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Catalytic and Immunochemical Detection of Hepatic and Extrahepatic Microsomal Cytochrome P450 1A1 (CYP1A1) in White-sided Dolphin (*Lagenorhynchus acutus*)

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Abstract

We have characterized microsomal systems and measured the levels of microsomal cytochrome P450 1A1 (CYP1A1) and ethoxyresorufin-O-deethylase activity in multiple internal organs of male and female white-sided dolphin (*Lagenorhynchus acutus*) from the northwest Atlantic Ocean. Internal organs were sampled within 24 hours of death, sometimes in a period of hours, collection times which are significantly less than usually seen for marine mammals. Tissue autolysis, as assessed by histological analysis of liver, was minimal to none in all individuals. Total P420 did not correlate with time from death to sampling, suggesting that it is a poor indicator of P450 degradation in cetacean tissues where perfusion isn't practical. The total hepatic microsomal P450 content, cytochrome b5 content, and NADPH-cytochrome c (P450) reductase (CPR) activity averaged 0.29 nmol mg⁻¹, 0.12 nmol mg⁻¹, and 238 nmol mg⁻¹ min⁻¹, respectively. Microsomal CPR activity in liver was higher than that in lung and kidney, and was higher than that reported in liver of most other cetacean species. Immunodetected CYP1A1 content was low in all organs, less than 3 pmoles CYP1A equivalents mg⁻¹. EROD activity ranged from 9 – 376 pmoles mg⁻¹ min⁻¹ and was greater in liver than in other tissues. Hepatic microsomal EROD activity and CYP1A1 content did not correlate. However, hepatic EROD activity, but not CYP1A1 protein content, was well correlated with both total PCB and Σmono-ortho PCB concentrations in blubber. Length, as a proxy for age, did not correlate with hepatic EROD activity or CYP1A1 protein levels, and sex did not influence the relationship between EROD and contaminant concentrations. We cannot easily control for the extent of tissue degradation in cetacean studies nor do we have a complete history of these animals. Therefore, other factors such as degradation or hormonal state may have a role in the observed relationships. Yet, as in other mammals, hepatic tissues appear to be a major site of CYP1A1 expression and probably of biotransformation of CYP1A substrates in white-sided dolphin. The expression of an EROD catalyst in liver likely reflects induction by PCBs, but the P450 enzyme catalyzing hepatic EROD activity in these whales may not be CYP1A1.

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Conflicts of Interest Statement

The authors have no conflicts of interest in this manuscript.

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Keywords

cytochrome P450 1A1; CYP1A1; white-sided dolphin; polychlorinated biphenyl; PCB

Introduction

The odontocetes, or toothed whales and dolphins, typically have higher concentrations of contaminants in their tissues than do mysticetes or baleen whales (O'Shea and Brownell, 1994). Coastal populations of odontocetes may have elevated exposure to contaminants and risk of toxicity from anthropogenic contaminants. Atlantic white-sided dolphins (*Lagenorhynchus acutus*) are found along the densely populated northeast coast of the United States and Canada, where there also are high rates of shipping traffic. Previous studies have shown that white-sided dolphins from this region have total polychlorinated biphenyl (PCB) concentrations of approximately 10–30 $\mu\text{g}\cdot\text{g}^{-1}$ wet weight in blubber (Weisbrod *et al.*, 2001; Tuerk *et al.*, 2005b), similar to levels seen in harbour porpoise (Westgate *et al.*, 1997) and higher than those seen in pilot whale (Weisbrod *et al.*, 2001) from the same region. Based on the similarity between PCB concentrations in white-sided dolphin and concentrations associated with altered immune function in seals (Ross *et al.*, 1995; de Swart *et al.*, 1996), it has been suggested that there is potential for toxic effects of PCBs in this species (Weisbrod *et al.*, 2001). Collectively, the wide distribution of white-sided dolphin, their exposure to and accumulation of potentially toxicologically relevant organochlorine concentrations, and availability of tissues via stranding events, indicate that white-sided dolphin are likely a good cetacean species for ecotoxicological studies.

Anecdotal and epidemiological studies of marine mammal populations have suggested that exposure to high levels of contaminants is associated with immune or reproductive dysfunction or cancer. High concentrations of organochlorine contaminants, usually including PCBs, have been related to mortality associated with viral and/or bacterial infections in seals (Ross *et al.*, 1996), bottlenose dolphin (Lipscomb *et al.*, 1994; Lahvis *et al.*, 1995; Schulman *et al.*, 1997), striped dolphin (Guitart *et al.*, 1996), common dolphin (Birkun *et al.*, 1999), and harbour porpoise (Jepson *et al.*, 1999). Exposure to polycyclic aromatic hydrocarbons has been proposed as one factor contributing to the unusually high gastro-intestinal cancer rate in St. Lawrence beluga whales (Martineau *et al.*, 1994). Reproductive abnormalities have been found in seals with exposure to high concentrations of organochlorines (Reijnders, 1986). These data suggest an association between contaminants and health impacts in marine mammal populations.

Cytochrome P450 (CYP) enzymes catalyze oxidative metabolism of a wide array of endogenous and exogenous compounds, including many environmental contaminants. CYP-mediated metabolism can result in either detoxification or activation and can determine the persistence of some xenobiotics, thereby influencing their toxicity (e.g. Uno *et al.*, 2006). Cytochrome P450 1A1 (CYP1A1) is an enzyme that is induced by and metabolizes planar halogenated aromatic hydrocarbons (PHAHs) such as the non-*ortho* and mono-*ortho* PCBs, and polycyclic aromatic hydrocarbons (PAHs), via activation of the aryl hydrocarbon receptor (AHR). The AHR pathway has been well documented in mammals (Whitlock, 1999), including cetaceans (Jensen and Hahn, 2001). Like other mammals, cetaceans have genes for CYP1A2 as well as CYP1A1 (Niimi *et al.*, 2005), and likely CYP1B1. CYP1A expression has been identified in cetacean tissues (Goksoeyr *et al.*, 1985; Goksoeyr *et al.*, 1988; Stegeman *et al.*, 1994; Watanabe *et al.*, 1989; White *et al.*, 1994; White *et al.*, 2000; Wilson *et al.*, 2005), usually by immunoblot, or, less frequently, inferred from ethoxyresorufin-O-deethylase (EROD) activity in liver microsomes. Congener-specific PCB contaminant profiles suggest that CYP1A expression is biologically important in cetaceans for metabolism and excretion (Tanabe *et*

al., 1988; Duinker *et al.*, 1989; Norstrom *et al.*, 1992). Studies of CYP1A1 expression primarily focus on liver or skin and blubber biopsies; CYP1A1 expression has been detected by immunoassay in extrahepatic organs of pilot whale and beluga whale (Stegeman *et al.*, 1994; Wilson *et al.*, 2005) only.

The white-sided dolphin is a species that commonly strands on Cape Cod, MA. Two large mass stranding events in 1998 and 1999, as well as a smaller event in 1997, allowed for a rare comprehensive collection of internal organs from multiple males and females from the same species. These samples were collected from animals within 24 hours post-mortem, some within a few hours; samples which represent one of the best preserved and comprehensive collections of organs from a cetacean species. Here we report the expression of CYP1A1 in various internal organs and the relationship of this expression to the levels of contaminants in blubber, age and sex. Identification of tissues with high expression of CYP1A1 could indicate relative capacity of tissues for PAH and PHAH metabolism, indicate possible target organs for toxicity and tissues that accumulate relatively high concentrations of inducing compounds.

Materials and Methods

Samples

A total of 12 dolphins, 5 males and 7 females, were sampled for this study from white-sided dolphins during 3 mass strandings on Cape Cod (Table 1). To obtain tissues that were not degraded, animals selected for sampling were those thought to have most recently died. Time between death and necropsy varied between samples because the exact time of death was not available prior to necropsy, due to logistical issues. In some cases, the exact time of death is not known due to missing data on the field necropsy forms. In these cases, we have given the maximum time (24 hours) possible but these samples were all likely sampled in significantly less time than the maximum. Some of these animals were euthanized. In all years, the strandings occurred during winter when cooler air temperatures would help to minimize degradation during the collection of samples. Necropsies were performed in the field under difficult circumstances, so that sampling was not identical for each animal. In spite of this, a large group of internal organs, including adrenal, heart, kidney, liver, lung, lymph node, spleen, and gonads, were collected whenever possible. Samples were collected from similar anatomical portions of each organ. One portion of each organ was flash frozen in liquid nitrogen during field necropsies. Approximately 300 g of blubber was taken just anterior and slightly ventral to the dorsal fin. The blubber samples extended the entire depth of the dermis, from the epidermis to the underlying muscle layer. Blubber samples were taken and archived at the National Institute for Standards and Technology (NIST, Charleston, SC) for contaminant analyses according to established NIST protocols (Becker *et al.*, 1999). Blubber samples were placed on ice and stored -20°C until shipped to NIST, where they were transferred to -150°C liquid nitrogen (LN_2) vapor freezers. Liver samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hemotoxylin and eosin and examined microscopically to assess tissue autolysis (Moore and Stegeman, 1996).

Morphometric information, including total length and girth, gender, and reproductive status were recorded for each animal. Body length was used as a proxy for sexual maturity, with females > 206 cm, and males > 212 cm considered sexually mature (Sergeant *et al.*, 1980; Palka *et al.*, 1997). Multiple teeth, usually 2 teeth from the middle of each of the upper and lower jaw, were collected for age determination. Ages have been published for some of these animals, including 98-005, 99-016, 99-017 and 00-013 (Tuerk *et al.*, 2005b), which were analyzed in this study.

Preparation of Microsomes

Microsomal fractions were prepared as previously described for beluga whales (White *et al.*, 1994), using approximately 1 gram of tissue. Samples were stored in liquid nitrogen as part of a long term cetacean tissue archive. Liver samples have been stored without loss of ethoxyresorufin-O-deethylase activity for periods up to 300 days (Kruner and Wesernhagen, 1999). Microsomal samples have been stored for over 3 years in liquid nitrogen without loss of EROD activity (Woodin and Stegeman, unpublished data). Tissues with a large amount of connective tissue or muscle were homogenized with a polytron homogenizer with a sawtooth generator to finely mince the tissues, prior to standard microsomal preparations. Microsomal protein concentrations were determined with the bicinchoninic acid assay (Smith *et al.*, 1985) adapted for a 96-well plate reader, using bovine serum albumin (Sigma, St. Louis MO) as the standard.

Cytochrome P450 and b5

The content of liver microsomal cytochrome P450 was determined from dithionite difference spectra of CO-treated samples (Stegeman *et al.*, 1979), assuming extinction coefficients of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ for P450 and $111 \text{ mM}^{-1} \text{ cm}^{-1}$ for P420 (Omura and Sato, 1964). Cytochrome b5 was measured using NADH difference spectra, assuming an extinction coefficient of $185 \text{ mM}^{-1} \text{ cm}^{-1}$, as described previously (Stegeman *et al.*, 1979).

Enzyme Assays and Immunoblots

NADPH cytochrome c (P450) reductase activity (CPR) was measured by monitoring absorbance at 550 nm, as previously described (Stegeman *et al.*, 1979). Ethoxyresorufin O-deethylase (EROD) activity was quantified using a spectrofluometric kinetic assay (Kennedy *et al.*, 1993). Immunoblot (western blot) analyses were performed as previously described (Klopper-Sams *et al.*, 1987; Stegeman *et al.*, 1991) with an enhanced chemiluminescent detection system (Matthews *et al.*, 1985). The primary and secondary antibodies were the monoclonal antibody 1-12-3 (3 ug ml^{-1} in 5% nonfat dry milk, 0.02 M Tris, 0.5 M NaCl, pH 7.5) and a goat anti-mouse horseradish peroxidase (BioRad, Richmond CA, 1 in 1000 dilution in 5% nonfat dry milk, 0.02 M Tris, 0.5 M NaCl, pH 7.5), respectively. CYP1A1 content was quantified by comparison of the intensities of individual bands with the linear response range of liver microsomes from β -naphthoflavone injected scup (*Stenotomus chrysops*), with known CYP1A1 content. Immunoblot (slot blot) analyses were performed with a Milliblot-S (Millipore, Bedford MA) apparatus with $\leq 7 \text{ }\mu\text{g}$ microsomal protein applied directly to a $0.2 \text{ }\mu\text{m}$ nitocellulose sheet (Protran BA 83, Schleicher and Schuell, Keene NH). Wells were washed with phosphate buffered saline and then processed as described for western blotting. CYP1A1 content was estimated densitometrically from developed X-ray audiograms of blots. MAb 1-12-3 is highly specific for CYP1A1 proteins, however, because cross-reactivity of the primary antibody for CYP1A proteins in different species still may vary, CYP1A1 concentrations deduced from the immunoblots represent relative rather than absolute concentrations and are expressed as CYP1A equivalents.

Content of Contaminants in blubber

The concentrations of organic chemical pollutants were measured by Tuerk in blubber samples of nine of the animals we examined (Tuerk *et al.*, 2005a; Tuerk *et al.*, 2005b). For our analyses, we used data generated by Tuerk for chlorinated pesticides; hexachlorobenzene (HCB), α -, β -, and γ -hexachlorocyclohexane (HCH), heptachlor and heptachlor epoxide, trans- and cis-chlordane, trans- and cis-nonachlor, oxychlordane, endosulfan I, dieldrin, Σ DDT congeners mirex, total toxaphene and toxaphene congeners 26, 32, 50, and 62; Σ PBDE congeners; total and Σ mono-*ortho* PCBs. Total PCB concentrations (Σ PCB) were calculated as the sum of individual peaks for all 56 *ortho*-substituted PCBs analyzed (IUPAC# 18, 28, 29, 31, 44, 45,

49, 52, 56, 60, 63, 66, 70, 74, 76, 81, 82, 84, 87, 89, 90, 92, 95, 99, 101, 105, 107, 110, 118, 123, 128, 132, 138, 146, 149, 151, 153, 154, 156, 157, 158, 163, 170, 171, 174, 180, 182, 183, 187, 193, 194, 195, 201, 202, 206, 209). Total DDT concentrations (Σ DDT) were calculated as the sum of the DDT compounds (2,4'-, and 4,4'-DDD, DDE, and DDT) and total PDBEs (Σ PBDE) were calculated as the sum of the PBDE congeners (26, 32, 50, 62). The Σ mono-*ortho* PCB concentrations were calculated as the sum of individual peaks of 17 mono-*ortho* substituted PCB congeners (IUPAC# 29, 28, 31, 56, 60, 63, 66, 70, 74, 76, 81, 105, 107, 118, 123, 156, 157) and four PCBs (IUPAC # 87, 149, 171, 202) with multiple (2–4) *ortho* substitutions that co-eluted with a mono-*ortho* substituted PCB.

Statistics—Statistics were performed using Statistica (StatSoft, Tulsa OK). Data were tested for normality using the Shapiro Wilks W test and heterogeneity with Levine's test. The impact of age (length) and gender on liver EROD activity was determined using a two-way ANOVA. Correlations between measurements were determined using a Pearson's r correlation. Statistical differences were determined when the p value was <0.05.

Results

A summary of biological and sampling information is in Table 1, for the 12 white-sided dolphin (7 females and 5 males), from which tissues were collected during field necropsies of mass strandings on Cape Cod, MA in 1997, 1998 and 1999. Six of these animals were euthanized, which gave a precise time of death; difficult field conditions resulted in incomplete field notes so the cause of death (euthanasia or stranding) was not known for four animals. The dolphins were necropsied within a maximum of 24 hours post-mortem, although most were necropsied within 12 hours and two within 3 hours of death. Hematoxylin and eosin stained liver sections were scored for the degree of autolysis and presence of bacteria, in case there was any post mortem decomposition, using the same criteria as used previously (Moore and Stegeman 1996). Two individuals (98-002 and 99-015) showed the very earliest stage of possible autolysis, with an enhanced cytoplasmic eosinophilia and fragmentation. Nuclear morphology was normal. Otherwise no signs of autolysis or decomposition were observed in any sample (data not shown).

The ages of four animals were determined from dentine growth layers on teeth. Morphometric measurements were taken, however, published age – length relationships for this species (Sergeant *et al.*, 1980) did not include a mathematical model that we could apply to our length data. Thus, ages were estimated based on length using equations generated from a limited data set of age and length (see Materials and Methods for details). Although these equations were based on only a few animals, the R^2 values were high (0.86 for males and 0.98 for females). Rough estimates of age based on the Sergeant *et al* age-length graph (1980), calculated ages based on the data in Tuerk *et al* (2005b), and the ages from dentine growth layers, together suggest that females ranged in age from 2 to 12 years and males from 3 to 12 years (Table 1). Mass strandings of white-sided dolphins involve herds whose composition is biased towards young and old animals (Sergeant *et al.*, 1980). Consistent with this, according to our estimates the animals we sampled were either reproductively mature or less than 3 years of age. Based on the age at separation from a breeding herd (Sergeant *et al.*, 1980), the youngest animals (age two years) may have been offspring of females in the herd from the prior breeding season (i.e. not young of the year). These animals likely were consuming the same food resources as the other stranded animals and not consuming milk (Sergeant *et al.*, 1980).

Two females and three males were considered sexually mature, based on their length. Both females that were considered sexually mature, but no other females, were lactating, as determined by visual inspection and collection of milk from the mammary gland at necropsy. Lactation state in these two animals has been confirmed by histology (unpublished data, J.

Wilson). Since females give birth from May to August, the sexually mature, lactating animals had most likely been lactating for approximately five to seven months, (one-third to one-half of the lactation period) (Sergeant *et al.*, 1980). The lactating females did not appear to be different in any measurement from the other animals in this study, except in having lower PCB concentrations than other females, and were therefore included in all analyses. Lower PCB concentrations are expected because of offloading of contaminants during lactation (for a detailed discussion of this in white-sided dolphin see Tuerk *et al.*, 2005b)

Total P450 Concentrations

The specific contents of spectrophotometrically-determined P450, P420 and cytochrome b5 in hepatic microsomal preparations are shown in Table 2. Specific content of total P450 ranged from 0.05 to 1.91 nmol mg⁻¹ but most samples had 0.10 to 0.23 nmol mg⁻¹ (Table 2). A significant P420 peak was present in all of the liver microsomal samples (Table 2), accounting on average for nearly 60% ($\pm 25\%$) of the total P450 plus P420 content. The relative P420 content was not related to the time between death and necropsy (data not shown). Activity of cytochrome P450 reductase was measured in kidney, liver and lung (Table 2) and rates were significantly higher in liver than the other two organs. The mean reductase activity was 238 (± 75 SD), 120 (± 15 SD), and 122 (± 14 SD) nmol mg⁻¹ min⁻¹ for liver, kidney, and lung, respectively.

CYP1A1 Expression and EROD Activity

The sample with the highest EROD activity (99-018) had 3-fold greater activity than the next highest sample, and was three standard deviations above the mean liver EROD activity. We considered this sample an outlier and removed it from the correlation analyses. The outlier is included in Figures 1 and 2 for comparison to the other samples. There was no correlation between the levels of EROD activity in liver and the P420 content in the liver (data not shown), or the time between death and necropsy (data not shown), nor was there a relationship between hepatic EROD and total P450 content (data not shown). Levels of EROD activity were highest in liver, ranging from 9 to 376 pmoles mg⁻¹ min⁻¹ (Table 3), while all other internal organs had EROD activities less than 13 pmoles mg⁻¹ min⁻¹ (Table 4). Levels of microsomal CYP1A1 equivalents were less than 3 pmol mg⁻¹, in all organs (Table 4). In contrast to EROD activities, where the highest activities were in liver, CYP1A1 content was not higher in liver than the other organs and the highest concentrations (22, 8, and 6 pmoles mg⁻¹) were observed in two lung samples and one spleen sample, all from different animals. CYP1A1 content and EROD activity were not well correlated, no matter whether all organs ($r=0.02$, $p>0.05$, Figure 1A) or just liver samples ($r=0.38$, $p>0.05$, Figure 1B) were included in the analysis.

EROD activity was significantly correlated with both total and Σ of mono-*ortho* PCBs (Figure 2A and B, Table 5), as well as additional individual mono-*ortho* PCB congeners (PCB66, PCB107, and PCB149) and heptachlor (Table 5). EROD activity was significantly correlated with levels of specific mono-*ortho* congeners PCB70, PCB156 and PCB157, as well as with HCB (Table 5). We have examined the pattern of contaminants in the blubber of these dolphins. Patterns of individual contaminant classes and specific PCB congeners do not vary between samples (data not shown). However, we do not have any contaminant data for the individual with the elevated EROD activity, so we cannot rule out that this animal had a higher burden of contaminants compared to the other individuals.

Discussion

Condition and Life-History of Animals

Mass strandings of cetaceans are not unusual on Cape Cod, MA and have included pilot whale, common dolphin and Atlantic white-sided dolphin (Sergeant *et al.*, 1980; Wiley *et al.*, 2001).

The animals involved in the 1997, 1998 and 1999 mass strandings, when sampling for this study took place, were apparently healthy, robust animals and there is currently no known cause of these strandings. There were no signs of illness (emaciation, presence of obvious parasites, abnormalities in major internal organs etc) and detailed necropsies were performed on each animal. These samples may be representative of the normal, healthy free-ranging population of white-sided dolphin in the northwest Atlantic Ocean.

Collecting tissues from stranded cetaceans can be quite challenging. Locating dead animals and relocation of carcasses to areas appropriate for necropsy can be time consuming. Tissue degradation will be a persistent issue. Moore and Stegeman (1996) showed autolytic changes in stranded pilot whale liver that were related to the time between death, and necropsy and tissue sampling. The quality of biopsy-derived RNA was of better quality than RNA isolated from skin from stranded cetaceans, which showed a pattern of degradation that appeared to be related to the time from death (Lapseritis, 2007). Thus, while we expected some level of tissue change and degradation in samples from stranded animals, attempts were made to limit this problem. First, animals included in this study were stranded and sampled during cold weather, mean air temperatures were 2–3°C and 2–5°C, for the 1998 and 1999 strandings respectively, when degradation could be slower. Second, preference was given to samples from animals that were known or suspected to have died within <3 hours, and particularly to euthanized animals for which an exact time death was recorded. However, the time between death and necropsy is likely to be on the order of hours and therefore, the issue of degradation remains and data must be interpreted with caution. Histological analyses of liver from these animals show no signs of autolysis or decomposition in all but two samples; the remaining two samples showed only the very earliest stage of possible autolysis suggesting that degradation issues was limited in this data set. Studies with systematic sampling of internal organs from cetacean species are very limited and most studies collect samples a longer time after death than those sampled in this study. This sample set, with both male and female animals, is rare and valuable and provides one our best opportunities to examine tissue biochemistry in a cetacean species.

Of the animals sampled, two females and three males were sexually mature. The two sexually mature females were lactating. Lactation is thought to have a large role in determining the accumulation of lipophilic contaminants in female animals and lactation is likely a time when the distribution of contaminants change and the relationship between blubber concentrations and concentrations in target tissues may differ. Clearly, hormonal differences between sexually mature and immature animals, lactating and non-lactating females, may have an impact on our measurements and bias the results. Yet, we saw no evidence of this in our data. Males and females were not different in any of our measurements, as we have previously seen (Wilson *et al.*, 2007), nor did the lactating females appear different than our other females. Future research may need to include measures of lactation and hormonal state into account. With only two lactating females, we do not have sufficient sample size to robustly test this.

Characterization of Microsomal Systems

There were significant amounts of putative cytochrome P420 present in all of the liver microsomal samples, suggesting degradation of P450 proteins. However, the amounts of native P450 or P420 did not relate either to EROD activity or time from death to necropsy. Likewise, EROD activity did not show any decrease correlated with time from death to necropsy. This suggests that the P420 peak observed may have been caused by interfering compounds such as oxyhemoglobin and was not degraded P450. Substantial P420 peaks were present in beluga whale liver microsomes (White *et al.*, 1994) even though the assays were performed in the presence of chemicals meant to eliminate the signal from oxyhemoglobin (Johannesen and DePierre, 1978). Hemoglobin contamination could be expected due to our inability to perfuse the liver to remove blood from the tissues prior to sampling. Measures of P420 may not be

useful for determining potential degradation in marine mammal samples because tissue perfusion is too difficult with such large livers and difficult field conditions.

For most animals the total P450 content was between 0.10 to 0.23 nmol mg⁻¹, a range similar or lower than that in liver of beluga (White *et al.*, 1994), (both long and short-finned species, Watanabe *et al.*, 1989; White *et al.*, 2000), killer whale, and striped dolphin (Watanabe *et al.*, 1989), and up to 3.5 fold times less than P450 content in minke whale liver (Goksoeyr *et al.*, 1986; Goksoeyr *et al.*, 1988). Thus, white-sided dolphins appear to have hepatic microsomal P450 levels similar to many other cetacean species.

Catalytic function of cytochrome P450 enzymes requires reduction of the heme iron via NADPH-cytochrome c (P450) reductase (CPR) and cytochrome b5. CPR activities in liver microsomes were greater than those seen in liver microsomes from beluga whale (White *et al.*, 1994), killer whale, short finned pilot whale, striped dolphin (Watanabe *et al.*, 1989), and minke whale (Goksoeyr *et al.*, 1986; Goksoeyr *et al.*, 1988). Microsomal CPR activities in kidney and lung were higher than those reported for liver in all other cetaceans except striped dolphin (Watanabe *et al.*, 1989) and 10 times higher than those reported for minke kidney microsomes (Goksoeyr *et al.*, 1988). Cytochrome b5 content was similar to or less than that seen in liver microsomes from beluga whale (White *et al.*, 1994), pilot whale (White *et al.*, 2000), and minke whale (Goksoeyr *et al.*, 1986; Goksoeyr *et al.*, 1988) although it did vary 5 fold between samples. The CPR activities and cytochrome b5 contents suggest a capacity for electron transfer to P450 enzymes in white-sided dolphin that is similar to that other cetacean species. Any decline in a specific P450 activity, compared to other cetacean species, is most likely related to differences in the content of the enzyme, and not insufficient electron transfer.

EROD Activity and CYP1A1 Expression in White-sided Dolphin

EROD activity was significantly greater in the liver than in any other organ as expected. Mean liver microsomal EROD activity was about 90 pmol mg⁻¹ min⁻¹, similar to rates reported for pilot whales (both long and short-finned species, Watanabe *et al.*, 1989; White *et al.*, 2000) and female beluga whale (Watanabe *et al.*, 1989). Liver microsomal EROD activities were much less than those reported for male beluga whale (White *et al.*, 1994), minke whale (Goksoeyr *et al.*, 1986; Goksoeyr *et al.*, 1988), striped dolphin, killer whale (Watanabe *et al.*, 1989), and sperm whale (Boon *et al.*, 2001). Only sperm whale and white-beaked dolphin have lower reported EROD activities (Boon *et al.*, 2001). Hepatic EROD activity, when expressed as pmol-nmol P450⁻¹ min⁻¹, is similar to that found in beluga whale, pilot whale (White *et al.*, 2000) and harbour seal (Troisi and Mason, 1997).

Comparison of EROD activities in extrahepatic organs of cetaceans is difficult since only one other study has reported such data. Mean EROD activity in minke whale kidney was 20 pmoles mg⁻¹ min⁻¹ (Goksoeyr *et al.*, 1988), a value higher than that found in the kidney of white-sided dolphin in this study. Measures of CYP1A1 related protein and activity in extrahepatic organs were low suggesting that PAH and PHAH metabolism by CYP1A is limited in extrahepatic organs, and extrahepatic target organs for PAH or PHAH toxicity are not easily identified in these dolphins, at least based on CYP1A levels.

Low CYP1A1 content might be expected, given the low EROD activities that we saw. By comparison, average CYP1A1 contents in liver of pilot whale (White *et al.*, 2000) and female beluga (White *et al.*, 1994) were six to seven times higher than the mean hepatic CYP1A1 content recorded in this study. White *et al.* (1994) reported hepatic CYP1A1 levels from male beluga whales had that were nearly 70 times higher than the levels we found here for white-sided dolphin liver. There could be differences in the cross-reactivity of the Mab 1-12-3 for CYP1A1 from different species. Yet, differences in cross-reactivity are not likely to account for such large differences in CYP1A1 content measured between these species, since the

epitope for this antibody is well conserved among vertebrate CYP1A1s (unpublished information) it strongly recognizes CYP1A enzymes in diverse vertebrate species (Stegeman and Hahn, 1994) including humans (Drahushuk *et al.*, 1998). Thus, CYP1A1 content in white-sided dolphin liver, like EROD activity, is significantly less than that reported for other cetacean species.

CYP1A1 levels did not correlate well with EROD activity, even when the analysis was limited to liver. This could be a reflection of the low and variable levels of CYP1A1. Certainly, a range of only 0–3 pmoles mg⁻¹ of CYP1A1 is very small. CYP1A1 content in liver has been strongly correlated to liver EROD activity in both pilot (White *et al.*, 2000) and beluga whale (White *et al.*, 1994), although the range of CYP1A1 content was 10 fold larger than that seen here. Degradation rates are likely different for immunodetectable CYP1A1 and catalytic activities and yet degradation is unlikely to account for the poor correlations seen here. The animals with the highest EROD activity do not have elevated CYP1A1 levels. Yet, catalytic function should be impacted prior to loss of immunodetectable protein. Indeed, beluga whales from the St. Lawrence estuary were found to have significant CYP1A1 protein but non-detectable EROD activity (McKinney *et al.*, 2004). The lack of correlation between CYP1A1 levels and EROD also could suggest that there are other EROD catalysts present. AHR agonists can induce both CYP1A2 and CYP1B1, and both can catalyze EROD activity in other mammals (e.g., Shimada *et al.*, 1997). The levels of expression of these other CYPs in white-sided dolphin are unknown.

Although CYP1A1 and EROD levels are low, contaminant residue analysis suggests that CYP1A-like biotransformation activity could be appreciable in this species, based on a significant negative relationship between length and the ratios of PCB105, 118 and 156 (possible CYP1A substrates) to PCB153 (not a CYP1A substrate) (Tuerk *et al.*, 2005b). Studies *in vitro* showed that both beluga and pilot whale liver microsomes were able to hydroxylate planar PCB congeners, apparently due to CYP1A activity (White *et al.*, 2000; McKinney *et al.*, 2006). In rodents, the pharmacokinetics of AHR ligands is altered by hepatic proteins, especially CYP1A2 (DeVito *et al.*, 1998). Such studies support a suggestion that CYP1A-like biotransformation is important in influencing concentrations of AHR ligands in these white-sided dolphins. The contribution of extrahepatic organs to biotransformation and clearance is not clear. As noted above, kidney EROD activities in minke whales (Goksoeyr *et al.*, 1988) were similar to the hepatic EROD rates we report here for white-sided dolphin. Beluga whale, a species for which CYP1A1 is expressed in extrahepatic tissues (Wilson *et al.*, 2005), may have significant extrahepatic biotransformation. CYP1A1 is expressed in integument from a large number of cetacean species (Angell *et al.*, 2004; Godard *et al.*, 2004; Wilson *et al.*, 2005; Wilson *et al.*, 2007) and this expression is correlated to PCB concentrations (Wilson *et al.*, 2007) and may play a role in biotransformation (Montie *et al.*, 2008), in at least bottlenose dolphin. However, the degree to which extrahepatic CYPs, particularly integument CYP1A1, contributes to overall rates of xenobiotic metabolism in cetaceans remains to be determined.

Correlations of CYP1A1 and PCB Concentrations

Concentrations of 56 PCB congeners and organochlorines in blubber previously determined for nine of the animals in this study, and of additional white-sided dolphin from this region, have been described in detail elsewhere (Tuerk, 2002; Tuerk *et al.*, 2005a; Tuerk *et al.*, 2005b). Total PCB concentrations averaged 25 µg g⁻¹ (±82.6 SD) and 18.5 g g⁻¹ (±3.6 SD) for males and females, respectively. PCB concentrations, including the Σmono-*ortho* PCBs, were well correlated with EROD activity but not CYP1A1 content in liver of white-sided dolphin. In beluga whale, the CYP1A1 content of liver microsomes was strongly correlated with the Σ of mono-*ortho* PCBs in blubber (White *et al.*, 1994). Since EROD activities and CYP1A1 content were also strongly correlated in that study (White *et al.*, 1994), it is likely that EROD activity also correlated with blubber PCB concentrations in the beluga whale. The

weights of the beluga were much less than expected based on their length (White *et al.*, 1994), which suggested that those animals had mobilized fat reserves. The white-sided dolphins in this study were robust and apparently healthy animals.

Blubber contaminant concentrations may not always be related to concentrations found in target organs. Studies of contaminant concentration in organs are rare but Karlson *et al.* (2000) showed a similar pattern of organochlorine contaminants in liver, muscle, brain, blubber and nuchal fat of harbour porpoise although the absolute concentrations varied between organs. The harbour porpoise study suggested there was an equilibrium distribution of contaminants between blood, organs and tissues in their samples. This would provide a physiological reason for why blubber PCB concentrations correlate with measures of CYP1A in liver (this study and White *et al.*, 1994). Lactation and starvation are clear conditions where equilibrium conditions may be disturbed. The animals in this study were robust, with thick blubber layers and no signs of starvation. Of the animals for which there is contaminant data, only one was a lactating female; measures of blubber contaminants may not be as relevant for comparison to organ CYP1A1 measures in this individual.

Conclusions

Sex does not appear to be an important determinant in CYP1A1 expression and/or activity as the values for both male and female samples fall on the same EROD activity - PCB curve. Similar conclusions were drawn from the CYP1A1 content – PCB curve with beluga (White *et al.*, 1994) and from immunohistochemical measures of CYP1A1 levels in bottlenose dolphin (Wilson *et al.*, 2007). Most animals sampled in this study were immature, which may be why the impact of sex on CYP1A1 expression or activity was negligible in this study. Furthermore, age was not a variable needed for the interpretation of CYP1A1 measurements. In contrast to other species including beluga, contaminant concentrations decrease with age in male white-sided dolphin (Tuerk, 2002) and yet the content of AHR agonists and levels of EROD activity are still positively correlated. If age was a factor, such positive correlations would not be expected in a species with an inverse age – contaminant relationship. Age also was not a factor for CYP1A1 measures in bottlenose dolphin (Wilson *et al.*, 2007). The influence of tissue autolysis, hormonal state or other factors cannot be excluded but in our data set, the contaminant concentrations appear to explain the levels of CYP1A1 expression.

Liver was the only internal organ with appreciable levels of EROD activity and in general, measures of CYP1A1 were low in white-sided dolphin compared to other cetacean species studied so far. This is in spite the relatively better quality of samples included in this study, suggesting that differences between species are likely larger. Yet, reductase and cytochrome b5 levels were similar to those reported in other cetaceans, suggesting that low EROD activity is reflective of CYP protein levels and not because of any insufficiency of co-enzyme/co-factor content in extrahepatic organs. Thus, liver seems to be the primary site of CYP1A1 expression in this species. EROD activity was not well correlated with CYP1A1 content, suggesting that other CYP enzymes may be contributing to this activity in the liver of white-sided dolphin.

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Figure 1. Ethoxyresorufin-O-deethylase activity versus the CYP1A1 content in microsomal samples

Panel A shows data from all organs of white-sided dolphin. Organs include adrenal, atrium, brain, heart, intestine, kidney, liver, lung, lymph node, mammary, muscle, ovary, pancreas, spleen, stomach, testis, thymus, uterus and ventricle. Panel B shows data from liver only. CYP1A1 content is relative to a scup standard (see materials and methods for details).

Figure 2. Relationship between blubber PCB concentrations and hepatic ethoxyresorufin-O-deethylase activity and CYP1A1 content in white-sided dolphin

Panel A shows total PCB concentrations, the sum of 56 PCB congeners and Panel B shows the Σ mono-ortho PCB concentrations, are the sum of 16 PCB congeners. Open symbols represent males; closed symbols represent females. Circles are for EROD activity; squares are for CYP1A1 content as determined by immunoblots. All PCB concentrations are in blubber of white-sided dolphin on a wet weight basis (see materials and methods for specific congeners, original data published in Tuerk *et al.*, 2005b). Ethoxyresorufin-O-deethylase activity, but not CYP1A1 content, was significantly correlated with both total ($r=0.74$, $p=0.05$) and Σ mono-ortho ($r=0.84$, $p=0.05$) PCB concentrations; please see Table 5 for more details of correlations between hepatic CYP1A1 measures and blubber contaminants.

Summary of Life History and Stranding Information for Dolphins Included in this Study.

Table 1

Dolphin	NIST Identification Number	Accession Number ¹	Sex	Length (cm) ²	Age (yr) ³	Sexual Maturity ^{4,9}	Euthanized ^{5,9}	Time from Death to Necropsy ⁶	Reproductive Status
97-228	NM7B135	MH-97-534	F	235	11.7 (NA) ⁸	Y	Y	10	Lactating
98-001	None ⁷	MH-98-414	F	227	9.0 (NA) ⁸	Y	U	24	Lactating
98-002	NM8B232	MH-98-415	F	182	2.1 (NA) ⁸	N	U	24	
99-014	NM10B104C	MH-99-537	F	187	2.5 (2)	N	N	24	
99-015	NM11B210C	MH-99-521	F	189	2.7 (2)	N	Y	12	
99-018	NM11B213C	MH-99-522	F	194	3.1 (2) ⁸	N	Y	12	
99-021	None ⁷	MH-99-563	F	180	2.0 (NA) ⁸	N	Y	12	
98-005	NM8B248	MH-98-456	M	230	7.7 (7)	Y	U	2	
99-013	NM10B101C	MH-99-536	M	205	4.4 (2)	N	N	24	
99-016	NM8B138	MH-99-520	M	243	11.5 (NA) ⁸	Y	Y	12	
99-017	NM10B107C	MH-99-519	M	225	6.6 (NA) ⁸	Y	U	12	
99-020	None ⁷	MH-99-549	M	204	3.4 (NA) ⁸	N	Y	3	

¹ Identification number given by the New England Aquarium;

² Total body length from snout to fluke notch,;

³ calculated by age-length relationships using data in Tuerk *et al* (2005b) (age determined by counting dentine growth layers, published in Tuerk *et al.*, 2005b);

⁴ based on total length;

⁵ Animals that were found alive and subsequently euthanized;

⁶ Animals with missing time of death data or were found dead were assigned the maximum possible time to necropsy (24 hours), actual time to necropsy is likely significantly less;

⁷ animals without blubber contaminant concentrations;

⁸ NA is not available;

⁹ N - no, Y - yes and U - unknown.

Table 2
Total P450, P420, Cytochrome b5 Content and NADPH Cytochrome P450 Reductase Activity in White-sided Dolphin Microsomes

Cytochrome b5, total P450, total P420 and relative P420 content were measured in liver microsomes. NADPH Cytochrome P450 reductase activity (reductase) was measured in liver, kidney and lung.

Study ID	Cytochrome b5 ¹	Total P450 ¹	Total P420 ¹	Relative P420/Content ²	Reductase ³		
					Liver	Kidney	Lung
97-228	0.25	0.09	0.51	0.85	159.4	102.7	104.3
98-001	0.11	0.18	0.16	0.47	220.6	133.7	122.8
98-002	0.07	0.14	0.29	0.67	234.5	119.3	121.8
98-005	0.05	0.10	0.13	0.55	233.1	152.1	131.3
99-013	NM ⁴	0.13	0.26	0.67	217.6	123.0	110.3
99-014	NM	0.16	0.20	0.55	119.1	133.9	111.9
99-015	NM	0.23	0.03	0.11	262.9	109.9	133.2
99-016	0.07	0.10	0.10	0.51	250.1	107.7	114.5
99-017	0.17	0.13	0.44	0.77	274.6	110.7	150.9
99-018	NM	0.22	1.61	0.88	404.3	110.1	122.1
99-020	0.11	0.05	0.28	0.86	NM	NM	NM
99-021	NM	1.91	0.53	0.22	NM	NM	NM

¹ nmol mg⁻¹;

² percent of total P420 and P450;

³ nmol mg protein⁻¹ min⁻¹,

⁴ NM is not measured in this sample

Table 3
Ethoxyresorufin-O-deethylase Activity and CYP1A1 Content in Liver Microsomes and Σ mono-ortho PCBs in blubber from White-sided dolphin

CYP1A1 content is measured relative to a scup standard (see material and methods for details). PCB concentrations are based on wet weight, in blubber (Tuerk *et al.*, 2005b).

Dolphin	Sex	Σ mono-ortho PCBs (ng g ⁻¹)	EROD Activity (pmoles mg ⁻¹ min ⁻¹)	CYP1A1 Content (pmoles mg ⁻¹)
97-228	F	1557	13.2	0.397
98-001	F	NA	9.2	0.567
98-002	F	3534	50.5	0.759
99-014	F	2852	96.6	0.680
99-015	F	3069	74.5	2.879
99-018	F	3469	376.0	1.638
99-021	F	NA	85.5	0.743
98-005	M	2136	37.7	0.689
99-013	M	4320	118.0	2.152
99-016	M	2940	42.6	0.249
99-017	M	4148	113.7	0.532
99-020	M	NA	66.5	0.410

Table 4

Mean CYP1A1 content (pmole mg⁻¹) and EROD activity (pmole mg⁻¹ min⁻¹) in Internal Organs of Male and Female White-sided dolphin

Mean values are reported with the standard deviation (SD) and N available for each organ. NA is not available, - is present for SD for groups where the N is 1.

	CYP1A1						EROD Activity					
	Female			Male			Female			Male		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
adrenal	0.66	0.26	7	0.66	0.25	2	1.59	0.40	7	0.62	0.33	2
atrium	0.62	0.39	3	NA		0	4.35	5.62	3	NA		0
brain	0.43	-	1	NA		0	0.51	-	1	NA		0
heart	NA		0	NA		0	2.04	1.08	4	1.32	0.32	2
intestine	1.25	0.92	5	0.57	0.46	2	1.48	1.03	5	4.80	6.49	2
kidney	0.64	0.32	7	0.46	0.43	3	2.10	1.28	7	2.84	1.70	3
liver	1.30	0.95	7	0.52	0.20	5	102.95	116.82	8	65.13	34.72	4
lung	4.76	8.03	7	0.86	0.60	3	3.04	2.56	7	2.78	3.06	3
lymph node	1.15	0.51	6	0.74	0.12	3	0.98	0.26	2	0.87	-	1
mammary	1.86	-	1	NA			0.83	-	1	NA		
muscle	1.22	-	1	NA		0	2.36	0.67	5	5.10	1.90	2
ovary	0.69	0.33	4	NA		0	3.16	3.14	4	NA		0
pancreas	1.06	0.90	4	0.74	0.76	2	1.28	0.78	4	1.70	0.98	2
spleen	1.78	1.81	7	1.64	1.22	3	1.66	1.06	7	1.52	1.00	3
stomach	0.91	0.32	3	1.96	0.29	2	0.74	0.02	2	6.78	7.21	2
testis	NA			1.04	0.17	2	NA			1.90	0.74	2
thymus	0.38	-	1	NA		0	6.97	-	1	NA		0
uterus	1.00	0.39	5	NA			3.64	4.91	5	NA		
ventricle	0.47	0.41	7	0.23	0.12	2	1.90	1.40	3	NA		0

Table 5
Correlation between CYP1A1 content or EROD activity in liver and contaminant concentrations in blubber of white-sided dolphin

Only contaminants that correlated with either CYP1A1 content or EROD activity are shown. The only individual PCB congeners tested were the mono-*ortho* PCBs. Correlations were performed without 99-018 included in the analysis. This sample had an EROD activity that was greater than three standard deviations above the mean. Correlation coefficients (r) are shown and significant correlations (p=0.05) are marked with a (*). Contaminant data was taken from Tuerk *et al.* (2005a,b).

Contaminant Concentrations	CYP1A1 Content	EROD Activity
Total PCBs	0.16	0.74 (*)
Σmono- <i>ortho</i> PCBs	0.35	0.84 (*)
<u>Select individual PCB congeners</u>		
tetrachlorobiphenyls –		
PCB66	0.58	0.71 (*)
PCB70 and PCB76 ¹	0.88 (*)	0.17
pentachlorobiphenyls –		
PCB107	0.44	0.73(*)
PCB123 ²	0.15	0.75(*)
hexachlorobiphenyls –		
PCB156 ³	0.74(*)	0.59
PCB157	0.93(*)	0.64
HCB	0.68	0.58
Heptachlor	0.41	0.71(*)
Total Toxaphene	0.53	0.81(*)

¹ PCB70 and PCB76 coeluted,

² PCB 123 coeluted with PCB149, a di-*ortho* substituted PCB,

³ PCB156 coeluted with PCB171 and PCB202, which have 3 and 4 *ortho* substitutions, respectively.