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Relationships between concentrations of selected Organohalogen Contaminants and thyroid hormones and vitamins A, E and D in Faroese pilot whales

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Abstract

Pilot whales (*Globicephala melas*) from the Faroe Islands, North-East Atlantic, have high body concentrations of organohalogenated compounds (OHCs), such as polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs) and brominated flame retardants (BFRs). The aim of the present study was to examine if and to what extent blood plasma and liver concentrations of several groups of these OHCs are related to concentrations of relevant nutritional and hormonal biomarkers in pilot whales. Thyroid hormones (THs: total and free thyroxine and total and free triiodothyronine) and vitamin A (retinol), D (25-hydroxyvitamin D_3) and E (α-tocopherol) were analysed in plasma (n=27) and vitamin A (total vitamin A, retinol and retinyl palmitate) and E (α- and γ-tocopherol) were analysed in liver (n=37) of Faroe Island pilot whales. Correlative relationships between the biomarkers and OHC concentrations previously analysed in the same tissues in these individuals were studied. The TH concentrations in plasma were significantly higher in juveniles than in adults. Vitamin D concentrations in plasma and α- and γ-tocopherol in liver were higher in adults than in juveniles. Multivariate statistical modelling showed that the age and sex influenced the relationship between biomarkers and OHCs. Some significant positive relationships were found between OHCs and thyroid hormone concentrations in the youngest juveniles ($p<0.05$). In plasma of juvenile whales α–tocopherol was also positively correlated with all the OHCs ($p<0.05$). Only few significant correlations were found between single OHCs and retinol and vitamin D in plasma within the age groups. There were significant negative relationships between hepatic PBDE concentrations and retinol (BDE-47) and γ–tocopherol (BDE-49, -47, -100, -99, -153) in liver. The relationships between OHCs and THs or vitamins suggest that in pilot whales OHCs seem to have minor effects on TH and vitamin concentrations.

Key words: Pilot whale; Thyroid hormone; Vitamin A; Vitamin E; POPs.

1 Introduction

Pilot whales (*Globicephala melas*) from the Faroe Islands, North-East Atlantic, belonging to the toothed whales (*Odontoceti*), have high body concentrations of organohalogenated compounds (OHCs), such as polychlorinated biphenyls (PCBs), organochlorinated pesticides (OCPs) and brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs) (Borrell and Aguilar, 1993; Dam and Bloch, 2000; Hoydal et al., 2015; van Bavel et al., 2001). Several OHCs are known to have disrupting effects on biological processes such as reproduction, immunefunction and neurodevelopment (Weisglas-Kuperus, 1998). Although the mechanisms behind these effects are not fully understood, some of these anthropogenic compounds are known to disrupt endocrine systems, and influence levels of enzymes, hormones and vitamins (Colborn et al., 1993; Letcher et al., 2010). These endocrine disrupting effects may in turn result in health effects that reduce reproductive success and survival of the afflicted populations.

It has been shown that OHCs, such as PCBs, interfere with the regulation and homeostasis of thyroid hormones (THs), vitamin A (retinoids) and vitamin E (tocopherols) in various marine mammals including toothed whale species, such as beluga (*Delphinapterus leucas*) (Desforges et al., 2013; Villanger et al., 2011b), seals and polar bears (*Ursus maritimus*) (Gabrielsen et al., 2015; Jenssen et al., 2003; Simms et al., 2000; Sørmo, 2005; Villanger et al., 2013). Furthermore,

vitamin D has been shown to be influenced by body concentrations of OHCs in grey seals (*Halichoerus grypus*) (Routti et al., 2008). Due to their physiological importance, these variables have been proposed to be relevant biomarkers of effects of OHC exposure (Borrell et al., 2002; Debier et al., 2005; Rolland, 2000; Simms and Ross, 2000). A biomarker is defined as a biological response that can be related to either exposure to, and/or toxic effects from environmental chemical or chemicals (Peakall, 1994).

In earlier studies of pilot whales OHC concentrations in blubber, liver and plasma were reported to be several times higher in juveniles than in adult females (Dam and Bloch, 2000; Hoydal et al., 2015), in line with earlier findings of mammalian OHC transfer from mother to offspring through gestation and lactation (Borrell et al., 1995). Thus there is an enhanced concern for the health of the juveniles since THs and vitamins are important for growth and development in young individuals, including that of the brain (Brouwer et al., 1998; Howdeshell, 2002; Zoeller et al., 2002).

Thyroid hormones consist of thyroxine (T4) and triiodothyronine (T3) and control metabolism, cell differentiation and growth and are essential for normal reproduction and important in thermoregulation (Gregory and Cyr, 2003; Rolland, 2000). The synthesis of THs is controlled through negative feedback by the HPT axis (hypothalamus-pituitary-thyroid axis) (Kirby, 1990) where thyrotropin releasing hormone (TRH) secreted from the hypothalamus triggers the release of thyroid stimulating hormone (TSH, thyrotropin) from the pituitary gland (St Aubin, 1987). TSH stimulates the production of the THs (T4 and T3) in the thyroid gland, which subsequently when released into the blood stream can inhibit the production of TSH from the pituitary (Kirby, 1990). Retinoids (Vitamin A and its metabolites) are essential for various physiological functions including growth and development, reproduction, vision, epithelial maintenance and immune function (Novak et al., 2008; Simms and Ross, 2000). In marine mammals retinoids are derived from the diet mostly as retinyl esters (RE) and are often referred to as “dietary hormones” (Simms and Ross, 2000). The vitamins E and D also control important organism functions. Vitamin E refers to a group of tocopherols that function as chain breaking antioxidants preventing the propagation of free radical reactions (Brigelius-Flohe and Traber, 1999) with α-tocopherol as the most active form in mammals. Vitamin D_3 has several important roles in the organism, including mineral homeostasis, and is involved in calcium metabolism and bone mineralization together with other endocrine hormones. The predominant form of vitamin D is 25-hydroxyvitamin D_3 (25(OH)D3). Vitamin D can be produced by cutaneous exposure to ultraviolet-b light in terrestrial mammals, but fish eating marine mammals like pilot whales are able to satisfy their vitamin D_3 requirements from the diet (Kenny et al., 2004). Similarly vitamin E is also derived from the diet (Debier and Larondelle, 2005).

Several mechanisms can be involved in the OHC-mediated disruption of THs and vitamins (Brouwer et al., 1998; Liu et al., 2014). Thyroxine (T4), produced by the thyroid gland, is transported in plasma to target tissues by binding to the thyroxine plasma transport protein (TTR), albumin or thyroxine binding globulin (TBG). When delivered at the target cell T4 is deiodinated by T4-monodeiodinase to triiodothyronine (T3), which is the active hormone. The T4-TTR complex is transported in the blood plasma in a complex with retinol bound to the retinol binding protein (RBP). Hydroxy metabolites of some OHCs like PCBs or PBDEs can inhibit thyroxine (T4) binding to thyroxine plasma transport protein (TTR) by competing with T4 for TTR binding sites with the result of loss in T4 and retinol-RBP from the body (Brouwer et al., 1989a; Bytingsvik et al., 2013; Murk et al., 1998). TBG is the principal transport protein in

marine mammals (St Aubin, 2001) and has been measured in cetaceans, including Short-finned pilot whale (*Globicephala macrorynchus (scammoni))* (Ridgway and Patton, 1971) whereas efforts on analyzing TTR in beluga have not been successful (St Aubin, 2001). OHCs can also interact with thyroid gland function and morphology, leading to effects on the synthesis and secretion of T4 (Brouwer et al., 1998), including effects on deiodination enzymes (Gabrielsen et al., 2015). Also thyroid metabolism can be affected by OHC exposure by induction of enzymes leading to increased glucuronidation of hepatic T4 and increased biliary secretion and elimination of T4 (Brouwer et al., 1998).

OHCs and their metabolites can reduce uptake of dietary vitamin A, decrease liver vitamin A stores, disrupt circulatory transport to tissues and/or increase glomerular filtration and excretion of vitamin A metabolites (Simms and Ross, 2000). In marine mammals, retinoids are stored mainly in the liver, but also in tissues like blubber, mainly as retinyl esters (Borrell et al., 2002). Although there is great variation in the vitamin supply from the diet and in the liver or extrahepatic tissue stores, vitamin A levels remain constant in plasma apparently due to homeostatic regulation. Thus, body depletion of retinoids can better be evaluated through concentrations in depot tissues such as liver and blubber (Borrell et al., 2002) and negative correlations have been found between OHC concentrations and retinoid levels in blubber (Nyman et al., 2003; Tornero et al., 2004b; V. Tornero et al., 2005a). However, relationships between contaminants and retinol levels in plasma have also been reported in marine mammals (Braathen et al., 2004; Brouwer et al., 1989b; Jenssen et al., 2003) and Greenland sharks (*Somniosus microcephalus)* (Molde et al., 2013). Analyses in beluga from Arctic Canada found that PCB correlated negatively with vitamin A in liver, but positively in blubber and plasma (Desforges et al., 2013). The negative relationship between vitamin A and PCB in liver was thought to be related to up-regulation of hepatic enzymes involved in vitamin A metabolism (Desforges et al., 2013).

In consideration of the high levels of OHCs that have been reported in Faroe Island pilot whales, the aim of the present study was to examine how the concentrations of several groups of OHCs are related to levels of THs, vitamin A, E and D status. Plasma and liver from pilot whales sampled in 2009 - 2011 on the Faroe Islands were analysed in the present study. Plasma samples were analysed for concentrations of thyroid hormones (total and free T4 and T3) and vitamin A (retinol), E (α-tocopherol) and D (25(OH)-D3). Liver samples were analysed for vitamin A (total vitamin A, retinol and retinyl palmitate) and E (α- and γ-tocopherol). Correlative relationships among these biomarkers and plasma and liver concentrations of the PCBs, PBDEs, OCPs and their metabolites were studied.

2 Materials and methods

Plasma and/or liver were sampled from 37 pilot whales 2009, 2010 and 2011 on the Faroe Islands. The sampling of liver and plasma from 27 individuals in 2010 and 2011 was previously described in Hoydal et al. (2015). In addition to these 27 individuals; 10 livers from pilot whales were sampled in 2009 and 2010. An overview of the individuals analysed and their biological data is given in Table S1.

The individuals were divided into adult females, adult males, sub-adults >2 years and calves 0-2 years according to their length and/or age as described in Hoydal et al. (2015). The grouping into adults and juveniles was done using the animal lengths based on the studies of age at sexual

maturity by Martin and Rothery (1993) and Desportes et al. (1993). The further division of the juveniles into the two age groups (0-2y and >2y) was based on their different feeding and exposure pattern, since the calves 0-2 years old are expected to be suckling and exposed to contaminants via milk, whereas the sub-adults >2 years old are expected to be weaned and exposed via their solid food based on analyses of stomach content by Desportes and Mouritsen (1993).

2.1 OHC concentrations

The 27 individuals, from which both liver and plasma were collected, were previously analysed for OHCs and metabolites and the results were reported in Hoydal et al. (2015). In the present study these results were calculated in molar concentrations (Table S2) and were used in the statistical analyses of relationships between contaminants and hormone and vitamin levels in the pilot whales.

2.2 Analysis of thyroid hormones in plasma

Measurements of plasma concentration of total thyroxine (TT4), total tri-iodothyronine (TT3), free thyroxine (FT4) and free tri-iodothyronine (FT3) were conducted at the Department of Biology, NTNU, using solid-phase ^{125}I radioimmunoassay (Coat-A-Count, Diagnostic Products, Los Angeles, CA, USA) and the analyses were carried out according to the protocols provided with the kits. The detection and quantification were performed on a gamma counter (Cobra Auto-Gamma, Packard Instruments Company, Dowers Grove, IL, USA). The samples were analysed in triplicates (TT4, FT4) or duplicates (TT3, FT3). The quality control material Lyphocheck Immunoassay Plus Control from Bio-Rad in three concentrations (level 1, 2 and 3) was analysed together with the samples. One of the controls (concentration level 2) and one of the 26 samples were analysed two times (in duplicate or triplicate) to measure the intra-assay stability of the analysis. If the differences between replicates were too large (CV >15%) the analyses were repeated for these samples. The inter-assay CVs for repeated analyses were 6.8-8.1% for TT4, 1.21-3.5% for TT3, 1.3-12.1% for FT4, and 5.3-9.0% for FT3.

2.3 Analysis of vitamin A, E and D in plasma

The plasma samples were analysed for retinol (vitamin A), α-tocopherol (vitamin E) and 25(OH)D3 (vitamin D) at Department of Analytical Chemistry, Gdańsk University of Technology, Poland. 500 µL of plasma was mixed with 0.5 mL of ethanolic internal standard mixture and vortexed for 30 seconds. Precipitated proteins were centrifuged at 6000 rpm for 1 minute and 1 mL of hexane was added. After vortexing for 45 seconds and centrifugation the hexane layer was collected. Hexane extraction was repeated two more times. Pooled hexane extract was evaporated to dryness in a gentle stream of nitrogen. Dry residue was dissolved in 0.5 mL of ethanolic 2,6-di-tert-butyl-4-methylphenol (BHA) solution (30 µg/mL) and analysed by HPLC-MS-MS.

The samples were analysed on an Agilent 1200 HPLC system coupled with an AB Sciex Instruments 4000 Q TRAP Mass spectrometer. The column used was an Agilent XDB C18 (1.8 µm, 4.6 × 50 mm). A gradient mobile phase was used (Component A: 10 mmol ammonium acetate in methanol-water mixture (90% methanol), Component B: 10 mmol ammonium acetate in methanol-methyl tert-butyl ether (80% methanol) and the flow rate of 1.0 mL/min. The injection volume was 5 µL.

2.3.1 Standards and calibration

Standards of retinol acetate, retinol palmitate, DL-α-tocopherol, δ-tocopherol, γ-tocopherol and DL-α-tocopherol acetate were supplied by Supelco, (Bellefonte, PA). Standard of retinol and BHT were purchased from Sigma-Aldrich, (St. Louis, MO, USA). HPLC-isocratic grade methanol and absolute ethanol were obtained from VWR Prolabo, (France). N-hexane used in extraction procedure was purchased from Merck, Lichrosolv, (Germany).

The primary solutions of standards were prepared by dissolution of pure substances in ethanol. The UV absorbances were measured and concentrations calculated using specific molar absorptivities (25(OH)D3 and 25(OH)D3-d6: ε=18300 at λ=265 nm, retinol: ε=52480 at λ=325 nm, retinyl-palmitate: ε=49260 at λ=325 nm, α -tocopherol and α -tocopherol -d6: ε=3265 at λ=292 nm).

A mixture of standards (25(OH)D3, retinol, retinyl-palmitate and α -tocopherol) was prepared by mixing of appropriate volumes of primary solutions and 0.5 mL of ethanolic BHA solution (3 mg/mL) and diluting with ethanol to a final volume of 50 mL. Separate mixture of internal standards (25(OH)D3-d6 and α -tocopherol -d6) was prepared in the same way.

Seven calibration solutions were prepared from the mixture of standards. Varying aliquots of the mixture together with 0.200 mL of internal standards mixture were added to ethanolic BHA solution (30 μg/mL). The concentration ranges for particular compounds were as follows: 1.7 – 170 ng/mL for 25(OH)D3, 10 – 950 ng/mL for retinol, 130 – 2500 ng/mL for retinyl-palmitate and 0.34 – 33 μg/mL for α -tocopherol.

External calibration curves were constructed by injecting calibration solutions in triplicate and calculating ratio of analyte's peak area to internal standard peak area as a function of analyte's concentration ($A/A_{IS}=f(c)$, where A – analyte's peak area, A_{IS} – internal standard peak area, c – analyte's concentration). 25(OH)D3-d6 was used as an internal standard for 25(OH)D3 and retinol, while α -tocopherol -d6 was used in case of α -tocopherol and retinyl-palmitate. Calibration curves were linear within the studied concentration ranges. Coefficients of determination were higher than 0.999 for all analytes except retinyl-palmitate (R^2=0.995). The performance of the method was examined analysing standard reference material SRM968e. The recoveries are given in the supplementary material (Table S3).

Limits of detection (LOD) were estimated as concentrations giving signal to noise ratio of 3. For 25(OH)D3, retinol, retinyl-palmitate and α -tocopherol the detection limits were 0.002, 0.005, 0.1 and 0.07 μg/mL, respectively.

2.4 Analysis of vitamin A and E in liver

2.4.1 Total vitamin A

Livers were analysed for total vitamin A (total retinol) at the Analytical Services of the University of Barcelona (Centres Científics i Tecnològics de la Universitat de Barcelona), by the method described in Tornero et al. (2004a). Pieces of liver were crushed with a mortar or scissors. Approximately 100 mg were taken from each homogenate and saponified overnight in 5 ml ethanolic KOH solution (1g KOH, 2 ml distilled water, 2 ml ethanol and 20mg ascorbic acid) in a mechanical shaker under a nitrogen atmosphere. The retinoids were extracted by adding 8 ml of diisopropyl ether and shaking again for 30 min. The aqueous phase was removed and the organic extract was washed three times by adding 5 ml of aqueous phosphate buffer and shaking the solution by hand and removing the aqueous phase. After the first wash 1 ml of internal

standard (retinyl acetate) was added to the extract. After this the organic extract was evaporated on a heating block under nitrogen flow and reconstituted by adding 1 ml of methanol with 0.05% butylated hydroxyl toluene (BHT). Then the samples were filtrated into eppendorf vials using a syringe with a filter (0.22μm) on the tip.

The samples were transferred to HPLC vials and analysed on a Waters Acquity Ultra Performance LC analyser with the software Empower PRO from Waters. The column used was a Phenomenex Kinetex 2.6um C18 100A (silica), 150 x 4.60 mm. Mobile phase was methanol and water and the flow rate 0.5 ml/min. The injection volume was 10 μl and the quantifying wavelength was 325nm. A seven point standard curve was made from synthetic retinol diluted in the internal standard solution. The samples with the highest concentrations were diluted 2, 3 or 5 times to get a concentration inside the standard curve. The CVs between replicates ranged from 0.74 to 38.2% with a mean of 8.7%. CVs for repeated analyses of standards were between 0.1 and 9.9%.

2.4.2 Vitamin A and E

The livers were analysed for different forms of vitamin A and E (retinol, retinyl palmitate, α-tocopherol and γ-tocopherol) by reverse-phase HPLC with fluorescence detection (PerkinElmer200 series, USA) at the Department of Biology, NTNU. Vitamins were extracted from liver samples with a modified liquid–liquid extraction technique described by Murvoll et al. (2005).

Briefly, following thawing, approximately 1 g of liver tissue was homogenized (Glas-Col homogenizer system, model: 099C K54, Terre Haute, IN, USA) with addition of deionized water (MilliQ) in an ice-bath. Subsequently, to 350-400 mg of liver:water homogenate 100 μL of internal standard solution (IS, retinol acetate 25 μg/mL) and 1000 μL of hexane with 0.04% of BHT (2,6-di-tert-butyl-4-methylphenol, Sigma Aldrich, St. Louis, MO, USA) were added. The samples were vortex mixed for 10 s and further homogenised with a 5 mm steel beads in a Qiagen TissueLyser (QIAGEN, CA, USA), run at 30 Hz for 1.5 min. Subsequently, the samples were sonicated (high-intensity ultrasonic processor, four microtips; Sonics and Materials, Newtown, CT). The ultrasound processor was set to give pulses of 2 s, followed by 0.5 s without any pulse. Total sonication time was 2 min. The amplitude was 21% of maximum for this instrument. After the sonication 400 μL of ethanol (with 0.04% of BHT) was added and the samples were again vortex mixed for 10 s. After a centrifugation for 3 min at 13100 rpm (Eppendorf centrifuge 5415D, Eppendorf AG, Hamburg, Germany), 900 μL of hexane layer was collected. Then, the entire extraction process was repeated with reduced (300 μL instead of 400 μL) volume of ethanol with 0.04% of BHT. The two extracts were pooled and evaporated to dryness at 40°C in an automated evaporation system (TurboVap® LV) under gentle stream of nitrogen (ca. 10 min). After evaporation, to each vial 1000 μL of 100% methanol was added. Samples were mechanically shaken (Vibramax 110, Heidolph,Germany) for 1 min and 150 μL of aliquot was transferred to HPLC amber vial. The whole extraction process was conducted under dim light conditions.

The chromatography was carried out using a HPLC instrument equipped with a fluorescence detector, an autosampler with a Peltier sample tray , a pump, vacuum degasser, and a column (Chrompack Intersil, ODS-3, 150×4.6 mm, 5 μm) from Varian, Inc. (Lake Forest,CA) connected to a guard column (ChromGuard SS 10×3 mm) also from Varian. Data were collected with Turbochrom Work station version 6.1.2 software. The chromatography was carried out using a

step gradient elution mode in which eluent A was methanol (Sigma-Aldrich) and eluent B methanol–water (98:2, v/v). The following gradient was used during the run: 100% A at a flow rate 1 mL/min (0-4 min), 1.2 mL/min (4-10 min) and 1.5 mL/min (10-15 min), 15-35 min 100% B at a flow rate 1.5 mL/min and 35-55 min 100 A at the flow rate 2.0 mL/min. The column was at 21 ºC. Retinol and retinol acetate were detected using an excitation wavelength of λ=325 nm and an emission wavelength of λ=470 nm at medium sensitivity. These settings were maintained from injection to 8 min. At 8 min, the excitation wavelength was changed to λ=295 nm and emission wavelength to λ=330 nm in order to optimize the detection of tocopherols. These conditions were maintained until 20 min. At 20 min the settings were changed to the initial fluorescence detector conditions in order to detect retinyl palmitate and they were maintained until the end of the chromatographic run. All vitamins were quantified on the base of peak area ratios over internal standards as obtained from calibration curves.

Standards consisted of retinol acetate (IS) retinyl palmitate, α-tocopherol and γ-tocopherol (Supelco, Bellefonte, PA,USA) and retinol (Sigma Aldrich). The standards were dissolved individually in 100 mL of absolute ethanol, while retinyl acetate was dissolved in 100 mL of ethanol with an addition of 0.04% BHT. Standard solutions were stored at −70 °C. The internal standard (IS) working solutions (WS) in methanol (retinol acetate 25 μg/mL) were prepared daily by appropriate dilution of the stock solution. Calibration curve consisted of a six-point linear calibration line (R^2>0.99, each measured in duplicate) derived from a mixture of analytes prepared by diluting standard solutions in methanol: retinol 0.02–15 μg/mL, retinyl palmitate 0.1–30 μg/mL, α- and γ-tocopherol 0.1–20 μg/mL. The final results were calculated as the mean of the concentrations determined for two replicate samples.

CVs for the replicate analyses of vitamin A and E in liver were within the range 0.2 – 9.0 (mean 3.4) for retinol, 0.3 – 7.7 (mean: 2.6) for α-tocopherol, 0.2 – 7.0 (mean: 2.34) for γ-tocopherol and 0.1 – 12.7 (mean: 2.18) for retinyl palmitate. The CVs were below 12.7 for all samples, except for sample 8 (11-31.3), 9 (19.0-24.4), US49 (15.9-25.3) and for retinyl palmitate also sample 4 (21.2) and US21 (22.4).

2.5 Statistics

The statistical programme SPSS (IBM, version 21) was used for univariate data analyses. Since most of the biomarkers and the contaminant concentrations (Hoydal et al., 2015) were not normally distributed, non-parametric analyses were used. Differences in hormone and vitamin concentrations among the age and sex groups were analysed with Kruskal-Wallis 1-way ANOVA with pairwise comparison. Correlations among the individual biomarkers and between biomarkers and OHCs were examined using Spearman correlations. Significant levels were set to $p \leq 0.05$.

Multivariate analyses were performed using the statistical program SIMCA-P+ (Umetrics, version 12.0, 2008). PCA analysis was used to study the relationship between individual contaminants and the biomarkers (THs and vitamins) and the biological factors (age, length). OPLS (orthogonal partial least squares regression) modelling was used to analyse the influence of the contaminants and the biological factors (X-variables) on the biomarkers (Y-variables). 27 OHCs in plasma, lipid%, length and age were included in the OPLS model to investigate their combined effect on the biomarkers in blood. Separate OPLS model for liver included 59 OHC variables, lipid%, length and age. For each OPLS model, R^2 and Q^2 values were calculated, where R^2 shows the goodness of fit (R^2X: variation of X explained by the model, R^2Y: variation of Y

explained by the model) and Q^2 shows the goodness of prediction (cross-validation of the model) (Eriksson et al., 2013). R^2 value >0.7 and a Q^2 value >0.4 denote a highly significant model when analysing biological data (Lundstedt et al., 1998). In addition the significance of the models was analysed by CV-ANOVA. If the initial model was not significant (CV-ANOVA, $p<0.05$) the least significant variables were removed one by one until a significant model was obtained. If significance was not obtained, the model was defined as not-significant. All variables were centred and scaled before the analysis. The contaminant variables were log transformed to approximate normal distribution.

3 Results

3.1 Thyroid hormones in plasma

The mean concentrations ± standard deviation of TT4, FT4, TT3 and FT3 were 62.78±29.2 nmol/L, 7.56±3.4 pmol/L, 1.21±0.6 nmol/L and 1.63±1.0 pmol/L, respectively. There were no significant differences in the concentrations of THs among the four age/gender groups (adult females, adult males, juv>2y, juv 0-2y: Table 1).

When comparing THs between juveniles (n=15) and adults (n=12, pooled adult males and females), the concentrations of all four THs were significantly higher in juveniles than in adults ($p<0.01$ for TT3 and FT3, $p<0.05$ for TT4 and FT4). Also the free active form of the hormone (FT3) in comparison to total T3 and free T4 was higher in juveniles than in adults ($p<0.05$).

Of the THs analysed; TT4, FT4, TT3, and FT3, were all highly inter-correlated ($p<0.001$). No significant correlations were found between TH concentrations and length or age of the animals (Figure 1).

3.2 Vitamin A, E and D in plasma

Concentrations of vitamins in plasma in relation to length of the individuals are shown in Figure 2. The concentrations of 25(OH)D3 were significantly higher in adult females than in juveniles 0-2 years ($p=0.02$), whereas the concentrations of retinol and α-tocopherol in plasma did not differ among the different age/gender groups (Table 1). Retinol and 25(OH)D3 in plasma were positively correlated with the age of the animals ($r_s=0.522$, $p=0.018$ and $r_s=0.529$, $p=0.024$, respectively). There were no differences in plasma vitamin concentrations when comparing all the juvenile animals (n=15) and the adults (n=12), except for 25(OH)D3 which was significantly higher in adults than in juveniles ($p=0.03$). In plasma, retinol was positively correlated to α-tocopherol ($r_s=0.614$, $p=0.001$) and to 25(OH)D3 ($r_s=0.437$, $p=0.033$). Furthermore, α-tocopherol and 25(OH)D3 were also significantly correlated ($r_s=0.544$, $p=0.006$). The vitamin concentrations in plasma were not correlated to the concentrations of THs in plasma.

3.3 Vitamin A and E in liver

The α-tocopherol concentrations in liver differed significantly between the age/sex groups (Kruskal-Wallis, $p=0.021$), but not in the pairwise comparison (Table 2). The liver concentrations of retinol, retinyl palmitate and γ-tocopherol in the pilot whales did not differ significantly between the four age/sex groups. However, α- and γ-tocopherol and retinol in liver were significantly higher in adults (n=12, pooled adult males and females) than in all the juveniles (n=15) ($p<0.05$). Furthermore, α- and γ-tocopherol in liver were significantly correlated to the length of the animals ($r_s=0.390$, $p=0.021$ and $r_s=0.346$, $p=0.042$, for α- and γ-

tocopherol respectively). Correlations between age and vitamins in liver were not significant. Figure 3 shows the relationship between vitamin concentrations in liver and the length of the individuals.

The hepatic concentrations of vitamins, except for α-tocopherol and retinyl palmitate, were inter-correlated in all animals ($p<0.01$). On a molar basis the retinyl palmitate constituted around 27% (25 - 75 percentiles: 22 – 31%) and retinol around 4% (25 - 75 percentiles: 2 – 5%) of the total vitamin A. Thus on a molar basis, the median retinyl palmitate concentration was 6 times higher than retinol concentration in liver (25 - 75 percentiles: 4 – 12 times). In the liver; concentration of α-tocopherol was on average 15 times higher than γ-tocopherol on a molar basis (25 - 75 percentiles: 12 – 17 times higher).

3.4 Relationship between vitamins in liver and plasma

The retinol concentrations in liver and plasma were significantly positively correlated ($r_s=0.418$, $p=0.038$). The retinol concentration in liver was on average 250 times higher than in plasma (25 - 75 percentiles: 80 – 400 times, assuming that the plasma density is 1.025g/mL). There were no other significant correlations between vitamins in liver and vitamins in plasma.

α-Tocopherol in plasma was not correlated to the concentrations in liver of either α-tocopherol or γ-tocopherol. The level of α-tocopherol in liver was on average 2 times higher than in plasma (range: 0.9 – 4.5 times).

3.5 Relationship between hormones, vitamins and OHCs

The PC1 explained 79% and 68% of the variation in liver and plasma respectively, while PC2 explained 7 and 10% of the variation in liver and plasma, respectively. The plot of scores representing liver samples (Figure 4A) showed a clear separation of the adult females group from the other age groups along the PC1 axis. The corresponding loading plot showed that the differences in the hepatic OHC concentrations, size and age and vitamin E concentrations between adult females and the rest of whales were responsible for the observed clustering of the samples along PC1 (Figure 4B). Differences in size and age as well as variation between different hepatic OHCs accumulation (e.g p,p'-DDE, CB-110, BDE-49) were responsible for separation of Juv>2y from 0-2y along the PC2 axis.

The plots of scores representing plasma samples, similarly as for the liver samples, showed a clustering and separation of the adult females from the other whales along the PC1 axis (Figure 5A). The loading plot (Figure 5B) showed that the clustering of the adult females was due to the differences in OHC concentration and THs and in the age and size between the adult females and the other whales. Also the relative levels of THs, TT3:FT3 and FT4:FT3, explained the clustering. Differences in TH concentrations and vitamin levels seemed to be responsible for the observed separation of the juvenile groups, Juv 0-2 and Juv >2, along the PC2 axis.

3.5.1 Relationships between OHCs and THs

Significant OPLS models (CV-ANOVA, $p<0.05$) were found for the THs (TT4: $R^2X=0.742$, $Q^2=0.292$; FT4: $R^2X=0.742$, $Q^2=0.259$; TT3: $R^2X=0.768$, $Q^2=0.387$; FT3: $R^2X=0.742$, $Q^2=0.372$) (Figure 6). The models showed that all the THs were negatively associated with the biological variables age and length. In contrast, the THs were positively associated with the plasma concentrations of the OHCs included in the OPLS. Spearman correlation analysis confirmed that

all PCBs, OCPs and PBDEs (BDE47) in plasma were significantly positively correlated with TT4, FT4, TT3 and FT3 ($p<0.05$).

Although no significant OPLS models were identified for the FT4:FT3 and the TT3:FT3 ratios, Spearman correlation analysis showed that the FT4:FT3 ratio was significantly negatively associated with all analysed contaminants ($p<0.05$), and that the TT3:FT3 ratio was significantly negatively associated with several PCB congeners (CB-74, -95, -99, -101/90, -118, -146, -149, -151, -153, -170/190, -174, -179, -180, -187), HCB, cis-chlordane and p,p'-DDE ($p<0.05$). The significant correlations between OHCs and THs are given in Table 3.

Since age and length were important variables in the OPLS models, further OPLS analyses were performed for the age groups separately (AdF: n=10 and Juveniles: n=15). However, no significant models were identified. When dividing the individuals into the four age/sex groups (and excluding the adult males since there were only two individuals), Spearman correlation analyses did identify correlations between some of the contaminants and THs mostly in the juveniles 0-2 years age group (Table 3). In adult females (n=10) only HCB was significantly positively correlated to TT3 and this was also found for all the juveniles (n=15).

In juveniles 0-2 years of age (n=8), TT4 correlated positively with CB-52, -74, -92, -95, -101/90, -105, -128, -149, -151, p,p'-DDE and BDE47 ($p<0.05$). TT3 correlated positively with CB-87, -92, -99 and trans-nonachlor ($p<0.05$), FT3 correlated positively with CB-87, -92, -99, p,p'-DDE and trans-nonachlor, FT3 ($p<0.05$) and TT4:FT4 ratio correlated positively with CB-74, -118 and -183. No correlations were found between THs and contaminants in plasma in juveniles >2 years (n=7).

A negative correlation was found between 4-OH-CB107/4'-OH-CB108 and the TT4:TT3 ratio in adult females ($p<0.05$), but not in the juvenile groups.

3.5.2 Relationships between vitamins and OHCs

The OPLS models were not significant for any of the vitamins in neither plasma nor liver. Spearman correlation analyses did, however, identify significant ($p<0.05$) correlations between the concentrations of some of the contaminants and vitamins. In the plasma, α-tocopherol was significantly positively correlated to concentrations of CB-87, -146 and -183 ($p<0.05$). There were no significant correlations between plasma retinol or 25(OH)D3 and any of the contaminants (Table 4). In liver, retinol was negatively correlated to BDE47, and γ-tocopherol was negatively correlated to BDE-47, -49, -100, -99 and -153 ($p<0.05$), whereas no significant correlations were found between α-tocopherol or retinyl palmitate and contaminants ($p>0.05$).

Within the age groups, there were also some statistically significant ($p<0.05$) relationships between vitamins and OHCs (Table 4). In plasma of adult females, retinol was positively correlated to HCB and negatively to CB-146. α-Tocopherol was negatively correlated to CB-74 and positively correlated to CB-87. In liver of adult females retinol was positively correlated to CB-74 and CB-196/203, retinyl palmitate was positively correlated to CB-196/203 but negatively correlated to BDE-28, -47, -49, and -153, and α-tocopherol was positively correlated to CB-171 and Mirex.

In plasma of the juveniles, in the 0-2y group α-tocopherol was positively correlated to all detected contaminants except for CB-118 and BDE-47 (Table 4). When pooling all the juveniles (0-2y and >2y groups) (n=15) α-tocopherol was positively correlated with all the detected

contaminants ($p<0.05$). 4-OH-CB107/4'-OH-CB108 was positively correlated with 25(OH)D3 in the 0-2y group (Table 4).

In liver of the juveniles in the 0-2y group there were no significant correlations between vitamins and contaminants. However, in the >2y group γ-tocopherol was negatively correlated to all measured contaminants, except for CB-44, -47/48, -56/60, -66, -97, -110, -141, PeCB, HCB, c-Chlordane, and BD-E49. When pooling all the juveniles (n=15) γ-tocopherol was negatively correlated to CB-172, BDE-47 and -100 ($p<0.05$).

4 Discussion

4.1 THs

The mean plasma thyroid hormone concentrations in Faroe Island pilot whales determined in this study were somewhat higher but mostly within the same ranges as those reported previously in pilot whale from the same area in 2003-04, except for FT3 which was higher than previously reported (Dam et al., 2010). However, compared to other odontoceti species such as beluga and bottlenose dolphin (*Tursiops truncatus*) plasma TH concentrations in the present pilot whales were mostly lower (Fair et al., 2011; Flower et al., 2015; Villanger et al., 2011b). Numerous variables can influence the TH concentrations, such as season, water temperature, geographical area, and biological variables such as age, sex, stress and pregnancy (Fair et al., 2011; Flower et al., 2015; St. Aubin and Geraci, 1989, 1988). It is therefore difficult to directly compare concentrations between different studies.

As shown in Figure 1 and the 25 -75 percentiles in Table 1, there were large variations in the TH concentrations, also within the age/sex groups and in particular for the juveniles 0-2 years group. While the 25 -75 percentile ranged by a magnitude of approximately 1.5 in adult females the difference was 2.5-3 magnitudes in the juveniles 0-2 years. The variation in juveniles >2 years was similar to that in adult females except for FT3 which had higher variation. FT3 was the TH with highest variation within the age groups.

The TH levels were found to be significantly higher in juveniles than in adults in the present study. Higher levels of THs in juveniles compared to adults or decreasing TH concentrations with age, have also been found in wild and captive bottlenose dolphins (Fair et al., 2011; West et al., 2014) beluga (Flower et al., 2015) and other mammals (Gabrielsen et al., 2011; Hall et al., 1998), as well as humans (Kapelari et al., 2008). This most likely reflects the importance of THs for processes like growth and development (Rolland, 2000) as well as heat production, since young individuals have a smaller size and thus smaller volume relative to surface. In the study of Fair et al. (2011) age was found to be a more important factor influencing circulating TH concentrations in bottlenose dolphins than sex, reproductive status, geographic location and ocean temperature. In the present study, the age and length were important variables in the OPLS models for the THs, in particular for TT4 and FT4, showing negative relationship, demonstrating the important influence of age on the TH levels.

The influence from sex on TH was not analysed in the present study since the males were mostly juveniles and the adults were mostly females. Differences in THs between sexes have been found in cetaceans showing higher TH concentrations in males (Flower et al., 2015), whereas other

studies have not found differences between the sexes or the results have been inconclusive (Fair et al., 2011; West et al., 2014).

4.1.1 Relationship between OHCs and THs

The OPLS models showed positive correlations between THs and plasma OHC concentrations but negative relationship between age and length and THs. The adult group of the pilot whales in the present study consisted mostly of adult females, which generally had four to ten times lower contaminant levels than the juveniles (Table 2S; Hoydal et al. 2015), whereas the TH levels were significantly higher in the juveniles. The positive relationship between contaminants and thyroid hormones found in the present study could thus be explained by the difference between these two age groups and not by an effect of the contaminants on the hormones. The previous analyses of pilot whales (Dam et al., 2010) also showed positive correlations between liver PCB TEQ values (calculated for mono- ortho-PCBs) and plasma TT3 and FT4. These analyses had not been corrected for age differences (Dam et al., 2010). However, when correcting for age in the present study, by treating the age groups separately, positive correlations between THs and several OHCs in plasma were still seen in the juvenile 0-2y group and between HCB and TT3 in both the adult females group and in the juveniles.

These positive associations are in contrast to the negative relationships between THs and OHCs previously reported in other odontocetes (Schwacke et al., 2012; Villanger et al., 2011b) and other mammals (Braathen et al., 2004; Debier et al., 2005; Tabuchi et al., 2006), and the lowered TH levels documented in wildlife mammals experimentally exposed to OHCs as compared to their controls (Brouwer et al., 1989b; Kirkegaard et al., 2011). In bottlenose dolphins from Georgia, US, with higher PCB contamination than the present pilot whales, negative correlations between ∑PCBs and TT4, FT4 and TT3 were found (Schwacke et al., 2012). Negative relationships between several PBDE congeners and CB-105 and TT4, FT4 and TT3 were also reported in a study of beluga whales from Svalbard (Villanger et al., 2011b), where lower OHC concentrations than in the present study were reported.

It should, however, be noted that significant positive correlations between OHCs and THs, as reported herein, also have been found in other marine mammals. In seals positive correlations were found between hydroxylated OHC metabolites and FT3 (Routti et al., 2010a) and between PBDEs and TT4 and TT3 (Hall et al., 2003). The concentrations of PBDEs in pilot whales in the present study were similar to those found in seals (Hall et al., 2003) and the concentrations of OH-metabolites were low, although the levels of PCBs and pesticides were relatively high (Hoydal et al., 2015). Furthermore, in polar bears from East Greenland with lower concentrations of PCBs than in the present pilot whales, positive correlations between ortho-chlorinated PCBs and TT4 were found, although negative correlations between OHCs and THs were also found (Villanger et al., 2011a).

The positive relationship between OHCs and THs could indicate stimulation of TSH secretion from the pituitary and/or disturbance of the feedback (T4 and T3 effect on the TSH release) by OHCs. Villanger et al. (2011b) reported negative correlation between TSH and FT4 in beluga whales from Svalbard reflecting the inhibition of TSH release from the pituitary by FT4.

The negative associations between TT3:FT3 and FT4:FT3 and the OHCs (Figure 5 and Table 3) reflect a relative increase in free T3 with higher OHC concentrations. The relative concentrations of FT3 to TT3 and FT4 were also found to be significantly higher (i.e. the TT3:FT3 and FT4:FT3

ratios were lower) in juveniles featuring higher OHC concentrations than adults. Thus, age related differences in TH concentration and OHC exposure could explain the observed relationships.

Higher concentration of free THs relative to total THs could result from the binding of contaminants or their metabolites to transport proteins. This in consequence may inhibit the binding of THs to proteins and the binding to the retinol:RBP complex which has been suggested as a mechanism responsible for TH disruption (Brouwer et al., 1998). However, this effect is mostly thought to be associated with PCBs and PBDEs OH-metabolites, due to their structural similarity to THs (Brouwer et al., 1998; Liu et al., 2014). In pups of hooded seals the TT3:FT3 ratio was found to be negatively correlated to OH-PCBs, particularly 3'-OH-CB138 (Gabrielsen et al., 2011; Villanger et al., 2013). On the contrary TT4:FT4 ratio was positively correlated to BDE-99 and 4-OH-CB107 in the same study. This mechanism does however seem unlikely for explaining the relatively higher free T3 levels in the present pilot whales since some of the PCBs, found to be negatively correlated to the TT3:FT3 ratio and important variables in the OPLS model for FT3, were recalcitrant compounds such as CB-146, -153, -180, and -187 (Structural group I) and DDE. These compounds are found not to be metabolized to any significant degree in cetaceans (Boon et al., 1997; Tanabe et al., 1988) and OH-metabolites would thus not be produced from these compounds. 4-OH-CB107/4'-OH-CB108 (co-eluting) were the only OH-metabolites detected in the pilot whales and it has been found to be the only or most prevalent OH-metabolite in other cetaceans as well (Houde et al., 2006; Kunisue et al., 2007; McKinney et al., 2006; Montie et al., 2009; Murata et al., 2007). The concentrations in pilot whales (mean: 0.32 ng/g ww = 0.9 pmol/g ww) were comparable or slightly lower than in the hooded seals, although the PCB concentrations were much higher in the pilot whales (Gabrielsen et al., 2011; Hoydal et al., 2015; Villanger et al., 2013). Compared to the TT4 concentrations of 22.2-118.2 pmol/g the binding affinity of the OH-metabolites would have to be 20 – 100 times higher than the T4 binding affinities to the transport proteins in order to cause disruption of the system. Analyses have shown binding affinities of hydroxylated PCBs and T4 to TTR to be of similar magnitude in humans, whereas only few OH-PCBs bind to TBG and then with 100 times lower binding affinity (Cheek et al., 1999).

Overall, the TH levels in the pilot whales in the present study do not seem to be negatively affected by the relative high OHC exposure. THs disruption by OHCs has been described as binding of OH-metabolites to thyroid hormone transport proteins or thyroid hormone receptors due to structural similarity (Brouwer et al., 1998; Liu et al., 2014). Hence, the very low concentrations OH-metabolites found in pilot whales (Hoydal et al., 2015) are probably below threshold concentration that could affect THs. Low concentrations of OH-metabolites in cetaceans results from their generally low ability to metabolise OHCs. Thus the negative effects of OHCs on THs that have been found in other cetacean species (Schwacke et al., 2012; Villanger et al., 2011b) indicate that other mechanisms than binding to transport proteins are involved and that other parameters such as TSH hormone concentrations and deiodinase activities are relevant and need to be considered when analysing the effects of OHCs on THs.

4.2 Vitamins

The total vitamin A levels in liver of pilot whales were somewhat higher than those reported in common dolphin (*Delphinus delphis*) from North-West Spain and harbour porpoises (*Phocoena phocoena*) from Canada (V. Tornero et al., 2005b; Tornero et al., 2004a) and similar to those found in beluga from the Northwest Territories in Canada (Desforges et al., 2013). The retinol

and retinyl palmitate concentrations in liver of the pilot whales were similar, or somewhat higher, than those found in the Canadian beluga and the retinol levels in plasma were similar to levels in beluga (Desforges et al., 2013) and in captive and free-ranging bottlenose dolphins from Sarasota, Florida in 1991-96 (Crissey and Wells, 1999).

In liver, the concentrations of α–tocopherol and γ-tocopherol (vitamin E) in pilot whale were higher than those in Canadian beluga (Desforges et al., 2013), while in plasma α–tocopherol concentrations were similar to those in bottlenose dolphins from Florida (Crissey and Wells, 1999).

The vitamin D concentration in plasma (mean: 79.6 ng/ml, range: 9.1 – 162.0 ng/ml) was higher than previously reported in two pilot whales (40.4 ng/ml) (Keiver et al., 1988), although the previously reported values were within the low range of the present study concentrations. Higher or similar levels of vitamin D3 in comparison to pilot whales in the present study have been reported in beluga (157.7 ng/ml) and bottlenose dolphin (293.1 ng/ml) (Keiver et al., 1988). Due to differences in analysing methods between these older analyses referred to and the present study the concentration comparisons have to be used with caution. To our knowledge no newer analyses of vitamin D3 in cetaceans have been reported.

4.2.1 Relationship between OHCs and vitamin levels

Since both contaminants and vitamins are derived from the diet, this has to be borne in mind when assessing correlative relationships between OHCs and vitamins. The inter-correlation between the vitamins in plasma and most of the vitamins in liver could be a reflection of a similar source from the diet. During gestation, placental transfer of vitamin A and E is limited and thus vitamin levels are low at birth (Debier and Larondelle, 2005). This is however followed by a rapid increase of vitamin A and E with milk ingestions as the concentrations in milk and especially colostrum are high (Debier and Larondelle, 2005; Debier et al., 2002). The apparent higher concentrations of retinol and α-tocopherol in liver of the three smallest individuals compared to the other small individuals (Figure 3) could be a reflection of this.

4.2.1.1 Vitamin A

In liver, BDE-47 was found to be negatively correlated to retinol, and when separating the age groups BDE-28, -49, -47 and 153 were negatively correlated to retinyl palmitate in adult females. CB-196/203 was, on the other hand, positively correlated to retinyl palmitate and CB-74 and -196/203 to retinol in liver in adult females. In plasma; HCB was positively and CB-146 negatively correlated to retinol in adult females.

PCB and vitamin A relationships in marine mammals have been investigated in cetaceans (Desforges et al., 2013; Tornero et al., 2006; V. Tornero et al., 2005a), seals (Debier et al., 2012; Jenssen et al., 2003; Nyman et al., 2003; Routti et al., 2010b, 2005; Vanden Berghe et al., 2010), and polar bears (Bechshøft et al., 2015, 2011; Braathen et al., 2004). These studies have shown both positive and negative relationships depending on tissue, exposure and biological factors. Desforges et al., (2013) found negative correlations between PCBs and vitamin A (retinyl esters) in liver, but positive correlations in plasma. Nyman et al. (2003) also found that PCBs in liver were negatively correlated to retinol and retinyl palmitate in grey seals. Moreover, PCBs in grey seal pups were negatively correlated to retinol or total vitamin A in plasma or serum (Jenssen et al., 2003; Vanden Berghe et al., 2010). However, Nyman et al. (2003) showed positive correlations between PCBs and retinol in grey seal plasma. Negative correlations between PCBs

and vitamin A in liver were not found in the present study although the PCB concentrations in the pilot whales were much higher than the integrated toxicity reference value on 1.6 mg/kg proposed by Desforges et al. (2013), based on 5% effect concentrations for both vitamin A and E.

Relationships between PBDE and vitamin A have been less investigated and to our knowledge, among marine mammals only seals have been studied (Vanden Berghe et al., 2013). Negative relationships between PBDEs and retinol have been found in laboratory rats and mice and in birds (Fernie et al., 2005). Negative correlation between BDE47 and hepatic retinol was found in captive PBDE exposed American kestrels and BDE99 and -100 were negatively correlated to retinol in plasma (Fernie et al., 2005). In the blood of snapping turtles (collected in 2001-2004) from 12 wetland sites on the Canadian side of the Laurentian Great Lakes of North America, some OHC concentrations were correlated with THs and/or vitamin A (Letcher et al., 2015). For example, significant negative (e.g. cis-chlordane) or positive (e.g. BDE-99) correlations were reported with TT4. Dehydroretinol concentrations were positively correlated with PCP, cis-chlordane, trans-nonachlor, CB-28, -44, -49, -52, -74 and -151. In contrast, 4'-OH-BDE49 and 4-OH-CB107/4'-MeO-CB108 concentrations were negatively correlated with dehydroretinol concentrations, and retinol was positively associated with Σ46PCB, Σ28OH-PCB, PCP, HCB, cis-chlordane, trans-nonachlor, 4-OH-CB187 and 6'-OH-BDE49 concentrations. In adult lactating female seals, vitamin A was found to be positively correlated to ∑PCBs and ∑PBDEs in inner blubber and serum (Vanden Berghe et al., 2013). BDE-47 and 4-OH-CB-107 were also positively correlated to vitamin A in serum in the same study.

The decreasing vitamin A levels in pilot whales associated with increasing PBDE levels could indicate a higher need for retinol (at least in adult females) due to exposure to this contaminant. However no changes in the plasma retinol levels were found. Furthermore, retinol in liver was positively correlated to retinol in plasma and retinyl palmitate in liver. This indicates that the vitamin A stores in the liver were not depleted by an increased requirement for retinol.

4.2.1.2 Vitamin D

Positive correlation between 4-OH-CB107/4'-OH-CB108 and CB-172 and 25(OH)D3 in 0-2y juveniles was the only association found between vitamin D and OHCs. Vitamin D_3, absorbed in the intestine, is metabolized in the liver to 25(OH)-vitamin D_3 (25(OH)D3) and then in the kidney to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$). The latter is the most active metabolite of vitamin D, involved in the regulation of serum calcium homeostasis (DeLuca, 2004). Levels of $1,25(OH)_2D$ have been shown to be negatively correlated with PCBs and DDT in Baltic grey seals (Routti et al., 2008). This could be due to either inhibition of the $1,25(OH)_2D$ metabolite formation or its enhanced renal clearance induced by the contaminants (Routti et al., 2008). In the present study the observed relation between 25(OH)D3 and OHCs could be explained by inhibition of the $1,25(OH)_2D$ metabolite formation (thus increasing 25(OH)D3 levels) by 4-OH-CB107/4'-OH-CB108 and CB-172. However, since very low concentrations of OH-CB107/4'-OH-CB108 were observed and correlations were only found in the 0-2y age group consisting of very few individuals it is likely that the correlative relationship found herein cannot be regarded as an effect of OHCs on vitamin D homeostasis.

4.2.1.3 Vitamin E

The most pronounced relationship between vitamins and OHCs was the positive correlation between α–tocopherol and all the analysed contaminants in plasma of juvenile pilot whales. α–Tocopherol has antioxidant properties and since several OHCs, including PCBs, OCPs and PBDEs

have abilities to induce oxidative stress (Abdollahi et al., 2004; Fernie et al., 2005; Valavanidis et al., 2006), the increase in plasma α–tocopherol with increasing OHC concentrations could be a result of organism defence against oxidative stress induction by OHCs. Previously α–tocopherol has been found to be positively correlated to PCB concentrations in plasma and blubber of ringed- and grey seals (Nyman et al., 2003) and PCBs in plasma of Greenland shark (Molde et al., 2013). However, the plasma α–tocopherol concentrations in juveniles were not significantly different from the concentrations in adult females, although the OHC levels in adult females were lower and not correlated with α–tocopherol concentrations. The higher plasma vitamin E levels would require a higher supply of vitamins from food or from vitamin E stores in the body. The α–tocopherol levels in the liver were however not correlated to the OHC concentrations or to the plasma α–tocopherol concentrations, indicating that the vitamin E amounts in the blood were sufficient to cope with this higher requirement.

The negative correlation between γ-tocopherol in liver and BDE-47, -49, -99, -100 and -153 and between γ–tocopherol and most of the contaminants analysed in juveniles >2 years could indicate depletion of γ-tocopherol stores in the liver due to contaminant exposure. In comparison with α–tocopherol, γ-tocopherol is found in much lower concentrations in the body and has distinct chemical reactivity and metabolism and biological activities (Jiang et al., 2001). γ-Tocopherol has higher anti-inflammatory properties compared to α–tocopherol (Jiang et al., 2001) and is much more easily metabolised in the liver and excreted with urine compared to α–tocopherol, which by binding to a transfer protein (α–TTP) is transported to peripheral tissues (Jiang et al. 2001). The catabolism of γ-tocopherol is cytochrome P450 mediated, most likely by CYP3A (Parker et al. 2000). Strong CYP3A protein expression has been reported in pilot whale liver (Celander et al., 2000). The decrease in liver γ-tocopherol with increasing contaminants levels could thus be a result of CYP enzyme induction. Some OHCs induce and are metabolised by CYP3A (Maurel, 1996) and the induction by OHCs could thus lead to increased CYP3A enzyme concentration and a higher metabolism of γ-tocopherol.

5 Conclusion

The present study reports concentrations of THs and vitamins A, E and D in pilot whales from the North-East Atlantic and the associations between OHC concentrations and these biomarkers. The results indicate that although pilot whales are highly exposed to OHCs the thyroid hormone system and the examined vitamins do not seem to be significantly disrupted. The multivariate analyses showed that both hormones and OHCs and to some extent also vitamins were influenced by the length or age of the animals and that the age/sex groups had to be treated separately when analysing for relationships with OHCs. Due to the resulting small number of individuals in each age/sex group the results have to be interpreted with caution. In juveniles there were significant positive relationships between plasma concentrations of α–tocopherol and all the OHCs analysed, indicating a response against oxidative stress. Vitamin A did not seem to be related to OHC concentrations in neither in liver nor plasma, except for negative correlations with PBDEs in adult females, indicating that some PBDEs (and/or other compounds) may affect hepatic function. In conclusions, it appears that the high body burdens of OHCs in Faroese pilot whales may have minor effects on levels of circulating THs and vitamin A, possibly due to the low capacity of pilot whales to form OH-PCBs.

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Figure captures

Figure 1 Plasma concentrations of total and free thyroxin (TT3 and FT4) and triiodothyronine (TT3 and FT3) in male and female pilot whales (*Glopicephala melas*) in relation to the length of the individuals.

Figure 2 Plasma concentrations of vitamin A (retinol), α–tocopherol (alfaTOC) and vitamin D (VitD) in male and female pilot whales (*Glopicephala melas*) in relation to the length of the individuals.

Figure 3 Liver concentrations of vitamin A (retinol, and retinyl palmitate), α–tocopherol (alfaToc) and γ-tocopherol (gammaToc) in male and female pilot whales (*Glopicephala melas*) in relation to the length of the individuals.

Figure 4 Score plot (A) and loading plot (B) of vitamins and the contaminants (Hoydal et al., 2015) analysed in pilot whale (*Glopicephala melas*) liver.

Figure 5 Score plot (A) and loading plot (B) of thyroid hormones and vitamins and the contaminants (Hoydal et al., 2015) analysed in pilot whale (*Glopicephala melas*) plasma.

Figure 6 Regression coefficient plots of the OPLS model showing regression coefficient (CoeffCS) values of each variable indicating the direction and strength of the relationships between individual X-variables and the Y-variable A: TT4, B: FT4, C: TT3 and D: FT3. The dark grey bars present CoeffCS values of variables with VIP values >1, which indicate high importance.