Spek et al., 2017); [7] (Nicolaides et al., 2010); [8] (Cristancho
497 and Lazar, 2011; Desvergne et al., 2006; Makowski et al., 2005; Ohashi et al., 2010); [9] (Capone et al., 2016;
498 Maltais et al., 2006; Matesanz-Isabel et al., 2011; Polson et al., 2006); [10] (Teft et al., 2006; Waterhouse et al.,
499 1996); [11] (Malek and Castro, 2010); 12 (Jofra et al., 2017); [13] (Elgueta et al., 2009); [14] (Ciofani and Zuniga-
500 Pflucker, 2005; Radtke et al., 1999); [15] (Malathi et al., 2007)

501

Table 2. Body length, tusk volume, plasma concentrations and ratios of thyroid hormones, and, plasma concentrations of Σ_5PFASs and blubber concentrations of Σ_{19}POPs in adult male walruses sampled from Svalbard in August 2014 and 2015 (n=38).

	mean ± SD	median	range
length (cm)	335 ± 29	340	226 - 390
tuskvolume (cm^3)	403 ± 175	374	135 - 894
TT3 (nmol/L)	1.31 ± 0.44	1.2	0.6 - 2.28
TT4 (nmol/L)	76 ± 24	73	38 - 137
rT3 (nmol/L)	2.28 ± 0.31	2.36	1.53 - 3.04
FT4 (pmol/L)	55 ± 29	56	3.14 - 107
FT3 (pmol/L)	19 ± 3.24	19	14 - 28
TT4:TT3	64 ± 28	59	21 - 154
TT3:FT3	67 ± 20	64	34 - 114
TT4:FT4	2127 ± 2192	1458	452 - 12230
FT3:rT3	8.73 ± 2.31	8.32	5.26 - 18
TT4:rT3	34 ± 12	30	18 - 69
FT4:FT3	2.90 ± 1.59	2.97	0.21 - 6.67
ΣPFAS (ng/g ww) [a]	6.25 ± 3.56	5.1	1.77 - 18
ΣPOP (ng/g ww) [b]	3336 ± 6458	1219	65 - 36822

[a] PCB74, -99, -101, -118, -153, -170, -180, -183, -194, PeCB, α-HCH, β-HCH, γ-HCH, oxychlordane, trans-nonachlor, Mirex, *p,p'*-DDE, BDE47 and BDE153

[b] PFHxS, PFOS, PFOA, PFNA and PFDA

Table 3. Transcript levels of genes of interest determined in a) blubber and b) blood cells explained by blubber concentrations of persistent organic pollutants (POP) and plasma concentrations of perfluoroalkyl substances (PFAS) in adult male walruses. Estimates from Bayesian analyses are given with 95% credible intervals (CI). Three outliers were removed from analyses of blubber genes. Significant results are in bold font.

	ln(ΣPOP ng/g ww)	ln(ΣPFAS ng/g ww)
	Estimate (95% CI)	Estimate (95% CI)
a) blubber		
THRA	0.01 (-0.05, 0.08)	**-0.06 (-0.11, -0.01)**[a]
RXRA	0.03 (-0.04, 0.1)	**-0.06 (-0.11, -0.01)**[b]
NR3C1	0.06 (-0.01, 0.12)	-0.04 (-0.09, 0.01)
HSD11B1	0.1 (-0.03, 0.21)	-0.01 (-0.1, 0.08)
PPARG	-0.04 (-0.21, 0.13)	0.03 (-0.09, 0.17)
ADIPOQ	0.12 (-0.003, 0.25)	0.04 (-0.05, 0.14)
FABP4	-0.09 (-0.3, 0.15)	-0.05 (-0.22, 0.13)
b) blood cells		
TSHR	-0.06 (-0.50, 0.37)	-0.12 (-0.45, 0.18)
CAPZB	-0.07 (-0.26, 0.12)	0.02 (-0.14, 0.16)
PDE8B	0.06 (-0.16, 0.25)	0.09 (-0.10, 0.24)
DIO1	-0.09 (-0.26, 0.11)	0.00 (-0.15, 0.16)
DIO2	-0.04 (-0.26, 0.21)	0.13 (-0.04, 0.30)
SLCO1C1	-0.01 (-0.24, 0.17)	-0.04 (-0.21, 0.12)
NR3C1	-0.06 (-0.24, 0.11)	0.06 (-0.07, 0.18)
HSD11B1	-0.07 (-0.24, 0.12)	0.05 (-0.07, 0.21)
PPARG	-0.28 (-0.59, 0.09)	0.01 (-0.27, 0.28)
ADIPOQ	-0.14 (-0.31, 0.02)	0.10 (-0.03, 0.24)
FABP4	-0.17 (-0.36, 0.07)	0.01 (-0.16, 0.17)
FCRL1	-0.09 (-0.26, 0.10)	0.06 (-0.08, 0.21)
FCRL2	0.05 (-0.12, 0.23)	**0.18 (0.05, 0.31)**
FCRL3	-0.06 (-0.31, 0.18)	0.11 (-0.08, 0.33)
FCRL5	0.09 (-0.09, 0.27)	**0.21 (0.07, 0.34)**
CTLA4	0.04 (-0.17, 0.25)	**0.15 (0.00, 0.30)**
Notch1	-0.09 (-0.26, 0.10)	0.12 (-0.02, 0.28)
PTPN22	0.01 (-0.17, 0.19)	**0.15 (0.01, 0.29)**
IL2RA	0.03 (-0.13, 0.18)	0.10 (-0.05, 0.22)
CD40	0.06 (-0.14, 0.25)	0.15 (-0.02, 0.29)
IFIH1	-0.13 (-0.36, 0.12)	0.14 (-0.05, 0.32)

[a] estimate with outliers: -0.03 (-0.18, 0.11)

[b] estimate with outliers: -0.05 (-0.11, 0.02)

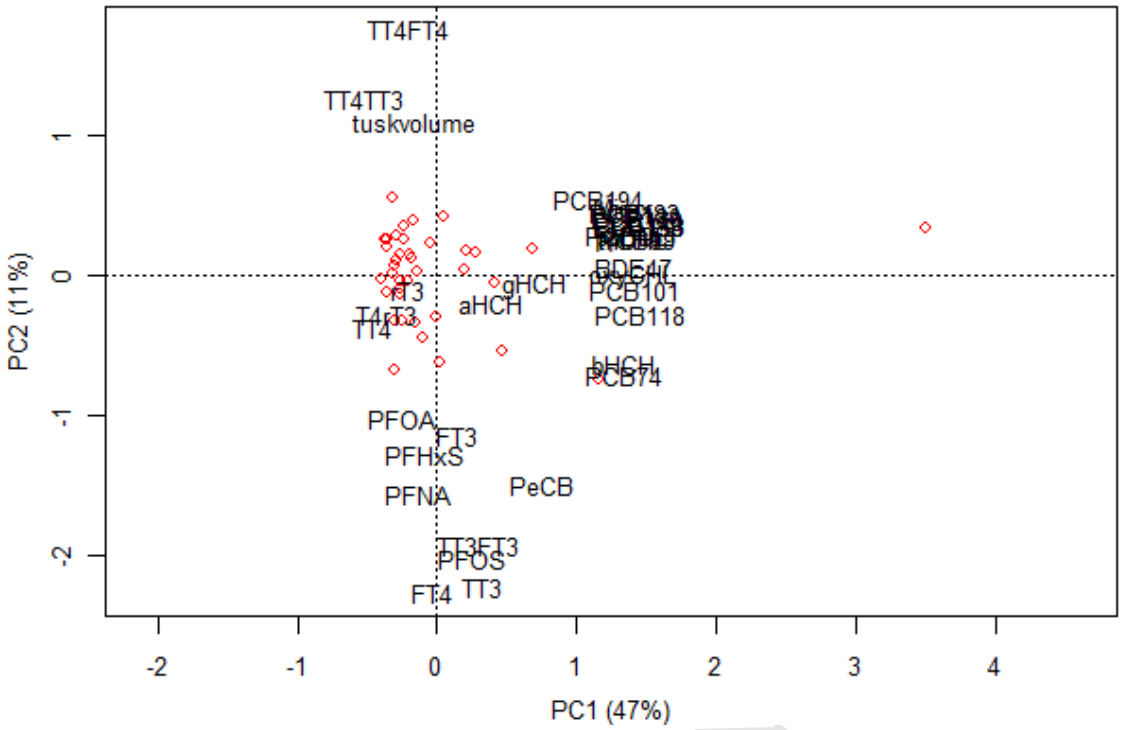
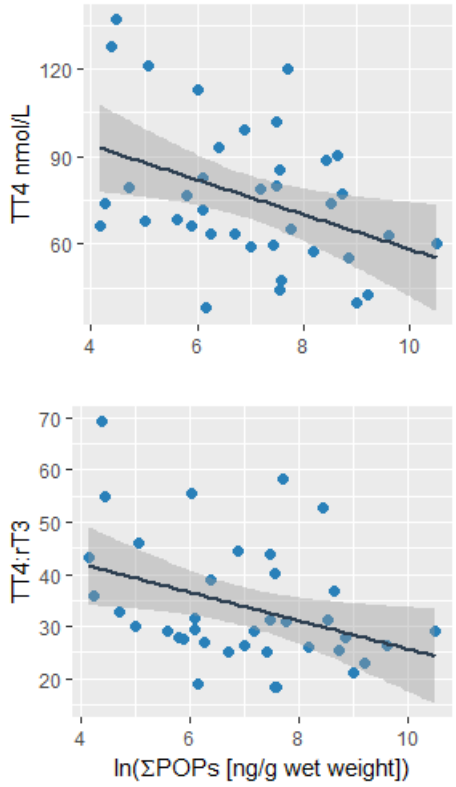


Figure 1. PCA biplot illustrating relationships between plasma concentrations and ratios of thyroid hormones and perfluoroalkyl substances, and blubber concentrations of lipophilic contaminants in adult male walruses sampled on Svalbard (red dots; n=38).



520

521 **Figure 2**. Concentrations of plasma total thyroxine (TT4), and, ratio of TT4 and reverse
522 triiodothyronine (rT3) plotted against blubber ΣPOPs in adult male walruses sampled on
523 Svalbard (n=38). Regression lines are shown with 95% confidence intervals.

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Highlights

- We investigated effects of contaminants in adult male walruses (n=38) from Svalbard
- We assessed five forms of thyroid hormones in plasma
- We studied transcript levels of seven genes in blubber and 23 genes in blood cells
- Plasma thyroxine concentrations were negatively related to POP levels in blubber
- Immune-related gene transcript levels in blood were related to plasma PFAS