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

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28 **Abstract**

29 Faroe Island pilot whales have been documented to have high body burdens of organohalogen
30 contaminants (OHCs), including polychlorinated biphenyls (PCBs) and polybrominated diphenyl
31 ethers (PBDEs), but low burdens of their hydroxylated metabolites (OH-PCBs and OH-PBDEs). The
32 present study investigated the hepatic expression and/or catalytic activity of phase I and II
33 biotransformation enzymes in relation to hepatic concentrations of target OHCs, including OH-PCBs
34 and OH-PBDEs, in long-finned pilot whales (*Globicephala melas*) from NE Atlantic. CYP1A, 2B,
35 2E and 3A protein expression were identified in juveniles and adult males, but not in adult females.
36 Ethoxyresorufin-O-deethylase (EROD) activity activity was significantly lower in adult females than
37 in juveniles and adult males. Using multivariate analyses to analyse relationships between biological
38 responses and OHC concentrations, a positive relationship was identified between EROD and OHCs.
39 The activity levels of phase II conjugating enzymes (uridine 5'-diphospho-glucuronosyltransferase
40 [UDPGT], and glutathione S-transferase [GST]) were low. The analyses of mRNA expression did
41 not show correlative relationships with OHC concentrations, but *cyp1a* and *ahr* transcripts were
42 positively correlated with EROD activity. We suggest that the low concentrations of OH-PCBs and
43 OH-PBDEs reported in pilot whales is probably due, to the identified low phase I biotransformation
44 activities in the species.

45

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

47

48 ## 1 Introduction

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

60 The cytochrome P450 (CYP) monooxygenase and transferase systems (glutathione S-
61 transferase [GST] and uridine 5'-diphospho-glucuronosyltransferase [UDPGT]) are the major enzyme
62 pathways involved in the xenobiotic phase I and II metabolism. In the phase I metabolism, CYP
63 enzymes introduce an oxygen atom to the molecule creating a functional group through which larger
64 endogenous molecules, such as sugars, sulphates, amino acids can be conjugated in the Phase II
65 reaction (Goksøyr, 1995). The CYP enzyme system consists of several isoform superfamilies that
66 exhibit substrate specificity and regulation after exposure to exogenous environmental contaminants
67 (Parke, 1990). The most important CYP families involved in metabolism of xenobiotic compounds
68 are CYP1, 2 and 3. The CYP 2 and 3 families are also involved in the metabolism of endogenous
69 compounds such as steroids and lipids (Li et al., 2003; McKinney et al., 2004; You, 2004). CYP1A
70 forms are induced by contaminants with a planar configuration, such as dioxins, PAHs and non- or
71 mono-*ortho* chlorine substituted PCB congeners by binding to the aryl hydrocarbon receptor (AhR)
72 (Stegeman and Hahn, 1994). Binding of such planar chemicals to the cytoplasmic AhR triggers the
73 formation of a complex with the AhR nuclear translocator (ARNT), subsequent translocation into the

74 nucleus and binding to the xenobiotic response elements (XRE) on the DNA to induce the
75 transcription of responsive genes in the AhR gene battery that include the CYP1 family (Stegeman
76 and Hahn, 1994). Xenobiotic compounds that bind to the AhR and exert their toxic effects through
77 this mechanism, have been allocated a toxic equivalent factor (TEF) by which the toxic equivalent
78 (TEQ) value can be calculated (Van den Berg et al., 2006). The toxicity of the compounds is assigned,
79 relative to the toxicity of the most potent AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)
80 and the TEQ is sometimes termed the dioxin toxicity value (Birnbaum, 1994; Van den Berg et al.,
81 2006).

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

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

100

101 ## 2 Materials and Methods

102 ### 2.1 Chemicals and reagents

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

112

113 ### 2.2 Sampling and organohalogenated contaminant (OHC) determination

114 Liver samples of 35 pilot whales were analysed for Phase I and II enzyme mRNA expression, protein
115 expression and/or catalytic activity. The livers were sampled in connection with traditional hunts on
116 the Faroe Islands, 7 samples in 2009, 12 samples in 2010 and 16 samples in 2011. Slices from the
117 distal part of the liver were sampled as soon as possible after the death of the whales, and the samples
118 were wrapped in heat-treated aluminium foil and frozen in liquid nitrogen immediately. The time
119 elapsed from the killing to the sampling (into liquid nitrogen) was between 1 and 3.5 h. The sampled
120 females and males were divided into groups of adults and juveniles according to their length and/or
121 age based on the studies of Martin and Rothery (1993) and Desportes et al. (1993). This resulted in
122 13 adult and 4 juvenile females and 7 adult and 11 juvenile males. See Hoydal et al. (2015) for further
123 details on the sampling procedures and age group division. Biometric measurements of the sampled
124 whales are given in Table S1.

125 A total of 25 of the sampled individuals were analysed for 175 individual OHCs (PCBs, 126 PBDEs and OCPs) and metabolites (e.g. OH-PCBs and OH-PBDEs), and 59 of these were detected 127 in the pilot whale liver. All of this data has been fully reported in Hoydal et al. (2015) and a summary 128 of the sum (Σ) concentrations of the contaminant groups (i.e. ΣPCB, ΣOCP and ΣPBDE/BFR) is 129 given in Table S2. The liver samples were analysed for Phase I and II enzymes as described below 130 and the relationship between these variables and the previously reported OHC concentrations were 131 evaluated for those individuals that had been analysed for OHCs. Toxic equivalency (TEQs) of 132 dioxin-like PCBs were calculated from toxic equivalent factors (TEFs) for the analysed non- or mono-133 ortho PCBs; PCB-105, -118, -156, -157, and -167, according to Van den Berg et al., (2006).

134

135 **2.3 Quantitative (real-time) PCR**

136 Liver samples from all individuals were homogenized in TRI-Reagent and RNA isolated using 137 Direct-zol RNA MiniPrep Kit according to the manufacturer's protocol (Zymo Research Corporation, 138 Irvine, CA USA). Total cDNA was generated from 1 μg total RNA using a combination of oligo (dT) 139 and random hexamer primers from iScript cDNA synthesis kit, as described by the manufacturer 140 (Bio-Rad, Oslo Norway). Real-time PCR were performed with specific primer pairs for cyp1a1, 141 cyp3a29 and AhR (Table S3), using Mx3000P real-time PCR system (Stratagene, La Jolla, CA). Each 142 25-μl DNA amplification reaction contained 12.5 μl iTaq SYBR Green supermix with ROX (Bio-143 Rad), 0.83 μl cDNA and 200 nM of each of forward and reverse primers. The three-step real-time 144 PCR program included an enzyme activation step at 95 °C (3 min) and 40 cycles of 95 °C (30 s), 60 145 °C (15 s) and 72 °C (15 s). Controls lacking cDNA template were included to determine specificity 146 of target cDNA amplification. Cycle threshold (Ct) values obtained from all target genes were 147 converted into copy number using standard plot of Ct-value versus log copy number as previously 148 described (Arukwe, 2006).

149

150 **2.4 Preparation of microsomes**

151 Liver microsomes were prepared following standard procedures. In brief, approximately 1 g of frozen
152 (-80 °C) liver was thawed on ice and homogenized in 4 ml of 0.1M sodium buffer (containing 0.15M
153 KCl, 1 mM EDTA and 1 mM DDT (dithiothreitol) at pH 7.4). The homogenate was centrifuged at
154 12,000 x *g* for 20 min at 4 ºC. The resulting supernatant (PMS fraction) was centrifuged at 100,000
155 x *g* (35,000 rpm) for 1 h at 4 ºC. The supernatant (cytosol fraction) was collected and stored at -80
156 ºC for GST analysis. 1 ml of the homogenization buffer containing 20 % glycerol at pH 7.4 was added
157 to the pellet (microsomal fraction) and homogenized. The resulting microsomes were stored at -80
158 ºC until used.

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

163

164 **2.5 Immunochemical assays**

165 The immunochemical assays for the CYP protein expression was performed using semi-quantitative
166 enzyme-linked immunosorbent assay (ELISA) and Western blotting, according to standard protocols
167 (Nilsen et al., 1998). Selected microsome samples representing the different age and sex groups were
168 analysed with Western blotting and all microsome samples were analysed using ELISA. In Western
169 blotting, proteins (50 µg/well) were separated using 4% stacking and 9% separating sodium dodecyl
170 sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) before
171 blotting. In ELISA, samples were diluted to 50 µg in coating buffer (0.05 M sodium-bicarbonate
172 buffer, pH 9.5). The diluted samples were adsorbed to microtiter wells (overnight at 4 °C). In both
173 cases, CYP protein levels were detected using polyclonal rabbit antibodies or monoclonal mouse
174 antibodies (Mouse anti-rat CYP2B1/2 (diluted 1:1000), mouse anti-human CYP3A4 (diluted 1:1000),
175 rabbit anti-human CYP2E1 (diluted 1:500), rabbit anti-human CYP2B6 (diluted 1:1000), mouse anti-
176 fish CYP1A (diluted 1:500), rabbit anti-fish CYP3A (PAb diluted 1:2000) and rabbit anti-fish

177 CYP1A (diluted 1:1000)). Goat anti-rabbit/mouse horseradish peroxidase-conjugated secondary
178 antibody (GAR/GAM-HRP, Bio-Rad) diluted 1:3000 was used as secondary antibody. H_2O_2/o-
179 phenylenediamine dihydrochloride (OPD) was used for ELISA detection at 492 nm using a Synergy
180 HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA). Western blot
181 immunoreactive CYP protein band was visualized using SuperSignal West Pico Chemiluminescent
182 kit (Pierce Biotechnology, Rockford, IL, USA).

183

184 **2.6 Enzyme activities and total protein content analysis**

185 Liver CYP enzyme activities were measured in 29 of the individuals by quantifying ethoxyresorufin-
186 , benzyloxyresorufin-, methoxyresorufin- and pentoxyresorufin-O-deethylase (EROD, BROD,
187 MROD and PROD respectively) activities. Liver microsomes (50 µl) were added to black
188 fluorometric well plate containing 100 µl of NAPDH (0.25 mg/ml) and incubated at 37 °C for 10
189 minutes. The reaction was then started by adding 1 µl of 250 µM of the substrates ethoxyresorufin
190 (EROD), benzyloxyresorufin (BROD), methoxyresorufin (MROD) or pentoxyresorufin (PROD)
191 dissolved in DMSO and the fluorescence was measured for 20 minutes (excitation: 535 nm; emission:
192 590 nm) at room temperature. The amount of substrate converted in each reaction was calculated
193 using known dilutions of resorufin that was used in preparing a standard curve (Mortensen and
194 Arukwe, 2007). Since the MROD, BROD and PROD enzyme activities showed higher replicate
195 variation, compared with EROD, only the EROD results are presented here and also used in the
196 statistical analyses. In some of the samples the replicate variation was too high or the concentration
197 was not quantifiable, and of the samples analysed for EROD only 19 were also analysed for OHCs.

198 UDPGT activity towards *p*-nitrophenol (*p*-NP) metabolism was measured in microsomal
199 samples as described by Andersson et al. (1985) and GST activity in hepatic cytosolic samples were
200 measured using 1-chloro-2,4-dinitrobenzene (CNDB) as substrate as described by Habig et al. (1974).
201 Both UDPGT and GST assays were adapted to 96-well plate reader and measured
202 spectrophotometrically using a microplate reader for absorbance reading. The reaction was run in

203 duplicates and enzyme activity values were normalized to protein content in the samples. As a quality
204 control in all enzyme assays, two known samples were assayed in parallel with all assay series, in
205 order to assure the consistency of the results obtained with unknown samples. All enzyme activities
206 were assayed at room temperature.
207

208 **2.7 Statistical analysis**

209 The statistics program SPSS (IBM, version 21) was used for univariate analyses. The results of GST
210 activity and CYP3A (ELISA) levels were normally distributed, whereas the results of the *cyp1a1*,
211 *cyp3a29* and *ahr* mRNA analysis, the UDPGT activity and EROD activity were not normally
212 distributed. All the non-normally distributed biomarkers were normally distributed when Log-
213 transformed. Differences among groups (age and sex) were tested with one-way ANOVA (all
214 pairwise) and Tukey *post hoc* test. Correlations between the biomarker variables were analysed with
215 Pearson correlation. Correlation between biomarker variables and biological variables (length and
216 age) were analysed with linear regression. Since the OHC concentrations were not normally
217 distributed (Hoydal et al., 2015), non-parametric Spearman correlation analyses were used for
218 examining relationships among OHCs and the biomarker variables. The level of significance was set
219 to $p < 0.05$. Multivariate analyses were performed with the statistical program SIMCA-P+ (Umetrics,
220 version 12.0, 2008). Relationships between the OHCs and biomarker variables and the biological
221 parameters (length, age) were examined using Principal Component Analysis (PCA). Since the
222 number of individuals sampled each year differed, and the sex and age distribution was not consistent
223 between the years, the year of sampling was not included as a parameter in the analysis. Furthermore,
224 the time span between 2009 and 2011 was considered small to be a major source of variation of the
225 contaminant exposure, compared to the within year variation. The association between the
226 contaminants and biological variables to each of the biomarkers was further analysed with Orthogonal
227 Projections to Latent Structures (OPLS) analysis and correlation analyses. PCA and PLS are able to
228 deal with data consisting of a lower number of observations (individuals analysed) than variables,

229 and with strongly correlated data and since the data are analysed simultaneously, it reduces the risk
230 of type I and II errors (Eriksson et al., 2013). The PCA identified one outlier (indv. US26), and this
231 individual was removed from all the results and statistical analyses.
232
233 **3 Results**
234
235 **3.1 Transcript (mRNA) expression**
236 Detectable and quantifiable levels of the targeted and expressed mRNA transcripts were observed.
237 With regard to abundance, *cyp1a1* showed the highest mRNA levels, while lower levels were
238 identified for *cyp3a29* and *ahr* mRNA (Figure 1). For each of the mRNA transcripts, there were large
239 variations within the age groups. There were no significant differences between the age/sex groups
240 for the *cyp* mRNAs and *ahr* mRNA levels analysed ($p>0.05$), except that *ahr* was significantly higher
241 in juvenile females than in juvenile males ($p = 0.022$). *Cyp1a1* and *cyp3a29* were significantly
242 correlated to length ($r^2 = 0.179$, $p = 0.013$ and $r^2 = 0.202$, $p = 0.008$, for *cyp1a1* and *cyp3a29*,
243 respectively), whereas *ahr* was not ($r^2 = 0.094$, $p = 0.077$). It should be noted that the r^2 values were
244 low and thus a larger sample size is needed for achieving a clear conclusion on these relationships.
245
246 **3.2 Protein expression**
247 The analyses of protein expression using Western blotting showed cross-reactions with antibodies for
248 CYP2E1, CYP2B6, polyclonal CYP3A and CYP1A (Figure 2). No protein cross-reactions were
249 observed with CYP2B1/2, and monoclonal CYP1A and CYP3A4 antibodies (data not shown).
250 Western blotting showed positive protein detections in young female individuals, whereas the adult
251 females did not show protein response (Figure 2). For the males, both juveniles and adults showed
252 protein responses (Figure 2). To compare the individual CYP3A protein expression, a semi-
253 quantitative ELISA analysis was used. No significant differences between sex and age groups were

254 observed in this analysis (Figure 3A) and the protein levels were not correlated to length or age of the
255 animals ($p > 0.05$).
256
257 ## 3.3 Enzyme activity
258 ### 3.3.1 Phase I enzymes
259 The only quantifiable Phase I enzyme activity was EROD, although the EROD activity level was low
260 (Figure 3B). EROD activity differed significantly between the age/sex groups, with the adult females
261 having significantly lower activity than the other groups ($p < 0.05$). Regardless of sex/age group,
262 logEROD activity was negatively correlated with age ($r^2 = 0.83$, $p < 0.001$, $n = 13$), but not with
263 length ($r^2 = 0.06$, $p = 0.194$, $n = 28$) of the whales. The discrepancy between these two biological
264 parameters is probably due to that age was not analysed in all individuals. In general, all CYP enzyme
265 activity analyses showed high variability between the analysed samples.
266
267 ### 3.3.2 Phase II enzymes
268 The UDPGT activity was between 0.273 and 0.753 nmol/min/mg protein. Although correlation
269 analyses showed that logUDPGT activity was positively correlated with length and age ($r^2 = 0.149$, p
270 $= 0.024$; $r^2 = 0.224$, $p = 0.047$, respectively), there were no significant differences between different
271 age or sex groups (Figure 3C). The GST activity was between 0.011 and 0.024 pmol/min/mg protein.
272 The GST activity did not differ between age/sex groups and was not correlated to animal length
273 (Figure 3D).
274
275 ## 3.4 Associations between OHC burden and biomarker responses
276 The score and loading plots from the PCA are shown in Figure 4, which included all quantifiable
277 OHCs and biomarker and biometric variables, age and length. The first two principal components
278 (PC1 and PC2) explained 76.6 % and 6.7 %, respectively, of the total variation in the dataset. The
279 score plot (Figure 4A) showed that adult females were grouped separately from the other age groups

280 (adult males, juveniles 0-2 years old and juveniles > 2 years old), along the PC1 axis. The loading
281 plot (Figure 4B) showed that the clustering of adult females along PC1 was because they were older
282 and larger (only two adult males were included in the analyses, since these were the only adult males
283 analysed for contaminants) and had lower hepatic contaminant concentrations as compared to the
284 other groups, which is in accordance with our previous findings (Hoydal et al., 2015). The sex
285 distribution was not equal in the group of juveniles, with three females and eleven males. However,
286 the juvenile females were distributed within the group of juvenile males, suggesting that sex is of less
287 importance for the variations in both hepatic OHC concentrations (see also Hoydal et al., 2015) and
288 for biomarker responses in the juvenile individuals (Figure 4A).
289 With respect to associations between the OHCs and the biomarkers, the PCA loading plot indicates
290 positive relationships between the hepatic concentrations of OHCs and the EROD activity, and
291 negative relationships between the OHCs and some of the other biomarkers, particularly UDPGT
292 (Figure 4B). The loading plot also indicated that age and length are confounding factors for biomarker
293 responses.
294 The associations between individual biomarkers, OHCs and biological parameters were
295 therefore further analysed using OPLS. The only significant model was observed for EROD (R^2X =
296 0.87, R^2Y = 0.71, Q^2 = 0.61, CV-ANOVA p = 0.01, Figure 5). In the model, age and length were the
297 most important biological predictors for the EROD activity in the sampled animals. Increasing age
298 and length had negative effects on the EROD activity. The most important contaminants that
299 predicted the EROD activity were BDE-49, PCB-110, PCB-97, BDE-153, PCB-44, BDE-28, BDE-
300 154, *cis*-chlordane and hexacholorobenzene. In the model, all these contaminants contributed
301 significantly in increasing the EROD activity (Figure 5). The positive relationship between EROD
302 and contaminants was confirmed by Spearman correlation analyses, which showed a significant
303 positive correlation between EROD and all analysed OHCs (r_s = 0.566 – 0.804, $p < 0.01$). Of the
304 OHCs analysed, TEFs have been defined for PCB-105, -118, -156, -157 and -167. The calculated
305 TEQ values correlated significantly with EROD activity (r_s = 0.723, p = 0.001, Figure S1).

306 Although no significant OPLS models were identified for the combined effects of the OHCs
307 and biological variables on any of the other biomarkers, correlation matrices identified some
308 significant relationships between the biomarkers and some of the OHCs (Table S4). UDPGT was
309 negatively correlated with PCB-44, -56/60, -130, -176, BDE-28 and -153. Further, GST activity was
310 negatively correlated to PCB56/60, -141 and cis-chlordane. *Ahr* mRNA was positively correlated
311 with PCB-44 and -97. As indicated in the PCA, there were significant correlations among some
312 biomarkers (Table S5). Although an inverse relationship was indicated between EROD and *cyp1a1*
313 mRNA in the PCA, these two variables correlated positively ($r_s = 0.432$, $p = 0.022$). *Cyp1a1* was also
314 positively correlated with *ahr*. However, there was no correlation between EROD and *ahr*. *Cyp3a29*
315 was positively correlated with *ahr* ($r_s = 0.418$, $p = 0.014$). There were no correlations between CYP3A
316 protein content, GST and UDPGT activities and any of the other biomarkers.
317
318
319 **4 Discussion**

320 Induction of CYP mRNA expression and activities, particularly CYP1, 2 and 3 families, are among
321 the most studied biological responses to OHC exposure. The ability of the studied pilot whales to
322 metabolize xenobiotics is tightly linked to their expression of phase I and II biotransformation
323 responses. Thus, the presence of phase I (CYP) biotransformation responses were analysed at
324 different biological levels, i.e. the transcript (mRNA), protein and enzyme activity levels. Overall,
325 we showed that pilot whales expressed hepatic CYP isoforms in the 1, 2 and 3 families. For CYP1,
326 there were positive correlations between EROD and OHCs. Furthermore, *Ahr* was positively
327 correlated with PCB-44 and -97, which also were important predictors for the EROD activity in the
328 OPLS model. Phase II enzyme activities (GST and UDPGT) were expressed in the pilot whales, and
329 both these enzymes were negatively associated with hepatic concentrations of some OHCs, although
330 the UDPGT activity increased with increasing age of the animal. While these responses were evident
331 in the pilot whales, the results apparently differed between the analytical methods, showing some

332 within enzyme discrepancy depending on the level of expression (mRNA, protein and enzyme
333 activity) and analytical method used. The induction of CYP enzymes involve ligand binding to the
334 AhR and subsequent formation of the AhR-ARNT complex and binding to consensus xenobiotic
335 response element (XRE) promoter region in DNA, resulting in increased mRNA transcription and
336 translation, followed by post-translational modification to yield a mature protein or enzyme. The
337 transcription of a gene does not always lead to functional proteins since protein expression is
338 influenced by factors, such as translational regulation and protein degradation and the protein function
339 can be altered by post translational modifications or proteolytic cleavage (Pradet-Balade et al., 2001).
340 Regardless of the above mentioned factors, it is obvious that pilot whales expressed Phase I and II
341 biotransformation enzymes, and that the pattern of these to some extent showed relationships to
342 contaminant burden with potential biomarker values.
343

344 **4.1 Biotransformation capacity**

345 **4.1.1 Influence of age and sex**

346 Generally, the CYP enzyme expression differed between age/sex groups at the protein and enzyme
347 activity levels, but not at the mRNA level. The qualitative protein expression (Western blotting)
348 showed a clear difference between sex/age groups with no visible expression in adult females, but
349 expression in most of the juvenile and adult male individuals. Similarly, EROD activity showed
350 lowest activity in the adult females, which was significantly lower than the activity observed in the
351 other sex/age groups. This difference between the adult females and the other sex/age groups was not
352 observed for the mRNA expression. Negative relationships with EROD were observed for age and
353 length variables, which can be explained by that most of the adults in the model were females (only
354 two were males) which had lower OHC and EROD concentrations, and not as a general lower EROD
355 expression with age. The higher expression of CYPs in young pilot whales using Western blot and
356 EROD analyses is in contrast to what is generally reported in mammals, which is that young
357 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

384 to a variety of different OHCs, including PCBs of which some have planar configuration. The EROD
385 activity was significantly lower in adult females compared to the other age/sex groups. In addition,
386 the proteins cross-reacting with CYP1A antibodies were detected at higher levels in juvenile and adult
387 males, showed lower expression in juvenile females and were not detected in adult females. It is
388 possible that the low CYP1A expression in the females is due to the much lower OHC burdens in
389 these individuals. The OHC concentrations were four to ten times lower in adult females than in the
390 juveniles (Hoydal et al., 2015) most likely as a result of maternal transfer of these compounds to their
391 offspring.
392 Of the analysed biomarkers, only the EROD activity correlated significantly with the OHC
393 exposure. Although, there were significant correlations between EROD and all the detected OHCs,
394 the most important variables in the OPLS model were BDE-49, PCB-110, PCB-97, BDE-153, PCB-
395 44, BDE-28, BDE-154, cis-chlordane and hexachlorobenzene, which all showed positive
396 relationships with EROD.The relationship between the important OHC variables and EROD could
397 indicate a possible induction of CYP1A by these contaminants. The substrates for the CYP1A
398 enzymes are compounds that have a planar configuration (Boon et al., 1992; Stegeman and Hahn,
399 1994) such as metabolic group III PCBs which are congeners with less than two *ortho*-chlorine atoms
400 and vicinal H atoms in the *ortho-meta* position (Boon et al., 1997, 1992; Stegeman and Hahn, 1994).
401 However, although Group III PCBs (PCB-60, -157, -156, -66, -118, -74) were found to be positively
402 correlated to EROD in the OPLS model, and some of these Group III PCBs were variables of high
403 importance (VIP values > 1), they were not important predictors of EROD in the model (Figure 5).
404 The most important PCBs for predicting the EROD activity were PCB-110, -97 and -44 which are
405 congeners with two *ortho*-chlorine atoms and vicinal H atoms in the *meta-para* position. These PCBs
406 belong to the Group IV PCBs, which are thought to be CYP2B inducers (Boon et al., 1997). In ringed
407 seals, the concentrations of group III and IV PCBs have been shown to be relatively lower, while
408 group I, II and V PCBs were relatively higher in seals from a highly contaminated area, compared to

409 those from a less contaminated area (Routti et al., 2008). These findings were explained by higher
410 biotransformation of group III and IV PCBs in the more contaminated area (Routti et al., 2008).

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

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

435 low CYP1A expression in females compared to in males (White et al., 1994), which is in accordance
436 with the present study on pilot whales.

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

442

443 **4.1.3 CYP2B and CYP2E**

444 In the present study, both CYP2B6 and CYP2E1 were expressed in pilot whales. CYP2B is known
445 to be induced by non-planar compounds (Boon et al., 1992), such as PCBs with > 2 *ortho*-chlorine
446 atoms (group IV or V), while CYP2E is induced by a variety of low molecular weight compounds
447 and involved in cell-toxicity and activation of pro-carcinogens through the production of reactive
448 oxygen species (ROS) (Cederbaum, 2015). Previous studies in cetaceans have proposed low activity
449 level for CYP2B or other CYP2 enzymes both in relation to PCB residues, proteins and enzyme
450 activities (Boon et al., 1997, 1992; Norstrom et al., 1992; Tanabe et al., 1988; White et al., 1994). In
451 pilot whales from Cape Cod, Massachusetts a 1990-91 study showed cross-reaction with anti-rabbit
452 CYP2B4 antibodies, but not with anti-scup P450B or anti-rat CYP2B1 (White et al., 2000). Herein
453 we showed that pilot whales are able to express CYP2B proteins, although the PROD and BROD
454 activities were either very low or below detection limits. In rodents, PROD and BROD were shown
455 as selective measures of CYP2B activity (Burke et al., 1994; Pustylnyak et al., 2007) and they have
456 been used for studies in marine mammals (Boon et al., 1992; Routti et al., 2008). However, several
457 species respond differently to these assays. For example, BROD and PROD have not been useful
458 markers of CYP2B induction in hamster (*Mesocricetus auratus*) and cynomologus monkey (*Macaca*
459 *fascicularis*) (Weaver et al., 1994). The use of PROD as a biomarker of CYP2B induction in marine
460 wildlife has been called into question as PROD has been found to be highly correlated to CYP1A

461 protein content (Letcher et al., 1996) and EROD activity, suggesting that CYP1A is a single and
462 common catalyst of these substrates (Ruus et al., 2002). In seals, it has been proposed that CYP2B
463 enzymes may be structurally different and have different substrate specificities than in rodent and
464 humans, and may not be a good biomarker for contaminants that induce CYP2B (Nyman et al., 2001).
465 Nevertheless, the expression in pilot whales did show the presence of both CYP2B and CYP2E in
466 juveniles and adult males and no expression in adult females, similar to the other CYPs that were
467 analysed in the present study.
468

469 **4.1.4 CYP3A**

470 The CYP3A is involved in metabolism of endogenous compounds such as steroids, but also
471 xenobiotics such as PCBs (Celander et al., 2000; Maurel, 1996). CYP3A was analysed at mRNA
472 (*cyp3a29*) and protein levels, both qualitatively using Western blot and semi-quantitatively using
473 ELISA technique. CYP3A genes are strongly inducible in response to xenobiotics in mammalian
474 species (Maurel, 1996). Induction is initiated by binding to the pregnane X receptor (PXR). PXR can,
475 however, bind a number of different ligands, including steroid hormones such as estrogen and
476 progesterone (Kliewer et al., 2002). In the present study, the qualitative protein expression analyses
477 were reflective of the OHC exposure with responses in the juveniles and adult males, which have
478 high OHC concentrations. No response was measured in adult females, which had very low OHC
479 concentrations. However, no correlation between OHC concentrations and *cyp3a29* mRNA or
480 CYP3A protein determined semi-quantitatively, was identified. The semi-quantitative protein results
481 did not correlate with mRNA expression, and qualitative and semi-quantitative protein analyses were
482 not consistent. For example, in the qualitative protein analyses, the CYP3A proteins were not
483 expressed in adult females, but in juvenile females, adult and juvenile males, whereas in the semi-
484 quantitative analysis and mRNA analysis, there were no differences between the age groups.
485 Although the Western blot analyses did not show cross-reactive protein bands for CYP3A in adult
486 females, in the ELISA analysis CYP3A was comparatively detected in adult females and the other

487 age/gender groups. This discrepancy may be attributed to a possible cross-reaction with other non-
488 target proteins in the samples. CYP3A has previously been measured in pilot whale tissues using
489 immunoblotting and immunohistochemical analyses, showing cross-reactions with antibodies raised
490 against rat CYP3A1 and trout CYP3A27 (Celander et al., 2000). The immunoblotting analyses in that
491 study showed one reacting protein band in each tissue, but the proteins expressed in liver and lung
492 tissues were of different molecular weight, compared with the proteins in other tissues such as kidney
493 and heart (Celander et al., 2000). Since CYP3A is also involved in metabolism of steroids and show
494 strong gender differences, the detection of this protein could definitely be influenced by fluctuations
495 in cellular steroid concentrations and thus, may not be a good biomarker for contaminant exposure.
496

497 **4.1.5 Phase II enzyme induction**

498 Phase II enzymes catalyse the conjugation of lipophilic compounds with endogenous molecules such
499 as glucuronic acid or glutathione. In the present study, the conjugating enzymes GST and UDPGT
500 were analysed showing that GST levels were low compared to previous observations in seals (Routti
501 et al., 2008; Wolkers et al., 1998). UDPGT was within the same levels as previously reported in
502 Canadian beluga (McKinney et al., 2004), but somewhat lower than reported in ringed seals from
503 Svalbard (Wolkers et al., 1998). The UDPGT catalyses the conjugation of glucuronic acid to
504 compounds such as OH-metabolites of PCBs or PBDEs (Safe, 1994). Very low concentrations of
505 OH-PCBs and OH-PBDEs were reported in the present pilot whales (Hoydal et al., 2015). Although
506 this could be due to high turnover rates of these OH-metabolites by phase II conjugating enzymes,
507 such as UDPGT, the relatively low UDPGT activity in the pilot whales suggests that this is not the
508 case. Thus, we suggest that the low concentrations of OH-PCBs and OH-PBDEs in pilot whales is
509 due to the identified low Phase I biotransformation activity in these pilot whales. Low Phase I
510 biotransformation activies have also been previously suggested for cetaceans (Boon et al., 1997;
511 Tanabe et al., 1988).
512

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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539

540 **References**

541 Andersson, T., Pesonen, M., Johansson, C., 1985. Differential induction of cytochrome P-450-
542 dependent monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyl
543 transferase activities in the liver of the rainbow trout by β-naphthoflavone or clophen A50.
544 Biochem. Pharmacol. 34, 3309–3314. doi:10.1016/0006-2952(85)90351-X

545 Arukwe, A., 2006. Toxicological housekeeping genes: Do they really keep the house? Environ. Sci.
546 Technol. 40, 7944–7949. doi:10.1021/es0615223

547 Beischlag, T. V., Perdew, G.H., 2005. ERα-AHR-ARNT protein-protein interactions mediate
548 estradiol-dependent transrepression of dioxin-inducible gene transcription. J. Biol. Chem. 280,
549 21607–21611. doi:10.1074/jbc.C500090200

550 Birnbaum, L.S., 1994. The mechanism of dioxin toxicity: Relationship to risk assessment. Environ.
551 Health Perspect. 102, 157–167.

552 Boon, J.P., Meer, J. Van Der, Allchin, C.R., Law, R.J., Klungsøyr, J., Leonards, P.E.G., Spliid, H.,
553 Storr-Hansen, E., Mckenzie, C., Wells, D.E., 1997. Concentration-Dependent Changes of PCB
554 Patterns in Fish-Eating Mammals: Structural Evidence for Induction of Cytochrome P450.
555 Arch. Environ. Contam. Toxicol. 33, 298–311. doi:10.1007/s002449900257

556 Boon, J.P., van Arnheim, E., Jansen, S., 1992. The toxicokinetics of PCBs in marine mammals with
557 special reference to possible interactions of individual congeners with the cytochrome P450-
558 dependent monooxygenase system: An overview, in: Walker, C.H., Livingstone, D.R. (Eds.),
559 Persistent Pollutants in Marine Ecosystems. Pergamon Press, p. 272.

560 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of
561 protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–54.

562 Burke, M.D., Thompson, S., Weaver, R.J., Wolf, C.R., Mayer, R.T., 1994. Cytochrome P450
563 specificities of alkoxyresorufin o-dealkylation in human and rat liver. Biochem. Pharmacol.
564 48, 923–936.

565 Cederbaum, A.I., 2015. Molecular mechanisms of the microsomal mixed function oxidases and
566 biological and pathological implications. Redox Biol. 4, 60–73.
567 doi:10.1016/j.redox.2014.11.008
568 Celander, M., Moore, M., Stegeman, J., 2000. Cellular localization of CYP3A proteins in various
569 tissues from pilot whale (Globicephala melas). Environ. Toxicol. Pharmacol. 8, 245–253.
570 Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting
571 chemicals in wildlife and humans. Environ. Health Perspect. 101, 378–84.
572 Dam, M., Bloch, D., 2000. Screening of mercury and persistent organochlorine pollutants in long-
573 finned pilot whale (Globicephala melas) in the Faroe Islands. Mar. Pollut. Bull. 40, 1090–
574 1099. doi:10.1016/S0025-326X(00)00060-6
575 Dam, M., Jenssen, B.M., Boon, J.P., van Bavel, B., Christiansen, D., Hoydal, K., 2010. Pilot study
576 on effects of POPs on long-finned pilot whales off the Faroe Islands. Poster SETAC, Sevilla,
577 Spain.
578 Desportes, G., Saboureau, M., Lacroix, A., 1993. Reproductive Maturity and Seasonality of Male
579 Long-Finned Pilot Whales, off the Faroe Islands, in: Donovan, G.P., Lockyer, C.H., Martin,
580 A.R. (Eds.), Report of the International Whaling Commission Special Issue; Biology of
581 Northern Hemisphere Pilot Whales. Cambridge, pp. 233–262.
582 Eriksson, L., Byrne, T., Johansson, E., Trygg, J., Vikström, C., 2013. Multi- and Megavariate Data
583 Analysis - Basic principles and applications, Third revi. ed. Umetrics Academy.
584 Goksøyr, A., 1995. Cytochrome P450 in marine mammals: isozyme forms, catalytic functions, and
585 physiological regulations, in: Blix, A.S., Walløe, L., Ulltang, Ø. (Eds.), Whales, Seals, Fish
586 and Man: Proceedings of the International Symposium on the Biology of Marine Mammals in
587 the North East Atlantic. Elsevier, Tromsø, p. 720.
588 Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Tansferases - The first enzymatic step
589 in mercapturic acid formation. J. Biol. Chem. 249, 7130–7140.
590 Hakk, H., Letcher, R.J., 2003. Metabolism in the toxicokinetics and fate of brominated flame

591 retardants--a review. Environ. Int. 29, 801–28. doi:10.1016/S0160-4120(03)00109-0

592 Hoydal, K.S., Letcher, R.J., Blair, D.A.D., Dam, M., Lockyer, C., Jenssen, B.M., 2015. Legacy and
593 emerging organic pollutants in liver and plasma of long-finned pilot whales (Globicephala
594 melas) from waters surrounding the Faroe Islands. Sci. Total Environ. 520, 270–285.
595 doi:10.1016/j.scitotenv.2015.03.056

596 Hoydal, K.S., Styrishave, B., Ciesielski, T.M., Letcher, R.J., Dam, M., Jenssen, B.M., 2017. Steroid
597 Hormones and Persistent Organic Pollutants in plasma from North-eastern Atlantic Pilot
598 whales. Environ. Res. 159, 613–621. doi:10.1016/j.envres.2017.09.003

599 Khan, R.A., Payne, J.F., 2002. Some factors influencing EROD activity in winter flounder
600 (Pleuronectes americanus) exposed to effluent from a pulp and paper mill. Chemosphere 46,
601 235–239. doi:10.1016/S0045-6535(01)00125-4

602 Kliewer, S.A., Goodwin, B., Willson, T.M., 2002. The nuclear pregnane X receptor: A key
603 regulator of xenobiotic metabolism. Endocr. Rev. 23, 687–702. doi:10.1210/er.2001-0038

604 Laemmli, U.K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of
605 Bacteriophage T4. Nature 227, 680–685. doi:10.1038/227680a0

606 Letcher, R.J., Bustnes, J.O., Dietz, R., Jenssen, B.M., Jørgensen, E.H., Sonne, C., Verreault, J.,
607 Vijayan, M.M., Gabrielsen, G.W., 2010. Exposure and effects assessment of persistent
608 organohalogen contaminants in arctic wildlife and fish. Sci. Total Environ. 408, 2995–3043.
609 doi:10.1016/j.scitotenv.2009.10.038

610 Letcher, R.J., Klasson-Wehler, E., Bergman, A., 2000. Methyl Sulfone and Hydroxylated
611 Metabolites of Polychlorinated Biphenyls, in: Paasivirta, J. (Ed.), The Handbook of
612 Environmental Chemistry Vol. 3 Part K. Springer-Verlag Berlin Heidelberg, pp. 315–359.
613 doi:10.1007/3-540-48915-0_11

614 Letcher, R.J., Norstrom, R.J., Lin, S., Ramsay, M. a, Bandiera, S.M., 1996. Immunoquantitation
615 and microsomal monooxygenase activities of hepatic cytochromes P4501A and P4502B and
616 chlorinated hydrocarbon contaminant levels in polar bear (Ursus maritimus). Toxicol. Appl.

617 Pharmacol. 137, 127–140. doi:10.1006/taap.1996.0065
618 Li, H., Boon, J.P., Lewis, W.E., van den Berg, M., Nyman, M., Letcher, R.J., 2003. Hepatic
619 microsomal cytochrome P450 enzyme activity in relation to in vitro metabolism/inhibition of
620 polychlorinated biphenyls and testosterone in Baltic grey seal (Halichoerus grypus). Environ.
621 Toxicol. Chem. 22, 636–44. doi:10.1002/etc.5620220324
622 Lindström-Seppä, P., Stegeman, J.J., 1995. Sex differences in cytochrome P4501A induction by
623 environmental exposure and β-naphthoflavone in liver and extrahepatic organs of recrudescent
624 winter flounder. Mar. Environ. Res. 39, 219–223. doi:10.1016/0141-1136(94)00054-S
625 Martin, A.R., Rothery, P., 1993. Reproductive Parameters of Female Long-Finned Pilot Whales
626 (Globicephala melas) Around the Faroe Islands, in: Donovan, G.P., Lockyer, C.H., Martin,
627 A.R. (Eds.), Report of the International Whaling Commission Special Issue; Biology of
628 Northern Hemisphere Pilot Whales. Cambridge, pp. 263–304.
629 Maurel, P., 1996. The CYP3 family, in: Ioannides, C. (Ed.), Cytochromes P450: Metabolic and
630 Toxicological Aspects. CRC Press, pp. 241–270.
631 McKinney, M.A., Arukwe, A., De Guise, S., Martineau, D., Béland, P., Dallaire, A., Lair, S.,
632 Lebeuf, M., Letcher, R.J., 2004. Characterization and profiling of hepatic cytochromes P450
633 and phase II xenobiotic-metabolizing enzymes in beluga whales (Delphinapterus leucas) from
634 the St. Lawrence River Estuary and the Canadian Arctic. Aquat. Toxicol. 69, 35–49.
635 doi:10.1016/j.aquatox.2004.04.010
636 Milsap, R.L., Jusko, W.J., 1994. Pharmacokinetics in the infant. Environ. Health Perspect. 102,
637 107–110. doi:10.1289/ehp.94102s11107
638 Moore, M.J., Stegeman, J.J., 1996. Hepatocyte vacuolation and autolytic changes in the liver of
639 pilot whales, Globicephala melas, stranded on Cape Cod, MA, USA. Sci. Total Environ. 186,
640 105–8.
641 Mortensen, A.S., Arukwe, A., 2007. Modulation of xenobiotic biotransformation system and
642 hormonal responses in Atlantic salmon (Salmo salar) after exposure to tributyltin (TBT).

643 Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 145, 431–41.
644 doi:10.1016/j.cbpc.2007.01.013
645 Nilsen, B.M., Berg, K., Goksøyr, A., 1998. Induction of Cytochrome P450 1A (CYP1A) in Fish - A
646 Biomarker for Environmental Pollution. Methods Mol. Biol. 107, 423–438.
647 Nomiyama, K., Murata, S., Kunisue, T., Yamada, T.K., Mizukawa, H., Takahashi, S., Tanabe, S.,
648 2010. Polychlorinated biphenyls and their hydroxylated metabolites (OH-PCBs) in the blood
649 of toothed and baleen whales stranded along Japanese coastal waters. Environ. Sci. Technol.
650 44, 3732–8. doi:10.1021/es1003928
651 Norstrom, R.J., Muir, D.C.G., Ford, C.A., Simon, M., Macdonald, C.R., Béland, P., 1992.
652 Indications of P450 monooxygenase activities in beluga (Delphinapterus leucas) and narwhal
653 (Monodon monoceros) from patterns of PCB, PCDD and PCDF accumulation. Mar. Environ.
654 Res. 34, 267–272. doi:10.1016/0141-1136(92)90119-7
655 Nyman, M., Raunio, H., Taavitsainen, P., Pelkonen, O., 2001. Characterization of xenobiotic-
656 metabolizing cytochrome P450 (CYP) forms in ringed and grey seals from the Baltic Sea and
657 reference sites. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 128, 99–112.
658 Ohtake, F., Fujii-Kuriyama, Y., Kawajiri, K., Kato, S., 2011. Cross-talk of dioxin and estrogen
659 receptor signals through the ubiquitin system. J. Steroid Biochem. Mol. Biol. 127, 102–107.
660 doi:10.1016/j.jsbmb.2011.03.007
661 Parke, D.V., 1990. Induction of cytochromes P-450 - General principles and biological
662 consequences, in: Ruckpaul, K., Rein, H. (Eds.), Frontiers in Biotransformation, Vol. 2:
663 Principles, Mechanisms and Biological Consequences of Induction. Taylor and Francis,
664 London, pp. 1–34.
665 Pradet-Balade, B., Boulmé, F., Beug, H., Müllner, E.W., Garcia-Sanz, J.A., 2001. Translation
666 control: Bridging the gap between genomics and proteomics? Trends Biochem. Sci. 26, 225–
667 229. doi:10.1016/S0968-0004(00)01776-X
668 Pustylnyak, V.O., Lebedev, A.N., Gulyaeva, L.F., Lyakhovich, V. V, Slynko, N.M., 2007.

669 Comparative study of CYP2B induction in the liver of rats and mice by different compounds.
670 Life Sci. 80, 324–8. doi:10.1016/j.lfs.2006.09.015
671 Rotander, A., van Bavel, B., Polder, A., Rigét, F., Auðunsson, G.A., Gabrielsen, G.W., Víkingsson,
672 G., Bloch, D., Dam, M., 2012. Polybrominated diphenyl ethers (PBDEs) in marine mammals
673 from Arctic and North Atlantic regions, 1986-2009. Environ. Int. 40, 102–9.
674 doi:10.1016/j.envint.2011.07.001
675 Routti, H., Letcher, R.J., Arukwe, A., Van Bavel, B., Yoccoz, N.G., Chu, S., Gabrielsen, G.W.,
676 2008. Biotransformation of PCBs in relation to phase I and II xenobiotic-metabolizing enzyme
677 activities in ringed seals (Phoca hispida) from Svalbard and the Baltic Sea. Environ. Sci.
678 Technol. 42, 8952–8. doi:10.1021/es801682f
679 Ruus, A., Sandvik, M., Ugland, K.I., Skaare, J.U., 2002. Factors influencing activities of
680 biotransformation enzymes, concentrations and compositional patterns of organochlorine
681 contaminants in members of a marine food web. Aquat. Toxicol. 61, 73–87.
682 doi:10.1016/S0166-445X(02)00043-7
683 Safe, S.H., 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic
684 responses, and implications for risk assessment. Crit. Rev. Toxicol. 24, 87–149.
685 doi:10.3109/10408449409049308
686 Stegeman, J.J., Hahn, M.E., 1994. Biochemistry and molecular biology of monooxygenases: current
687 perspectives on forms, functions, and regulation of cytochrome P450 in aquatic species, in:
688 Malins, D.C., Ostrander, G.K. (Eds.), Aquatic Toxicology: Molecular, Biochemical, and
689 Cellular Perspectives. Lewis Publishers, Boca Raton, pp. 87–203.
690 Tanabe, S., Watanabe, S., Kan, H., Tatsukawa, R., 1988. Capacity and mode of PCB metabolism in
691 small cetaceans. Mar. Mammal Sci. 4, 103–124. doi:10.1111/j.1748-7692.1988.tb00191.x
692 Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H.,
693 Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C.,
694 Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., Peterson, R.E., 2006. The 2005 World

695 Health Organization reevaluation of human and Mammalian toxic equivalency factors for
696 dioxins and dioxin-like compounds. Toxicol. Sci. 93, 223–41. doi:10.1093/toxsci/kfl055
697 Watanabe, S., Shimada, T., Nakamura, S., Nishiyama, N., Tanabe, S., Tatsukawa, R., Yamashita,
698 N., Tanabe, S., Tatsukawa, R., 1989. Specific profile of liver microsomal cytochrome P-450 in
699 Dolphin and Whales. Mar. Environ. Res. 27, 51–65. doi:10.1016/0141-1136(89)90018-4
700 Weaver, R.J., Thompson, S., Smith, G., Dickins, M., Elcombe, C.R., Mayer, R.T., Burke, M.D.,
701 1994. A comparative study of constitutive and induced alkoxyresorufin o-dealkylation and
702 individual cytochrome P450 forms in cynomolgus monkey (Macaca fascicularis), human,
703 mouse, rat and hamster liver microsomes. Biochem. Pharmacol. 47, 763–773.
704 Weijs, L., van Elk, C., Das, K., Blust, R., Covaci, A., 2010. Persistent organic pollutants and
705 methoxylated PBDEs in harbour porpoises from the North Sea from 1990 until 2008: Young
706 wildlife at risk? Sci. Total Environ. 409, 228–37. doi:10.1016/j.scitotenv.2010.09.035
707 White, R.D., Hahn, M.E., Lockhart, W.L., Stegeman, J.J., 1994. Catalytic and immunochemical
708 characterization of hepatic microsomal cytochromes P450 in beluga whale (Delphinapterus
709 leucas). Toxicol. Appl. Pharmacol. 126, 45–57. doi:10.1006/taap.1994.1089
710 White, R.D., Shea, D., Schlezinger, J.J., Hahn, M.E., Stegeman, J.J., 2000. In vitro metabolism of
711 polychlorinated biphenyl congeners by beluga whale (Delphinapterus leucas) and pilot whale
712 (Globicephala melas) and relationship to cytochrome P450 expression. Comp. Biochem.
713 Physiol. C. Toxicol. Pharmacol. 126, 267–84.
714 Wilson, J.Y., Moore, M.J., Stegeman, J.J., 2010. Catalytic and immunochemical detection of
715 hepatic and extrahepatic microsomal cytochrome P450 1A1 (CYP1A1) in white-sided dolphin
716 (Lagenorhynchus acutus). Aquat. Toxicol. 96, 216–224. doi:10.1016/j.aquatox.2009.10.021
717 Wilson, J.Y., Wells, R., Aguilar, A., Borrell, A., Tornero, V., Reijnders, P., Moore, M., Stegeman,
718 J.J., 2007. Correlates of cytochrome P450 1A1 expression in bottlenose dolphin (Tursiops
719 truncatus) integument biopsies. Toxicol. Sci. 97, 111–119. doi:10.1093/toxsci/kfm031
720 Wolkers, H., Boily, F., Fink-Gremmels, J., van Bavel, B., Hammill, M.O., Primicerio, R., 2009.

721 Tissue-specific contaminant accumulation and associated effects on hepatic serum analytes and
722 cytochrome P450 enzyme activities in hooded seals (Cystophora cristata) from the Gulf of St.
723 Lawrence. Arch. Environ. Contam. Toxicol. 56, 360–70. doi:10.1007/s00244-008-9186-6
724 Wolkers, H., Burkow, I.C., Hammill, M.O., Lydersen, C., Witkamp, R.F., 2002. Transfer of
725 polychlorinated biphenyls and chlorinated pesticides from mother to pup in relation to
726 cytochrome P450 enzyme activities in harp seals (Phoca groenlandica) from the gulf of St.
727 Lawrence, Canada. Environ. Toxicol. Chem. 21, 94–101.
728 Wolkers, J., Witkamp, R.F., Nijmeijer, S.M., Burkow, I.C., De Groene, E.M., Lydersen, C., Dahle,
729 S., Monshouwer, M., 1998. Phase I and phase II enzyme activities in Ringed seals (Phoca
730 hispida): Characterization of hepatic cytochrome P450 by activity patterns, inhibition studies,
731 mRNA analyses, and western blotting. Aquat. Toxicol. 44, 103–115. doi:10.1016/S0166-
732 445X(98)00063-0
733 You, L., 2004. Steroid hormone biotransformation and xenobiotic induction of hepatic steroid
734 metabolizing enzymes. Chem. Biol. Interact. 147, 233–46. doi:10.1016/j.cbi.2004.01.006
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738 **Figure 1** Concentrations of the initial quantity of mRNA for enzymes and receptors measured by
739 qPCR in liver of pilot whale (*Glopicephala melas*) versus the length of the animals divided into
740 different age/sex groups.

741

742 **Figure 2** Western blot responses for pilot whale liver proteins separated by SDS PAGE and treated
743 with CYP antibodies. For the females no. 1-4 are juveniles and no. 5-9 are adults. For the males no.
744 1-5 are juveniles and no. 6-9 are adults.

745

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

748

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

752

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

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