Hepatic Phase I and II Biotransformation Responses and Contaminant Exposure in Long-Finned Pilot Whale from the Northeastern Atlantic

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Abstract

Faroe Island pilot whales have been documented to have high body burdens of organohalogen contaminants (OHCs), including polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), but low burdens of their hydroxylated metabolites (OH-PCBs and OH-PBDEs). The present study investigated the hepatic expression and/or catalytic activity of phase I and II biotransformation enzymes in relation to hepatic concentrations of target OHCs, including OH-PCBs and OH-PBDEs, in long-finned pilot whales (*Globicephala melas*) from NE Atlantic. CYP1A, 2B, 2E and 3A protein expression were identified in juveniles and adult males, but not in adult females. Ethoxyresorufin-O-deethylase (EROD) activity activity was significantly lower in adult females than in juveniles and adult males. Using multivariate analyses to analyse relationships between biological responses and OHC concentrations, a positive relationship was identified between EROD and OHCs. The activity levels of phase II conjugating enzymes (uridine 5'-diphospho-glucuronosyltransferase [UDPGT], and glutathione S-transferase [GST]) were low. The analyses of mRNA expression did not show correlative relationships with OHC concentrations, but cyp1a and ahr transcripts were positively correlated with EROD activity. We suggest that the low concentrations of OH-PCBs and OH-PBDEs reported in pilot whales is probably due, to the identified low phase I biotransformation activities in the species.

Keywords: Marine mammals, OHCs, Liver, Enzyme activity, mRNA expression, CYP
1 Introduction

Marine mammals are continuously exposed to environmental contaminants such as organohalogenated compounds (OHCs) through their diet (Letcher et al., 2010). Long-finned pilot whales (Globicephala melas) from Northeast (NE) Atlantic waters have high tissue concentrations of polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs) and polybrominated diphenyl ether (PBDE) flame retardants (Dam and Bloch, 2000; Hoydal et al., 2015; Rotander et al., 2012). These high tissue contaminants levels raise concern for the health of the animals, as these contaminants are known to interfere with several biological systems with serious effects on the individual as a result (Colborn et al., 1993). In addition to dietary intake, the tissue concentrations of environmental contaminants in these animals are dependent on their ability to metabolize and excrete these contaminants through phase I and II biotransformation systems (Boon et al., 1992; Hakk and Letcher, 2003; Tanabe et al., 1988).

The cytochrome P450 (CYP) monooxygenase and transferase systems (glutathione S-transferase [GST] and uridine 5'-diphospho-glucuronosyltransferase [UDPGT]) are the major enzyme pathways involved in the xenobiotic phase I and II metabolism. In the phase I metabolism, CYP enzymes introduce an oxygen atom to the molecule creating a functional group through which larger endogenous molecules, such as sugars, sulphates, amino acids can be conjugated in the Phase II reaction (Goksøyr, 1995). The CYP enzyme system consists of several isoform superfamilies that exhibit substrate specificity and regulation after exposure to exogenous environmental contaminants (Parke, 1990). The most important CYP families involved in metabolism of xenobiotic compounds are CYP1, 2 and 3. The CYP 2 and 3 families are also involved in the metabolism of endogenous compounds such as steroids and lipids (Li et al., 2003; McKinney et al., 2004; You, 2004). CYP1A forms are induced by contaminants with a planar configuration, such as dioxins, PAHs and non- or mono-ortho chlorine substituted PCB congeners by binding to the aryl hydrocarbon receptor (AhR) (Stegeman and Hahn, 1994). Binding of such planar chemicals to the cytoplasmic AhR triggers the formation of a complex with the AhR nuclear translocator (ARNT), subsequent translocation into the
nucleus and binding to the xenobiotic response elements (XRE) on the DNA to induce the transcription of responsive genes in the AhR gene battery that include the CYP1 family (Stegeman and Hahn, 1994). Xenobiotic compounds that bind to the AhR and exert their toxic effects through this mechanism, have been allocated a toxic equivalent factor (TEF) by which the toxic equivalent (TEQ) value can be calculated (Van den Berg et al., 2006). The toxicity of the compounds is assigned, relative to the toxicity of the most potent AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and the TEQ is sometimes termed the dioxin toxicity value (Birnbaum, 1994; Van den Berg et al., 2006).

Measuring the induction of biotransformation systems such as CYP enzymes and subsequent formation of metabolites, is an established biomarker for exposure and effects of OHCs (Wolkers et al., 2009). The induction of CYP enzymes by OHCs have shown relationships with contaminant burden in many orders of species, including cetaceans (White et al., 1994; Wilson et al., 2010, 2007). The ability and capacity to metabolize OHCs differs between species, and in general, cetaceans are known to have low ability to metabolize OHCs, especially PCB with vicinal H-atoms in the meta- and para positions and with two ortho-chlorines, compared to other marine mammals such as seals and terrestrial mammals (Boon et al., 1997; Tanabe et al., 1988). This has been explained by lower or the absence of CYP2B activity (i.e. phenobarbital-type activity) in cetaceans (Tanabe et al., 1988). Nevertheless, analyses in pilot- and beluga whales (Delphinapterus leucas) have demonstrated that PCBs are metabolized by liver enzymes, shown by the induction of CYP1A and CYP3A families (White et al., 2000). However, it is still unclear whether CYP2B expression occurs or functions in cetaceans (McKinney et al., 2004; White et al., 2000).

The aims of this study were to analyse the expression and/or catalytic activities of hepatic Phase I enzymes (CYP1A, CYP2B and CYP3A) and Phase II conjugating enzymes (Glutathione S-transferase [GST] and Uridine 5'-diphospho-glucuronosyltransferase [UDPGT]) in long-finned pilot whales harvested in Faroe Island waters, and to examine possible associations between these enzymes and hepatic concentrations of OHCs, previously reported in these animals (Hoydal et al., 2015).
2 Materials and Methods

2.1 Chemicals and reagents

Direct-zol™ RNA MiniPrep RNA isolation kit was purchased from Zymo Research Corporation (Irvine, CA, USA). iScript cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Mouse anti-rat CYP2B1/2, mouse anti-human CYP3A4, rabbit anti-human CYP2E1 and rabbit anti-human CYP2B6 antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA), mouse anti-fish CYP1A and rabbit anti-fish CYP1A antibodies were purchased from Biosense Laboratory (Bergen, Norway). Rabbit anti-fish CYP3A was a gift from Dr. Malin Celander. Goat anti-rabbit/mouse horseradish peroxidase-conjugated secondary antibody (GAR/GAM-HRP) was purchased from Bio-Rad (Hercules CA, USA). All other chemicals were of highest commercial grades.

2.2 Sampling and organohalogenated contaminant (OHC) determination

Liver samples of 35 pilot whales were analysed for Phase I and II enzyme mRNA expression, protein expression and/or catalytic activity. The livers were sampled in connection with traditional hunts on the Faroe Islands, 7 samples in 2009, 12 samples in 2010 and 16 samples in 2011. Slices from the distal part of the liver were sampled as soon as possible after the death of the whales, and the samples were wrapped in heat-treated aluminium foil and frozen in liquid nitrogen immediately. The time elapsed from the killing to the sampling (into liquid nitrogen) was between 1 and 3.5 h. The sampled females and males were divided into groups of adults and juveniles according to their length and/or age based on the studies of Martin and Rothery (1993) and Desportes et al. (1993). This resulted in 13 adult and 4 juvenile females and 7 adult and 11 juvenile males. See Hoydal et al. (2015) for further details on the sampling procedures and age group division. Biometric measurements of the sampled whales are given in Table S1.
A total of 25 of the sampled individuals were analysed for 175 individual OHCs (PCBs, PBDEs and OCPs) and metabolites (e.g. OH-PCBs and OH-PBDEs), and 59 of these were detected in the pilot whale liver. All of this data has been fully reported in Hoydal et al. (2015) and a summary of the sum (Σ) concentrations of the contaminant groups (i.e. ΣPCB, ΣOCP and ΣPBDE/BFR) is given in Table S2. The liver samples were analysed for Phase I and II enzymes as described below and the relationship between these variables and the previously reported OHC concentrations were evaluated for those individuals that had been analysed for OHCs. Toxic equivalency (TEQs) of dioxin-like PCBs were calculated from toxic equivalent factors (TEFs) for the analysed non- or mono-ortho PCBs; PCB-105, -118, -156, -157, and -167, according to Van den Berg et al., (2006).

### 2.3 Quantitative (real-time) PCR

Liver samples from all individuals were homogenized in TRI-Reagent and RNA isolated using Direct-zol RNA MiniPrep Kit according to the manufacturer’s protocol (Zymo Research Corporation, Irvine, CA USA). Total cDNA was generated from 1 µg total RNA using a combination of oligo (dT) and random hexamer primers from iScript cDNA synthesis kit, as described by the manufacturer (Bio-Rad, Oslo Norway). Real-time PCR were performed with specific primer pairs for cyp1a1, cyp3a29 and AhR (Table S3), using Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Each 25-µl DNA amplification reaction contained 12.5 µl iTaq SYBR Green supermix with ROX (Bio-Rad), 0.83 µl cDNA and 200 nM of each of forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (30 s), 60 °C (15 s) and 72 °C (15 s). Controls lacking cDNA template were included to determine specificity of target cDNA amplification. Cycle threshold (Ct) values obtained from all target genes were converted into copy number using standard plot of Ct-value versus log copy number as previously described (Arukwe, 2006).

### 2.4 Preparation of microsomes
Liver microsomes were prepared following standard procedures. In brief, approximately 1 g of frozen (-80 °C) liver was thawed on ice and homogenized in 4 ml of 0.1M sodium buffer (containing 0.15M KCl, 1 mM EDTA and 1 mM DDT (dithiothreitol) at pH 7.4). The homogenate was centrifuged at 12,000 x g for 20 min at 4 °C. The resulting supernatant (PMS fraction) was centrifuged at 100,000 x g (35,000 rpm) for 1 h at 4 °C. The supernatant (cytosol fraction) was collected and stored at -80 °C for GST analysis. 1 ml of the homogenization buffer containing 20 % glycerol at pH 7.4 was added to the pellet (microsomal fraction) and homogenized. The resulting microsomes were stored at -80 °C until used.

Total protein concentration was determined with the method of Bradford (1976), using bovine serum albumin (BSA) as standard. All enzymes and protein concentrations were analyzed using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA) for absorbance readings.

2.5 Immunochemical assays

The immunochemical assays for the CYP protein expression was performed using semi-quantitative enzyme-linked immunosorbent assay (ELISA) and Western blotting, according to standard protocols (Nilsen et al., 1998). Selected microsome samples representing the different age and sex groups were analysed with Western blotting and all microsome samples were analysed using ELISA. In Western blotting, proteins (50 µg/well) were separated using 4% stacking and 9% separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) before blotting. In ELISA, samples were diluted to 50 µg in coating buffer (0.05 M sodium-bicarbonate buffer, pH 9.5). The diluted samples were adsorbed to microtiter wells (overnight at 4 °C). In both cases, CYP protein levels were detected using polyclonal rabbit antibodies or monoclonal mouse antibodies (Mouse anti-rat CYP2B1/2 (diluted 1:1000), mouse anti-human CYP3A4 (diluted 1:1000), rabbit anti-human CYP2E1 (diluted 1:500), rabbit anti-human CYP2B6 (diluted 1:1000), mouse anti-fish CYP1A (diluted 1:500), rabbit anti-fish CYP3A (PAb diluted 1:2000) and rabbit anti-fish...
CYP1A (diluted 1:1000). Goat anti-rabbit/mouse horseradish peroxidase-conjugated secondary antibody (GAR/GAM-HRP, Bio-Rad) diluted 1:3000 was used as secondary antibody. H$_2$O$_2$/o-phenylenediamine dihydrochloride (OPD) was used for ELISA detection at 492 nm using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA). Western blot immunoreactive CYP protein band was visualized using SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL, USA).

2.6 Enzyme activities and total protein content analysis
Liver CYP enzyme activities were measured in 29 of the individuals by quantifying ethoxyresorufin-, benzyloxyresorufin-, methoxyresorufin- and pentoxyresorufin-O-deethylase (EROD, BROD, MROD and PROD respectively) activities. Liver microsomes (50 µl) were added to black fluorometric well plate containing 100 µl of NAPDH (0.25 mg/ml) and incubated at 37 °C for 10 minutes. The reaction was then started by adding 1 µl of 250 µM of the substrates ethoxyresorufin (EROD), benzyloxyresorufin (BROD), methoxyresorufin (MROD) or pentoxyresorufin (PROD) dissolved in DMSO and the fluorescence was measured for 20 minutes (excitation: 535 nm; emission: 590 nm) at room temperature. The amount of substrate converted in each reaction was calculated using known dilutions of resorufin that was used in preparing a standard curve (Mortensen and Arukwe, 2007). Since the MROD, BROD and PROD enzyme activities showed higher replicate variation, compared with EROD, only the EROD results are presented here and also used in the statistical analyses. In some of the samples the replicate variation was too high or the concentration was not quantifiable, and of the samples analysed for EROD only 19 were also analysed for OHCs.

UDPGT activity towards $p$-nitrophenol ($p$-NP) metabolism was measured in microsomal samples as described by Andersson et al. (1985) and GST activity in hepatic cytosolic samples were measured using 1-chloro-2,4-dinitrobenzene (CNDB) as substrate as described by Habig et al. (1974). Both UDPGT and GST assays were adapted to 96-well plate reader and measured spectrophotometrically using a microplate reader for absorbance reading. The reaction was run in
duplicates and enzyme activity values were normalized to protein content in the samples. As a quality
control in all enzyme assays, two known samples were assayed in parallel with all assay series, in
order to assure the consistency of the results obtained with unknown samples. All enzyme activities
were assayed at room temperature.

2.7 Statistical analysis

The statistics program SPSS (IBM, version 21) was used for univariate analyses. The results of GST
activity and CYP3A (ELISA) levels were normally distributed, whereas the results of the cyp1a1,
cyp3a29 and ahr mRNA analysis, the UDPGT activity and EROD activity were not normally
distributed. All the non-normally distributed biomarkers were normally distributed when Log-
transformed. Differences among groups (age and sex) were tested with one-way ANOVA (all
pairwise) and Tukey post hoc test. Correlations between the biomarker variables were analysed with
Pearson correlation. Correlation between biomarker variables and biological variables (length and
age) were analysed with linear regression. Since the OHC concentrations were not normally
distributed (Hoydal et al., 2015), non-parametric Spearman correlation analyses were used for
examining relationships among OHCs and the biomarker variables. The level of significance was set
to p < 0.05. Multivariate analyses were performed with the statistical program SIMCA-P+ (Umetrics,
version 12.0, 2008). Relationships between the OHCs and biomarker variables and the biological
parameters (length, age) were examined using Principal Component Analysis (PCA). Since the
number of individuals sampled each year differed, and the sex and age distribution was not consistent
between the years, the year of sampling was not included as a parameter in the analysis. Furthermore,
the time span between 2009 and 2011 was considered small to be a major source of variation of the
contaminant exposure, compared to the within year variation. The association between the
contaminants and biological variables to each of the biomarkers was further analysed with Orthogonal
Projections to Latent Structures (OPLS) analysis and correlation analyses. PCA and PLS are able to
deal with data consisting of a lower number of observations (individuals analysed) than variables,
and with strongly correlated data and since the data are analysed simultaneously, it reduces the risk of type I and II errors (Eriksson et al., 2013). The PCA identified one outlier (indv. US26), and this individual was removed from all the results and statistical analyses.

3 Results

3.1 Transcript (mRNA) expression

Detectable and quantifiable levels of the targeted and expressed mRNA transcripts were observed. With regard to abundance, cyp1a1 showed the highest mRNA levels, while lower levels were identified for cyp3a29 and ahr mRNA (Figure 1). For each of the mRNA transcripts, there were large variations within the age groups. There were no significant differences between the age/sex groups for the cyp mRNAs and ahr mRNA levels analysed (p>0.05), except that ahr was significantly higher in juvenile females than in juvenile males (p = 0.022). Cyp1al and cyp3a29 were significantly correlated to length ($r^2 = 0.179$, $p = 0.013$ and $r^2 = 0.202$, $p = 0.008$, for cyp1al and cyp3a29, respectively), whereas ahr was not ($r^2 = 0.094$, $p = 0.077$). It should be noted that the $r^2$ values were low and thus a larger sample size is needed for achieving a clear conclusion on these relationships.

3.2 Protein expression

The analyses of protein expression using Western blotting showed cross-reactions with antibodies for CYP2E1, CYP2B6, polyclonal CYP3A and CYP1A (Figure 2). No protein cross-reactions were observed with CYP2B1/2, and monoclonal CYP1A and CYP3A4 antibodies (data not shown). Western blotting showed positive protein detections in young female individuals, whereas the adult females did not show protein response (Figure 2). For the males, both juveniles and adults showed protein responses (Figure 2). To compare the individual CYP3A protein expression, a semi-quantitative ELISA analysis was used. No significant differences between sex and age groups were
observed in this analysis (Figure 3A) and the protein levels were not correlated to length or age of the animals (p > 0.05).

3.3 Enzyme activity

3.3.1 Phase I enzymes

The only quantifiable Phase I enzyme activity was EROD, although the EROD activity level was low (Figure 3B). EROD activity differed significantly between the age/sex groups, with the adult females having significantly lower activity than the other groups (p < 0.05). Regardless of sex/age group, logEROD activity was negatively correlated with age ($r^2 = 0.83$, $p < 0.001$, $n = 13$), but not with length ($r^2 = 0.06$, $p = 0.194$, $n = 28$) of the whales. The discrepancy between these two biological parameters is probably due to that age was not analysed in all individuals. In general, all CYP enzyme activity analyses showed high variability between the analysed samples.

3.3.2 Phase II enzymes

The UDPGT activity was between 0.273 and 0.753 nmol/min/mg protein. Although correlation analyses showed that logUDPGT activity was positively correlated with length and age ($r^2 = 0.149$, $p = 0.024$; $r^2 = 0.224$, $p = 0.047$, respectively), there were no significant differences between different age or sex groups (Figure 3C). The GST activity was between 0.011 and 0.024 pmol/min/mg protein. The GST activity did not differ between age/sex groups and was not correlated to animal length (Figure 3D).

3.4 Associations between OHC burden and biomarker responses

The score and loading plots from the PCA are shown in Figure 4, which included all quantifiable OHCs and biomarker and biometric variables, age and length. The first two principal components (PC1 and PC2) explained 76.6 % and 6.7 %, respectively, of the total variation in the dataset. The score plot (Figure 4A) showed that adult females were grouped separately from the other age groups.
(adult males, juveniles 0-2 years old and juveniles > 2 years old), along the PC1 axis. The loading plot (Figure 4B) showed that the clustering of adult females along PC1 was because they were older and larger (only two adult males were included in the analyses, since these were the only adult males analysed for contaminants) and had lower hepatic contaminant concentrations as compared to the other groups, which is in accordance with our previous findings (Hoydal et al., 2015). The sex distribution was not equal in the group of juveniles, with three females and eleven males. However, the juvenile females were distributed within the group of juvenile males, suggesting that sex is of less importance for the variations in both hepatic OHC concentrations (see also Hoydal et al., 2015) and for biomarker responses in the juvenile individuals (Figure 4A).

With respect to associations between the OHCs and the biomarkers, the PCA loading plot indicates positive relationships between the hepatic concentrations of OHCs and the EROD activity, and negative relationships between the OHCs and some of the other biomarkers, particularly UDPGT (Figure 4B). The loading plot also indicated that age and length are confounding factors for biomarker responses.

The associations between individual biomarkers, OHCs and biological parameters were therefore further analysed using OPLS. The only significant model was observed for EROD ($R^2_X = 0.87$, $R^2_Y = 0.71$, $Q^2 = 0.61$, CV-ANOVA $p = 0.01$, Figure 5). In the model, age and length were the most important biological predictors for the EROD activity in the sampled animals. Increasing age and length had negative effects on the EROD activity. The most important contaminants that predicted the EROD activity were BDE-49, PCB-110, PCB-97, BDE-153, PCB-44, BDE-28, BDE-154, cis-chlordane and hexachlorobenzene. In the model, all these contaminants contributed significantly in increasing the EROD activity (Figure 5). The positive relationship between EROD and contaminants was confirmed by Spearman correlation analyses, which showed a significant positive correlation between EROD and all analysed OHCs ($r_s = 0.566 - 0.804$, $p < 0.01$). Of the OHCs analysed, TEFs have been defined for PCB-105, -118, -156, -157 and -167. The calculated TEQ values correlated significantly with EROD activity ($r_s = 0.723$, $p = 0.001$, Figure S1).
Although no significant OPLS models were identified for the combined effects of the OHCs and biological variables on any of the other biomarkers, correlation matrices identified some significant relationships between the biomarkers and some of the OHCs (Table S4). UDPGT was negatively correlated with PCB-44, -56/60, -130, -176, BDE-28 and -153. Further, GST activity was negatively correlated to PCB56/60, -141 and cis-chlordane. Ahr mRNA was positively correlated with PCB-44 and -97. As indicated in the PCA, there were significant correlations among some biomarkers (Table S5). Although an inverse relationship was indicated between EROD and cyp1a1 mRNA in the PCA, these two variables correlated positively ($r_s = 0.432$, $p = 0.022$). Cyp1a1 was also positively correlated with ahr. However, there was no correlation between EROD and ahr. Cyp3a29 was positively correlated with ahr ($r_s = 0.418$, $p = 0.014$). There were no correlations between CYP3A protein content, GST and UDPGT activities and any of the other biomarkers.

4 Discussion

Induction of CYP mRNA expression and activities, particularly CYP1, 2 and 3 families, are among the most studied biological responses to OHC exposure. The ability of the studied pilot whales to metabolize xenobiotics is tightly linked to their expression of phase I and II biotransformation responses. Thus, the presence of phase I (CYP) biotransformation responses were analysed at different biological levels, i.e. the transcript (mRNA), protein and enzyme activity levels. Overall, we showed that pilot whales expressed hepatic CYP isoforms in the 1, 2 and 3 families. For CYP1, there were positive correlations between EROD and OHCs. Furthermore, Ahr was positively correlated with PCB-44 and -97, which also were important predictors for the EROD activity in the OPLS model. Phase II enzyme activities (GST and UDPGT) were expressed in the pilot whales, and both these enzymes were negatively associated with hepatic concentrations of some OHCs, although the UDPGT activity increased with increasing age of the animal. While these responses were evident in the pilot whales, the results apparently differed between the analytical methods, showing some
within enzyme discrepancy depending on the level of expression (mRNA, protein and enzyme activity) and analytical method used. The induction of CYP enzymes involve ligand binding to the AhR and subsequent formation of the AhR-ARNT complex and binding to consensus xenobiotic response element (XRE) promoter region in DNA, resulting in increased mRNA transcription and translation, followed by post-translational modification to yield a mature protein or enzyme. The transcription of a gene does not always lead to functional proteins since protein expression is influenced by factors, such as translational regulation and protein degradation and the protein function can be altered by post translational modifications or proteolytic cleavage (Pradet-Balade et al., 2001). Regardless of the above mentioned factors, it is obvious that pilot whales expressed Phase I and II biotransformation enzymes, and that the pattern of these to some extent showed relationships to contaminant burden with potential biomarker values.

4.1 Biotransformation capacity

4.1.1 Influence of age and sex

Generally, the CYP enzyme expression differed between age/sex groups at the protein and enzyme activity levels, but not at the mRNA level. The qualitative protein expression (Western blotting) showed a clear difference between sex/age groups with no visible expression in adult females, but expression in most of the juvenile and adult male individuals. Similarly, EROD activity showed lowest activity in the adult females, which was significantly lower than the activity observed in the other sex/age groups. This difference between the adult females and the other sex/age groups was not observed for the mRNA expression. Negative relationships with EROD were observed for age and length variables, which can be explained by that most of the adults in the model were females (only two were males) which had lower OHC and EROD concentrations, and not as a general lower EROD expression with age. The higher expression of CYPs in young pilot whales using Western blot and EROD analyses is in contrast to what is generally reported in mammals, which is that young individuals have a lower ability of CYP enzyme induction (Weijs et al., 2010; Wolkers et al., 2002).
In humans, the hepatic phase I reactions are low in neonates, but develop rapidly to the same level as in adults during the first 6 months (Milsap and Jusko, 1994). In the present study, the age of the juvenile whales was between < 1 and 13 years of age.

CYP induction can be influenced by estrogen concentrations due to cross-talk between the AhR and estrogen receptor (ER). When compounds bind to the AhR, the complex can either impair or activate the expression of estrogen responsive genes (Ohtake et al., 2011). The reproductive state of the individual can also affect the expression of AhR activated genes. For example, in fish several studies have demonstrated the impairment of CYP1A induction during periods of gonadal development (Khan and Payne, 2002; Lindström-Seppä and Stegeman, 1995). The lower CYP expression in adult female pilot whales could possibly be attributed to potentially high cellular estrogen concentrations (Beischlag and Perdew, 2005). However, analyses of steroid levels in the same whale individuals as analyzed in the present study did not show higher plasma estrogen concentrations in adult females compared to the other age groups (Hoydal et al., 2017). Therefore, it is possible that the regulation of CYP enzymes expression pattern in pilot whale are controlled by complex biological processes that are yet to be well-understood, and different from what is known in other marine mammals. Nevertheless, as CYP1 expression and activity is expected to be positively related to OHC concentrations, and the adult females had the lowest OHC concentrations, it is likely that the low OHC concentrations contributed to low CYP expression in the females.

4.1.2 CYP1A

The CYP1A enzyme can be induced by planar compounds such as dioxin, PCBs and PAHs, by binding to the AhR. In the present study, CYP1A expression was analysed at mRNA, protein and EROD activity levels. The EROD assay is known to be a selective marker for CYP1A1 enzyme activity (Burke et al., 1994). The mRNA expression of cyp1a1 and the ahr were positively correlated, and the cyp1a1 mRNA expression was also positively correlated with EROD activity, indicating induction of the CYP1A gene in the liver of pilot whale. The pilot whales were potentially exposed
to a variety of different OHCs, including PCBs of which some have planar configuration. The EROD activity was significantly lower in adult females compared to the other age/sex groups. In addition, the proteins cross-reacting with CYP1A antibodies were detected at higher levels in juvenile and adult males, showed lower expression in juvenile females and were not detected in adult females. It is possible that the low CYP1A expression in the females is due to the much lower OHC burdens in these individuals. The OHC concentrations were four to ten times lower in adult females than in the juveniles (Hoydal et al., 2015) most likely as a result of maternal transfer of these compounds to their offspring.

Of the analysed biomarkers, only the EROD activity correlated significantly with the OHC exposure. Although, there were significant correlations between EROD and all the detected OHCs, the most important variables in the OPLS model were BDE-49, PCB-110, PCB-97, BDE-153, PCB-44, BDE-28, BDE-154, cis-chlordane and hexachlorobenzene, which all showed positive relationships with EROD. The relationship between the important OHC variables and EROD could indicate a possible induction of CYP1A by these contaminants. The substrates for the CYP1A enzymes are compounds that have a planar configuration (Boon et al., 1992; Stegeman and Hahn, 1994) such as metabolic group III PCBs which are congeners with less than two ortho-chlorine atoms and vicinal H atoms in the ortho-meta position (Boon et al., 1997, 1992; Stegeman and Hahn, 1994). However, although Group III PCBs (PCB-60, -157, -156, -66, -118, -74) were found to be positively correlated to EROD in the OPLS model, and some of these Group III PCBs were variables of high importance (VIP values > 1), they were not important predictors of EROD in the model (Figure 5).

The most important PCBs for predicting the EROD activity were PCB-110, -97 and -44 which are congeners with two ortho-chlorine atoms and vicinal H atoms in the meta-para position. These PCBs belong to the Group IV PCBs, which are thought to be CYP2B inducers (Boon et al., 1997). In ringed seals, the concentrations of group III and IV PCBs have been shown to be relatively lower, while group I, II and V PCBs were relatively higher in seals from a highly contaminated area, compared to
those from a less contaminated area (Routti et al., 2008). These findings were explained by higher biotransformation of group III and IV PCBs in the more contaminated area (Routti et al., 2008).

It should, however, be noted that when assigned TEF values to calculate the total TEQ of the PCBs, there was a significant positive correlation between TEQ and the EROD activity in the present study. Of the PCBs analysed in the present study which have assigned TEF values, all are group III PCBs (PCB-105, -118, -156, and -157) except one (PCB-167, group I). This indicates that group III PCBs clearly play a role in inducing CYP1A1 activity, even though group IV PCBs in general appeared to be more important predictors of the EROD activity. Given that the CYP expression (mRNA, protein and activity) levels were low in the pilot whales, it is possible that other endogenous responses triggered by group IV PCBs may have affected the EROD activity. Thus, it is possible that the high importance of the group IV PCBs on the EROD activity may reflect indirect effects on EROD by these PCBs through other endogenous processes.

CYP enzyme catalysed biotransformation may lead to the formation of persistent hydroxylated (OH)-metabolites (Letcher et al., 2000). The only OH-PCB detected in the plasma of the studied pilot whales was 4-OH-CB107/4'-OH-CB108 (co-eluting) (Hoydal et al., 2015). The possible precursors of 4-OH-CB107 are the group III PCBs PCB-107, PCB-105 and PCB-118 (Nomiyama et al., 2010), which were not important variables in the OPLS model. Nevertheless, when assigned TEF-values PCB-105 and PCB-118, contributed to the significant positive relationship between TEQ and EROD activity. The EROD activity was generally very low in the pilot whales, as was the concentration of OH-PCBs (Hoydal et al., 2015). This indicates that low CYP induction response in pilot whales most likely is the cause of low concentrations of OH-PCBs in pilot whales. Previous analyses in pilot whales have shown cross-reaction with antibodies raised against scup CYP1A1, while EROD activities were in the range 67 ± 76 and 93 ± 182 pmol/min/mg in male and female pilot whales, respectively (White et al., 2000). Another study in short-finned pilot whales (Globicephala macrorhynchus) reported EROD activities at 42 pmol/min/mg protein (Watanabe et al., 1989) without significant sex difference. Analyses in beluga whale have, on the other hand, shown
low CYP1A expression in females compared to in males (White et al., 1994), which is in accordance with the present study on pilot whales.

Pilot whales from the Faroe Islands have previously been analysed for EROD activity (Dam et al., 2010). The measured activity in those analyses ranged from less than detectable limit to 95.5 pmol/min/mg protein (Dam et al., 2010). In the present study the highest EROD activity was 18.4 pmol/min/mg protein. As in the present study, Dam et al. (2010) reported a positive significant correlation between EROD activity and dioxin toxicity (TEQs), particularly for mono-ortho PCBs.

4.1.3 CYP2B and CYP2E

In the present study, both CYP2B6 and CYP2E1 were expressed in pilot whales. CYP2B is known to be induced by non-planar compounds (Boon et al., 1992), such as PCBs with > 2 ortho-chlorine atoms (group IV or V), while CYP2E is induced by a variety of low molecular weight compounds and involved in cell-toxicity and activation of pro-carcinogens through the production of reactive oxygen species (ROS) (Cederbaum, 2015). Previous studies in cetaceans have proposed low activity level for CYP2B or other CYP2 enzymes both in relation to PCB residues, proteins and enzyme activities (Boon et al., 1997, 1992; Norstrom et al., 1992; Tanabe et al., 1988; White et al., 1994). In pilot whales from Cape Cod, Massachusetts a 1990-91 study showed cross-reaction with anti-rabbit CYP2B4 antibodies, but not with anti-scup P450B or anti-rat CYP2B1 (White et al., 2000). Herein we showed that pilot whales are able to express CYP2B proteins, although the PROD and BROD activities were either very low or below detection limits. In rodents, PROD and BROD were shown as selective measures of CYP2B activity (Burke et al., 1994; Pustylnyak et al., 2007) and they have been used for studies in marine mammals (Boon et al., 1992; Routti et al., 2008). However, several species respond differently to these assays. For example, BROD and PROD have not been useful markers of CYP2B induction in hamster (Mesocricetus auratus) and cynomologus monkey (Macaca fascicularis) (Weaver et al., 1994). The use of PROD as a biomarker of CYP2B induction in marine wildlife has been called into question as PROD has been found to be highly correlated to CYP1A.
protein content (Letcher et al., 1996) and EROD activity, suggesting that CYP1A is a single and common catalyst of these substrates (Ruus et al., 2002). In seals, it has been proposed that CYP2B enzymes may be structurally different and have different substrate specificities than in rodent and humans, and may not be a good biomarker for contaminants that induce CYP2B (Nyman et al., 2001). Nevertheless, the expression in pilot whales did show the presence of both CYP2B and CYP2E in juveniles and adult males and no expression in adult females, similar to the other CYPs that were analysed in the present study.

4.1.4 CYP3A

The CYP3A is involved in metabolism of endogenous compounds such as steroids, but also xenobiotics such as PCBs (Celander et al., 2000; Maurel, 1996). CYP3A was analysed at mRNA (cyp3a29) and protein levels, both qualitatively using Western blot and semi-quantitatively using ELISA technique. CYP3A genes are strongly inducible in response to xenobiotics in mammalian species (Maurel, 1996). Induction is initiated by binding to the pregnane X receptor (PXR). PXR can, however, bind a number of different ligands, including steroid hormones such as estrogen and progesterone (Kliewer et al., 2002). In the present study, the qualitative protein expression analyses were reflective of the OHC exposure with responses in the juveniles and adult males, which have high OHC concentrations. No response was measured in adult females, which had very low OHC concentrations. However, no correlation between OHC concentrations and cyp3a29 mRNA or CYP3A protein determined semi-quantitatively, was identified. The semi-quantitative protein results did not correlate with mRNA expression, and qualitative and semi-quantitative protein analyses were not consistent. For example, in the qualitative protein analyses, the CYP3A proteins were not expressed in adult females, but in juvenile females, adult and juvenile males, whereas in the semi-quantitative analysis and mRNA analysis, there were no differences between the age groups. Although the Western blot analyses did not show cross-reactive protein bands for CYP3A in adult females, in the ELISA analysis CYP3A was comparatively detected in adult females and the other
age/gender groups. This discrepancy may be attributed to a possible cross-reaction with other non-target proteins in the samples. CYP3A has previously been measured in pilot whale tissues using immunoblotting and immunohistochemical analyses, showing cross-reactions with antibodies raised against rat CYP3A1 and trout CYP3A27 (Celander et al., 2000). The immunoblotting analyses in that study showed one reacting protein band in each tissue, but the proteins expressed in liver and lung tissues were of different molecular weight, compared with the proteins in other tissues such as kidney and heart (Celander et al., 2000). Since CYP3A is also involved in metabolism of steroids and show strong gender differences, the detection of this protein could definitely be influenced by fluctuations in cellular steroid concentrations and thus, may not be a good biomarker for contaminant exposure.

4.1.5 Phase II enzyme induction

Phase II enzymes catalyse the conjugation of lipophilic compounds with endogenous molecules such as glucuronic acid or glutathione. In the present study, the conjugating enzymes GST and UDPGT were analysed showing that GST levels were low compared to previous observations in seals (Routti et al., 2008; Wolkers et al., 1998). UDPGT was within the same levels as previously reported in Canadian beluga (McKinney et al., 2004), but somewhat lower than reported in ringed seals from Svalbard (Wolkers et al., 1998). The UDPGT catalyses the conjugation of glucuronic acid to compounds such as OH-metabolites of PCBs or PBDEs (Safe, 1994). Very low concentrations of OH-PCBs and OH-PBDEs were reported in the present pilot whales (Hoydal et al., 2015). Although this could be due to high turnover rates of these OH-metabolites by phase II conjugating enzymes, such as UDPGT, the relatively low UDPGT activity in the pilot whales suggests that this is not the case. Thus, we suggest that the low concentrations of OH-PCBs and OH-PBDEs in pilot whales is due to the identified low Phase I biotransformation activity in these pilot whales. Low Phase I biotransformation activities have also been previously suggested for cetaceans (Boon et al., 1997; Tanabe et al., 1988).
5 Conclusion

The present study showed that pilot whales are able to express CYP1A, 2B, 2E and 3A. Although pilot whales are highly exposed to OHCs, they do not show high enzyme catalytic activities. The positive relationship between CYP1A (EROD) activity and OHCs indicate that these compounds cause induction of CYP1A enzymes. The analyses of mRNA expression provided important information on the responses to xenobiotic compounds in pilot whales, although no correlative relationship with the compound concentrations were identified. We suggest that the low concentrations of OH-PCBs and OH-PBDEs in pilot whales are due to the identified low Phase I biotransformation activities in pilot whales. Further analyses of enzyme expressions by analysing more individuals of each age/sex group and with different levels of contaminant exposure are needed for further understanding of the biotransformation capacity of pilot whales.

Supplemental data

Tables S1-S5 Biometric data and sampling information, summary of OHC concentrations in plasma, primer sequences and tables of significant Spearman rank correlations.

Figure S1 correlation between EROD and PCB TEQs.

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Figure 1 Concentrations of the initial quantity of mRNA for enzymes and receptors measured by qPCR in liver of pilot whale (*Globicephala melas*) versus the length of the animals divided into different age/sex groups.

Figure 2 Western blot responses for pilot whale liver proteins separated by SDS PAGE and treated with CYP antibodies. For the females no. 1-4 are juveniles and no. 5-9 are adults. For the males no. 1-5 are juveniles and no. 6-9 are adults.

Figure 3 CYP3A protein content analysed by ELISA, EROD activity, GST activity, and UDPGT activity versus length in pilot whale liver for different age and sex groups.

Figure 4 Scores plot (A) and loading plot (B) of PCA analysis of OHC concentrations and biomarkers for Cyp induction and phase II induction in livers of pilot whales from the Faroe Islands.

Figure 5 Regression coefficient plot of the OPLS model showing regression coefficient (CoeffCS) values of each variable indicating direction and strength of the relationship between individual X variables and the Y variable EROD. The dark grey bars present CoeffCS values of variables with VIP values > 1, which indicate high importance.