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Characterization and profiling of hepatic cytochromes P450 and phase II xenobiotic-metabolizing enzymes in beluga whales (*Delphinapterus leucas*) from the St. Lawrence River Estuary and the Canadian Arctic

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Abstract

Cytochromes P450 (CYP, phase I) and conjugating (phase II) enzymes can be induced by and influence the toxicokinetics (metabolism) and toxicity of xenobiotic contaminants in exposed organisms. Beluga whale (*Delphinapterus leucas*) from the endangered St. Lawrence (SL) River Estuary population exhibit deleterious health effects and various severe pathologies that have been associated with contaminant exposure. In contrast, such effects (e.g. reproductive and immunological impairment) are generally less frequent in less exposed populations in the Canadian Arctic (CA). In the present study, opportunistic sampling resulted in the collection immediately after death of liver tissue from a single female neonate SL beluga (SL6) and male and female CA beluga ($n = 10$) from the Arviat region of western Hudson Bay, in addition to sampling of stranded carcasses of male and female SL beluga ($n = 5$) at least 12 h postmortem. We immunologically characterized cross-reactive proteins of hepatic microsomal CYP1A, CYP2B, CYP3A, CYP2E, epoxide hydrolase (EH) and uridine diphosphoglucuronosyl transferase (UDPGT) isozymes. Cross-reactive proteins were found in all SL and CA beluga using anti-rat CYP1A1, anti-rainbow trout CYP3A, anti-human CYP2E1, anti-rabbit EH and anti-human UDPGT1A1 polyclonal antibodies (Abs), whereas faintly cross-reactive CYP2B proteins were only found in SL6 and the CA samples using an anti-rabbit CYP2B1 Ab. In corresponding catalytic activity assessments, only SL6 and all CA beluga microsomal samples exhibited CYP1A-mediated 7-ethoxyresorufin *O*-deethylase (EROD) activity (51–260 pmol/mg/min), CYP3A-mediated activity (113–899 pmol/mg/min) based on the formation of 6β-hydroxytestosterone using a testosterone hydroxylase assay, and UDPGT activity (830–4956 pmol/mg/min) based on 1-naphthylglucuronide formation. The marginal cross-reactivity with the anti-CYP2B1 Ab and lack of catalytically measurable

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hydroxytestosterone isomers associated with CYP2B-type activity in all the SL and CA animals is suggestive of low CYP2B-type enzyme expression in beluga. The absence of measurable total P450 enzyme levels and catalytic activities in samples from the stranded SL belugas suggested catalytically inactive enzymes as a consequence of tissue degradation related due to the time delay of sample collection after death. However, all SL and CA animals demonstrated similar, immunologically cross-reactive phase I and II hepatic enzyme profiles, which is suggestive of the importance of metabolism in the toxicokinetics and fate of xenobiotics in animals from both populations.

Keywords: Cytochrome P450; Epoxide hydrolase; UDPGT; Xenobiotic-metabolizing enzymes; St. Lawrence; Canadian Arctic; Beluga whale; *Delphinapterus leucas*

1. Introduction

Marine mammals are particularly susceptible to contamination by organohalogens due to their large lipid reserves, relatively long life spans and elevated positions in the aquatic food web. A small, isolated and endangered population of beluga whales (*Delphinapterus leucas*) inhabits the St. Lawrence (SL) Estuary in Quebec, Canada, an area downstream of many sources of industrial and agricultural contaminants. Polychlorinated biphenyls (PCBs), organochlorine pesticides and other organohalogen contaminants have been detected in SL beluga tissues (e.g. Martineau et al., 1988; Muir et al., 1996; Gauthier et al., 1998; Metcalfe et al., 1999; Hickie et al., 2000; Hobbs et al., 2003) not only at elevated levels in comparison to Canadian Arctic (CA) belugas (Norstrom and Muir, 1994; Metcalfe et al., 1999), but also at levels known to elicit toxic effects in laboratory animals (Béland et al., 1993). A link has been suggested between tissue organochlorine levels and the health of the SL population (Martineau et al., 1994). Reproductive and immunological impairment and a high incidence of neoplasms are all strongly associated with pollutant exposure (De Guise et al., 1995).

Metabolic capacity is an important determinant in the bioaccumulation, biomagnification, toxicokinetics and potential toxicity of lipophilic organohalogen contaminants. The cytochrome P450 monooxygenases (CYPs) play a central role in the oxidative biotransformation (phase I) of a wide range of xenobiotic and endogenous compounds (Goksøyr and Förlin, 1992; Stegeman and Hahn, 1994; Lewis et al., 1998). Products from phase I metabolism are conjugated to larger endogenous molecules via catalytic mediation by phase II enzymes such as glutathione-*S*-tranferase (GST) and uridine diphosphoglucuronosyl transferase (UDPGT) (George, 1994; Wolkers et al., 1998). Phase I and II enzymes, most abundant in hepatic tissues, may transform lipophilic compounds into either detoxified or bioactivated forms. As there are qualitative and quantitative differences in the levels and inducibility of individual CYP isozymes among species and populations of species (Boobis et al., 1990; Smith, 1991), it is important to determine the ability of exposed animals to metabolically influence the toxicokinetics and fate of accumulated anthropogenic contaminants. There is limited documentation on the metabolic capacity of marine mammals towards xenobiotic compounds, and most reports are from seal populations of various species (Boon et al., 1992; Nyman et al., 2001). However, immunochemical characterization of CYP isozymes has been reported for some whale species and populations, mainly for families from the odontocete suborder. White et al. (1994, 2000) found homologues of CYP1A, CYP2B and CYP2E in the liver of CA beluga, and a CYP1A isoform has been characterized in various tissues of SL beluga (Wilson et al., 2000). CYP1B, CYP3A-like and CYP4A isozymes have been discovered in striped dolphin (*Stenella coeruleoalba*), pilot whale (*Globicephala melas*), minke whale (*Balaenoptera acutorostrata*) and/or sperm whale (*Physeter macrocephalus*) (Goksøyr et al., 1988, 1989; Goksøyr, 1995; Celander et al., 2000; Godard et al., 2000; Boon et al., 2001). In beluga, catalytic characterization has been limited to CYP1A-mediated EROD, PROD, MROD (7-ethoxy-, pentoxy- and methoxyresorufin *O*-deethylase, respectively) and aryl hydrocarbon hydroxylase (AHH) activities (White et al., 1994, 2000; Addison et al., 1998). These studies rely on enzymatically-active hepatic tissues, but in

contrast to laboratory specimens, it is often difficult to obtain well-preserved, enzymatically-viable tissues from stranded free-ranging animals. Therefore, catalytic activity as a quantitative indicator of metabolic potential is questionable in situations where liver preservation cannot be assured. Immunological profiling is a more robust technique to measure phase I and II enzymes, providing suitably cross-reactive Ab interactions are found. On the other hand, immunochemical characterization is not necessarily fully representative of existing protein profiles as identification depends on the constitutive and induced levels, and on the cross-reactivity of the selected antibodies for specific microsomal proteins (Goksøyr, 1995; Letcher et al., 1996; Lewis, 2000).

To more completely determine the metabolic potential of beluga whale, a more comprehensive assessment is required of hepatic xenobiotic-metabolizing enzymes in individuals from populations of contrasting exposure to contaminants that are capable of enzyme induction or suppression. In the present study, major phase I and II isozymes, CYP1A, CYP2B, CYP3A, CYP2E, EH and UDPGT, known to be involved in xenobiotic metabolism, were immunologically and catalytically characterized in the hepatic microsomes of belugas from the SL (tissue collected from stranded individuals) and from the Arviat region of western Hudson Bay in the Canadian Arctic (fresh tissue collected as part of native subsistence hunting). The results were used to evaluate the use of immunologic expression and catalytic activity of xenobiotic-metabolizing enzymes in the optimally preserved liver of beluga from a CA population as a model of metabolic potential in SL and other beluga populations.

2. Materials and methods

2.1. Reagents

Bovine serum albumin (BSA), Coomassie Brilliant Blue G-250, potassium chloride, sodium dithionite, L-ascorbic acid, phenazine methosulfate, 7-ethoxyresorufin, resorufin, sodium dodecyl sulfate (SDS), bromophenol blue, 4-chloro-1-naphthol, Brij-58, 1-naphthol, 1-naphthyl β-D-glucuronide sodium salt and uridine 5′-diphosphoglucuronic acid trisodium salt were purchased from Sigma (St. Louis, MO, USA). Disodium ethylenediaminetetraacetate (Na_2EDTA), acrylamide/bisacrylamide solution (30%, w/v, monomer to cross-linker ratio of 37.5:1), ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), β-mercaptoethanol, glycine, polyoxyethylene sorbitan monolaurate (Tween-20) and gelatin were acquired from Bio-Rad Laboratories (Mississauga, Ont., Canada). Dithiothreitol (DTT), glycerol, 95% ethanol, 85% phosphoric acid, sodium dihydrogen phosphate, HPLC grade methanol, concentrated HCl, NaOH pellets and hydrogen peroxide (30%, w/v) were obtained from EM Science (Gibbstown, NJ, USA). HPLC-grade dichloromethane and acetonitrile were obtained from Merck (Darmstadt, Germany). Sodium chloride and β-nicotinamide adenine dinucleotide phosphate (NADPH) reduced, tetrasodium salt were acquired from Fluka (Buchs, SG, Switzerland). NADPH regenerating system solutions A ($NADP^+$, glucose-6-phosphate, $MgCl_2$) and B (glucose-6-phosphate dehydrogenase) were supplied by Gentest (Woburn, MA). Tris(hydroxymethyl)-aminomethane was purchased from Aldrich (Milwaukee, WI, USA). Dipotassium hydrogen orthophosphate (K_2HPO_4) was obtained from BDH Chemicals (Toronto, Ont., Canada). Carbon monoxide was procured from BOC (Mississauga, Ont., Canada). Testosterone, androstenedione, 2α-, 6α-, 6β-, 7α-, 11β-, 15α-, 16α- and 16β-hydroxytestosterone were obtained from Steraloids (Wilton, NH). Water was supplied by a Milli-Q system (Millipore, San Jose, CA) with a 0.22 μm filter.

The following primary polyclonal antibodies were used for immunochemical detection of phase I and II proteins: goat anti-rat CYP1A1 (1:1000 dilution, Gentest), rabbit anti-rat CYP2B1 (1:1000 dilution, Research Diagnostics, Flanders, NJ), rabbit anti-rainbow trout CYP3A *iso*-enzyme (1:1000 dilution, kindly donated by Dr. Malin Celander, University of Göteborg, Göteborg, Sweden), rabbit anti-human CYP2E1 (1:1000 dilution, Research Diagnostics), goat anti-rabbit EH (1:1000 dilution, Oxford Biomedical Research, Oxford, MI) and rabbit anti-human UDPGT1A1 (1:500 dilution, Gentest). The secondary antibodies were donkey anti-goat IgG (1:2500 dilution, Research Diagnostics) for CYP1A and EH and goat anti-rabbit IgG (1:3000 dilution, Bio-Rad Laboratories) for detection of all other proteins. A

pre-stained molecular weight standard was purchased from Bio-Rad Laboratories and a specific human UDPGT1A1 molecular weight standard was acquired from Gentest.

2.2. Sample collection

Liver tissues were obtained from five SL belugas stranded in the summers of 2000 and 2001. The samples, identified as SL1, SL2, SL3, SL4 and SL5, were excised 12–18 h after discovery of the carcass and immediately stored in liquid nitrogen. A sixth individual, SL6, was stranded alive in the summer of 2003 and had to be euthanized; hepatic tissue was taken within 15 min after death and frozen on dry ice for 2 days until it could be stored at −80 °C. Tissue degradation in these stranded SL animals is ranked by the extent of autolysis, which was measured histologically (Table 1). Autolysis was minimal in SL6 and mild in SL4; however, all other SL samples were moderately to severely autolysed. Liver tissues from ten CA belugas were obtained during native subsistence hunting in August of 2002 (CA1, CA2, CA3, CA4, CA5) and 2003 (CA6, CA7, CA8, CA9, CA10). All carcasses were sampled within 30–90 min of death, and liver samples were immediately transferred in the gas phase of a liquid nitrogen dry shipper. After transport, all samples were frozen at −80 °C. Biological and physiological details on the SL and CA individuals are provided in Table 1.

The age classification of the SL and CA belugas, regardless of precise age as in the case of the CA animals, was based on established practices. Based on data collected from two Arctic beluga populations, sexual maturity of beluga to adults occurs at approximately 7 years of age for females and 8 years of age for males as indicated by a transition of the skin color from grey to white (Hobbs et al., 2003). Therefore, SL1, SL3 and SL4 were juveniles and SL2 and SL5 were older adults. With the exception of CA2 and CA9 (juveniles) and CA4 (neonate), all the CA belugas were adults.

Table 1
Sample collection, biological and physiological data for beluga whale

(A) From the St. Lawrence River Estuary (Quebec)

Sample ID	Sex	Age (year)	Length (cm)	Weight (kg)	Extent of autolysis	Date stranded	Location stranded
SL1	F	4.5	316.0	625.0	Extreme	26 July 2000	Tadoussac
SL2	M	27	410.0	410.0	Extreme	29 July 2001	Ile au Caribou, Côte Nord
SL3	M	5.5	298.0	342.0	Moderate	28 September 2001	Ile Verte
SL4	F	3.5	300.0	334.5	Mild	2 October 2001	Pointe-au-Père
SL5	M	27.5	385.0	839.5	Marked	19 November 2001	Baie du HaHa, Bic
SL6	F	Neonate (≤2 days)	144.0	41.5	None	31 July 2003	Ste-Luce-sur-Mer

(B) From the Canadian Arctic (Nunavut)

Sample ID	Sex	Age[a]	Length (cm)	Weight (kg)	Date collected	Location collected
CA1	F	Adult	ND	ND	11 August 2002	Arviat, w. Hudson Bay
CA2	F	Juvenile	ND	ND	11 August 2002	Arviat, w. Hudson Bay
CA3	F	Adult	ND	ND	12 August 2002	Arviat, w. Hudson Bay
CA4	F	Neonate	ND	ND	12 August 2002	Arviat, w. Hudson Bay
CA5	F	Adult	ND	ND	12 August 2002	Arviat, w. Hudson Bay
CA6	F	Adult	ND	ND	6 August 2003	Arviat, w. Hudson Bay
CA7	F	Adult	ND	ND	6 August 2003	Arviat, w. Hudson Bay
CA8	M	Adult	ND	ND	6 August 2003	Arviat, w. Hudson Bay
CA9	M	Juvenile	ND	ND	8 August 2003	Arviat, w. Hudson Bay
CA10	M	Adult	ND	ND	9 August 2003	Arviat, w. Hudson Bay

ND: not determined.

[a] Estimated from skin color (see Section 2).

2.3. Preparation of hepatic microsomes

Microsomes were prepared by differential ultracentrifugation as described by Nilsen et al. (1998). Briefly, liver tissues were thawed on ice. Three volumes (liver weight per volume) of buffer (0.1 M K_2HPO_4, 0.15 M KCl, 1 mM Na_2EDTA, 1 mM DTT, 20% glycerol, pH 7.4) were added and the mixture was homogenized with an Ultra-Turrex. The homogenate was centrifuged at 12,500 × *g* (20 min, 4 °C). The supernatant was centrifuged at 100,000 × *g* (60 min, 4 °C). The pellet was resuspended in one volume of buffer (0.1 M K_2HPO_4, 1 mM Na_2EDTA, 1 mM DTT, 20% glycerol, pH 7.4) and stored at −80 °C.

2.4. Microsomal protein and total cytochrome P450 content

Microsomal protein content was determined by the spectrophotometric method of Bradford (1976) using BSA as the standard (0.2–1.0 mg/ml). Absorbance was measured using a Dynatech Labs MRX UV-Vis microplate reader equipped with a 570 ± 5 nm filter. Total CYP content was initially assessed by the dithionite-difference spectra of carbon monoxide-treated microsomes (Omura and Sato, 1964) using a Cary 300 Bio dual-beam UV-Vis spectrophotometer. However, a large peak at 420 nm suggested possible CYP degradation or hemoglobin and methemoglobin contamination. Treatment with ascorbic acid and phenazine methosulfate allowed for measurement of cytochrome P450 content in the presence of these contaminating proteins (Johannesen and DePierre, 1978).

2.5. Catalytic assays

The 7-ethoxyresorufin-*O*-deethylase (EROD) activity was assessed using the fluorescence microplate assay of Eggens and Galgani (1992). EROD activity in the beluga microsomes was measured under optimized assay conditions (e.g. pH 8.0 and 37 °C) based on previously published procedures (White et al., 1994, 2000). Testosterone hydroxylase activity was determined as per the method of Li and Letcher (2002) including the use of 6α-hydroxytestosterone as internal standard, with a few exceptions. The buffer was set to pH 8.0 and the incubation mixtures were pre-incubated for 5 min at 37 °C. At time $t = 0$ min, one addition of NADPH regenerating system was added (50 μl solution A, 10 μl solution B). Testosterone metabolism was analyzed using a Waters 2695 Alliance HPLC system equipped with a Zorbax Eclipse XDB-C18 column (5 μm, 250 mm × 4.6 mm i.d.) and a Waters 2487 UV-Vis detector. Phase II glucuronidation was assessed using 1-naphthol as the substrate (Wolkers et al., 1998). In a total reaction volume of 200 μl, 100 mM phosphate buffer (pH 8.0, 3 mM $MgCl_2$), 250 μM 1-naphthol, 1 mg/ml microsomes and Brij-58 (1 mg/mg protein) were pre-incubated for 5 min at 37 °C. The reaction was initiated by UDP-glucuronic acid (3 mM) and was terminated by cold acetonitrile (200 μl) after 20 min. Mixtures were vortexed, left on ice for 30 min, then centrifuged (2000 rpm, 10 min). The supernatent was filtered through a 0.45 μm syringe filter and 5 μl was analyzed by HPLC using a Jasco FP-920 fluorescence detector ($\lambda_{ex} = 290$ nm, $\lambda_{em} = 330$ nm). A 200 μM 1-naphthylglucuronide (1-NG) standard was prepared in methanol, and calibration curve standards were prepared by serial dilution. 1-NG was eluted with 10 mM NaH_2PO_4 (pH 2.5, 17.5% acetonitrile) (Wortelboer et al., 1992). The flow rate was set to 2.0 ml/min for a total run time of 20 min (retention time of 1-NG was 15.4 min).

2.6. Immunochemical analysis

Microsomal proteins (45 μg per well) were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and electroblotted from the gel onto a nitrocellulose membrane (Towbin et al., 1979). Proceeding application of the primary antibody, an appropriate horseradish peroxidase (HRP)-labeled secondary antibody was incubated with the membranes, followed by an HRP-color reagent (4-chloro-1-naphthol plus hydrogen peroxide solution) to visualize the cross-reactive bands. Molecular weights were approximated by comparison with a simultaneously run pre-stained molecular weight standard (5 μl in well).

2.7. Data analysis

The precision of all assays was assessed by intraday ($n \geq 3$) replicate analysis for each individual beluga. It was not possible to assess the statistical significance

of the differences in specific catalytic activities for the CA and SL animals as a function of sex or age due to the small number of individual belugas and the inherent difficulty and cost in obtaining samples. In the case of the SL animals, catalytic activities were generally not detectable. However, among adult CA animals it was possible to statistically assess the significance of the simple linear regression between pairings of EROD, testosterone hydroxylase and UDPGT activities. All hypotheses tested were two-tailed and were based on a maximum probability of a type-I error (α) that was set at 0.05 ($P < 0.05$).

3. Results

3.1. Microsomal protein and total CYP content

Microsomal protein and total CYP contents of the beluga liver samples are given in Table 2. Dithionite difference spectra of SL1 to SL5 showed a peak at 420 nm, but not at 450 nm, even when treated with ascorbic acid and phenazine methosulfate (PMS) to remove the interfering CO-hemoglobin peak at 420 nm. Spectra of SL6 and the CA individuals showed a large 420 nm peak and no detectable 450 nm peak using the conditions of Omura and Sato (1964). However, when the SL6 and CA1 to CA10 microsomes were treated with ascorbic acid and PMS, a clear P450 peak was revealed and detected (spectra not shown).

3.2. Phase I and II enzyme activities

Microsomal CYP1A activities, as measured by EROD activity, were not detectable for the SL1, SL2, SL3 and SL5 belugas, whereas activity in the SL4 animal was low but measurable (12 ± 1 pmol/mg/min). In contrast, the freshly sampled SL6 neonate had a mean EROD activity of 87 ± 15 pmol/mg/min. The average EROD rates in the hepatic microsomes of the CA individuals were 68 ± 34 pmol/mg/min in the three juveniles, 73 ± 30 pmol/mg/min in the five adult females, and highest at 175 ± 85 pmol/mg/min in the two adult males (Table 2).

Fig. 1 presents the measurable testosterone hydroxylase activities in the hepatic microsomes. The major hydroxytestosterone metabolite formed in all of the CA microsomes, and in SL6, was 6β-hydroxytestosterone. In SL6, the mean rate of 6β-testosterone hydroxylase was 351 ± 42 pmol/mg/min, and was 343 ± 235, 613 ± 304, 450 ± 115 pmol/mg/min in the CA juveniles, females and males, respectively.

Table 2
Microsomal protein content, CYP content and monooxygenase activity in the livers of stranded St. Lawrence and captured Canadian Arctic beluga whale

Sample ID	Microsomal yield (mg microsomal protein/g tissue)	Total CYP[a] (pmol/mg protein)	EROD (pmol/mg protein/min)
SL1	2.9 ± 0.1	ND	ND
SL2	1.6 ± 0.1	ND	ND
SL3	2.9 ± 0.4	ND	ND
SL4	6.3 ± 0.2	ND	12 ± 1
SL5	3.1 ± 0.4	ND	ND
SL6	8.4 ± 0.3	170	87 ± 15
CA1	11.0 ± 2.0	180	107 ± 6
CA2	13.0 ± 1.0	140	51 ± 3
CA3	11.2 ± 0.4	130	61 ± 2
CA4	16.0 ± 2.0	130	107 ± 10
CA5	8.2 ± 0.5	60	33 ± 2
CA6	7.7 ± 0.1	170	99 ± 5
CA7	6.7 ± 0.2	80	66 ± 10
CA8	8.1 ± 0.2	180	90 ± 13
CA9	6.3 ± 0.2	90	47 ± 10
CA10	5.5 ± 0.1	240	260 ± 20

Values are mean ± S.D. ($n \geq 3$ replicates). ND: not detectable.
[a] Replicate analyses were not possible due to limited available tissue (microsomes).

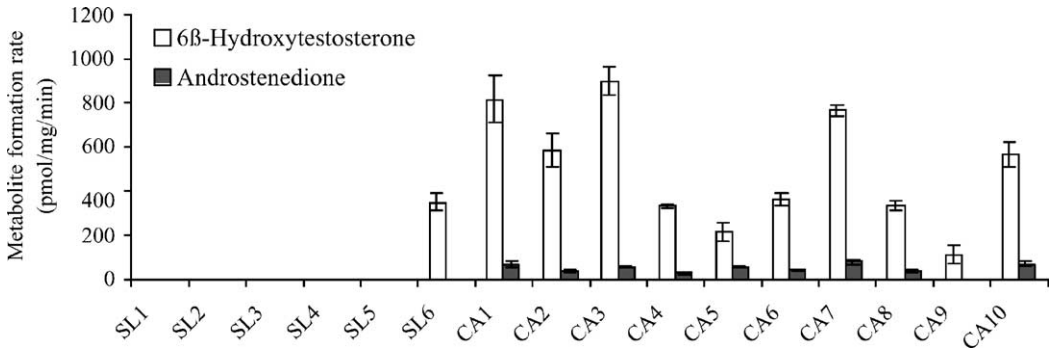

Fig. 1. Testosterone metabolite formation in the hepatic microsomes of St. Lawrence River Estuary and Canadian Arctic beluga whales. Microsomes (1 mg/ml) were incubated with 250 μM testosterone at 37 °C in PBS (100 mM, pH 8.0, 3 mM $MgCl_2$, 1 mM EDTA), and the reaction was initiated by the addition of NADPH regenerating solution. Each bar is the mean of $n = 3$ replicates. Error bars represent the standard deviation.

The other minor metabolite detected in 9 of the 10 CA assay samples was identified as androstenedione, whereas 2α-, 6α-, 7α-, 15α-, 16α- and 16β-hydroxytestosterone metabolites were not detectable. No measurable hydroxytestosterone metabolite formation was found for SL1 to SL5. There was no significant correlation between the rates of 6β-hydroxytestosterone formation ($P < 0.05$, $r^2 = 0.02$, $n = 10$ samples) or androstenedione formation ($P < 0.05$, $r^2 = 0.07$, $n = 9$ samples) with CYP1A-mediated EROD activity in the CA animals. However, there was a significant positive correlation ($P < 0.05$, $r^2 = 0.35$, $n = 9$) between the rates of 6β-hydroxytestosterone and androstenedione formation in the CA belugas.

UDPGT activity in the beluga microsomes as measured by the rate of 1-NG formation is given in Fig. 2. The formation rate of 1-NG was comparable for the CA juveniles, adult females and adult males, at values of 1922 ± 1063, 3076 ± 1237 and 2791 ± 1102 pmol/mg/min, respectively. For SL6, the 1-NG formation rate was 830 ± 244 pmol/mg/min.

3.3. Immunodetection of phase I and II proteins

Western blotting of the microsomal fractions with the anti-rat CYP1A1 antibody revealed two distinct cross-reactive protein bands (around 50 and 53 kDa) in all the SL and CA samples comparable to the characteristic molecular weight range for CYP proteins.

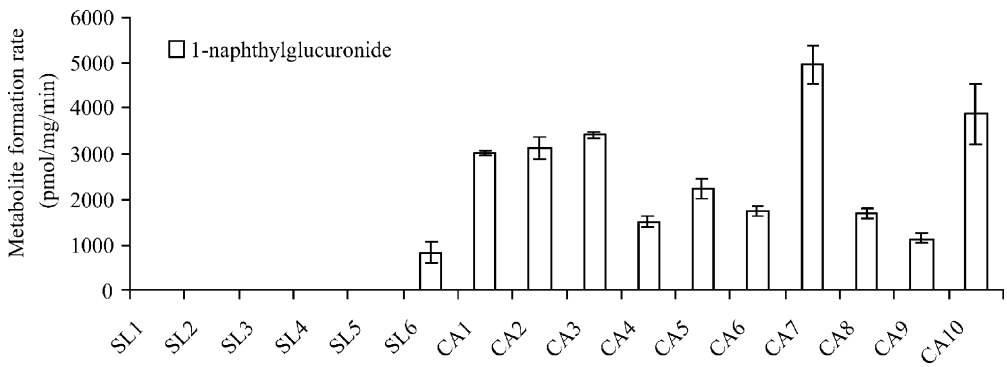

Fig. 2. Formation of 1-naphthylglucuronide in the hepatic microsomes of St. Lawrence River Estuary and Canadian Arctic beluga whales. Microsomes (1 mg/ml) were incubated with 250 μM 1-naphthol and 1 mg/mg (protein) Brij-58 at 37 °C in PBS (100 mM, pH 8.0, 3 mM $MgCl_2$). The reaction was initiated by the addition of UDP-glucuronic acid (3 mM). Each bar is the mean of $n = 3$ replicates, and error bars indicate the standard deviation.

(Fig. 3A). Using the CYP2B1 Ab, two weak bands at 48 and 55 kDa were seen for SL6 and the CA beluga (Fig. 3B). A band cross-reactive with anti-rainbow trout P450con (a putative CYP3A homologue) was visible at a molecular weight of approximately 52 kDa (Fig. 3C) in the SL and CA samples. Blotting with anti-CYP2E resulted in one distinct band for all samples at a molecular weight near 53 kDa (Fig. 3D). Addition of anti-rabbit EH resulted in a strong band at around 52 kDa in the SL and CA belugas (Fig. 3E).

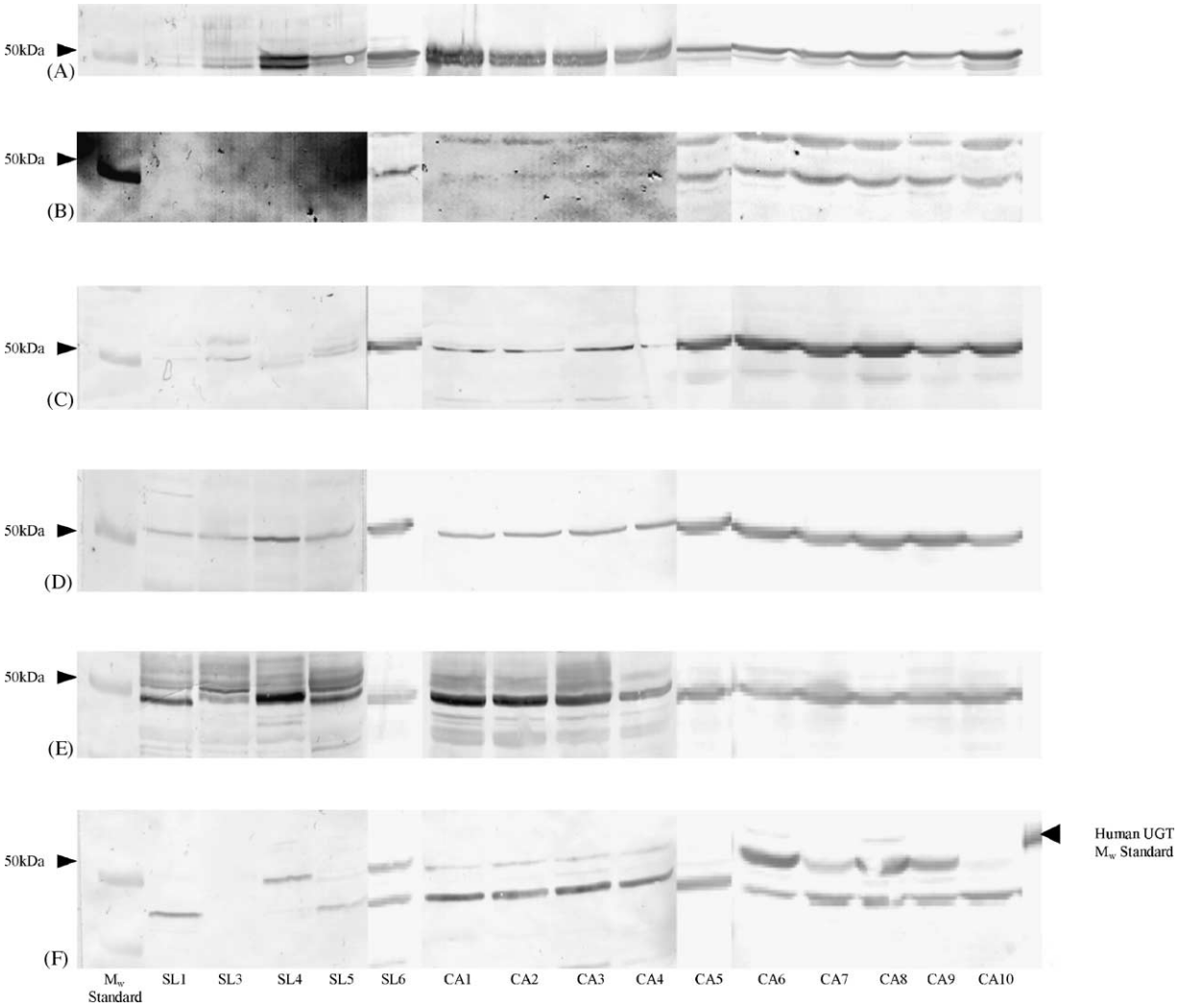

Fig. 3. Immunoblot of St. Lawrence River Estuary and Canadian Arctic beluga whale hepatic microsomes cross-reacted with (A) goat anti-rat CYP1A1 polyclonal antibody (PAb) at a 1:1000 dilution; (B) rabbit anti-rat CYP2B1 PAb at a 1:1000 dilution; (C) rabbit anti-rainbow trout CYP3A PAb at a 1:1000 dilution; (D) rabbit anti-human CYP2E1 PAb at a 1:1000 dilution; (E) goat anti-rabbit EH PAb at a 1:1000 dilution; (F) a rabbit anti-human UDPGT antibody at a 1:500 dilution. An HRP-conjugated donkey anti-goat secondary antibody at a 1:2500 dilution was used to visualize CYP1A- and EH-type protein bands. An HRP-conjugated goat anti-rabbit secondary antibody at a dilution of 1:3000 was applied to visualize CYP2B-, CYP3A-, CYP2E- and UDPGT-type bands. All sample wells were loaded with 45 μg of beluga hepatic microsomal protein. The first lane was loaded with 5 μl of molecular weight standard (M_w Std.). All membranes were scanned at the same resolution and exposure except for the CYP2B membranes, which were scanned at a higher exposure to ameliorate visualization of faint cross-reactive CYP2B-type bands.

Detectable cross-reaction with anti-human UDPGT1A was seen for SL4, SL5 and SL6 (Fig. 3F). The mobility of these approximately 50 kDa bands was comparable to that of a concurrently run human UDPGT1A molecular weight standard (about 56 kDa, Fig. 3F). In SL1, SL5 and SL6 a cross-reactive band was seen at around 45 kDa. Further investigation is required to identify this protein, as the mobility is within the appropriate range of a UDPGT protein. All ten of the CA samples exhibited both of the 45 and 50 kDa bands.

Two general points regarding the blots should be noted. Firstly, the CA beluga protein bands were generally more intense (except in comparison to SL4 and SL6) and the intensities varied less between individuals than the band intensities of the SL individuals. Secondly, although putative P450 cross-reactive protein bands in the MW range for CYP proteins were detected in all CA lanes, often proteins in only a few of the SL samples exhibited cross-reactivity when incubated with the same antibodies under identical immunoblotting conditions. It should also be noted that samples for each of Fig. 3A–F were not all run in the same gel (due to a limited number of lanes in each gel) or in gels with the same lane width (i.e. 5 lanes versus 10 lanes). Therefore, membranes in the figure were scaled as closely as possible to the same size, and bands from each population were grouped together for clarity. It may thus appear that there are variations in band mobilities; however, the band mobilities were calculated using the molecular weight standard run with every gel (not all shown in Fig. 3) and showed no major differences when comparing the same bands run on different gels.

4. Discussion

Although biotransformation is often a major factor in the toxicokinetics and fate of organic contaminants, little is known regarding characterization and catalytic activity of phase I and II xenobiotic-metabolizing enzymes in marine mammals. A greater understanding of metabolic potential is particularly important in marine mammals that are exposed to high levels of contaminants, such as those living in the St. Lawrence River Estuary. In this study, antibodies against different phase I and II isozymes showed cross-reactivity with homologous proteins in beluga liver microsomes. EROD, testosterone hydroxylase and UDPGT catalytic activities measured mainly in the CA animals were consistent with the immunologically characterized enzyme proteins. Our study reports for the first time in any cetacean species the catalytic and immunochemical detection of hepatic UDPGT and the immunochemical detection of a hepatic EH protein. Despite the specificity of the different antibodies toward the respective proteins, there were artifactual and non-specific cross-reactivities of some antibodies due to the less specific nature of polyclonal antibodies (Luzio and Jackson, 1988).

4.1. CYP1A

The expression of CYP1A is highly inducible across taxa, including marine mammals, via an aryl hydrocarbon receptor-mediated mechanism as a consequence of exposure to AhR-active substrates such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) and other planar, dioxin-like contaminants (e.g. benzo[*a*]pyrene and non-*ortho*-chlorinated PCBs). Beluga whales from SL and Arctic populations are exposed to contrasting levels and patterns of AhR agonists (Metcalfe et al., 1999). However, an immunologic comparison of CYP1A in individual belugas from SL and CA populations has not been reported. Previously, the only published study on hepatic CYP enzymes in beluga whale (White et al., 1994) revealed the presence of one CYP1A band at 53 kDa using a monoclonal anti-scup antibody and a polyclonal anti-mouse antibody that is cross-reactive with CYP1A1 and 1A2 in mice and rats. Using the anti-rat antibody, which recognizes both rat CYP1A1 and 1A2, two distinct bands were detected in both the SL and CA samples. We postulate that the 53 kDa band is a CYP1A1 homologue based on electrophoretic migration (White et al., 1994). The 50 kDa band may suggest the presence of a CYP1A2 homologue, according to previous studies of CYP1A1 and 1A2 type proteins in other marine mammals using polyclonal anti-CYP1A1 antibodies. In 1998, Goksøyr et al. using an anti-cod CYP1A1, reported two bands at 54 and 51–52 kDa in minke whales which were thought to be CYP1A1 and CYP1A2-like. Bandiera et al. (1995) reported the cross-reactivity of a polyclonal anti-rat CYP1A1 antibody with two separate

proteins in polar bear microsomes, which the authors suggested were CYP1A1 and CYP1A2. The anti-rat CYP1A1 we used cross-reacts with rat CYP1A1 (52 kDa) and 1A2 (50 kDa). White et al. (1994) had previously suggested that expression of CYP1A2 may have been too low to detect or that the enzyme did not exist in the individual beluga (i.e. from the population in the MacKenzie River Estuary) that were examined. A further explanation may be that the affinity of the anti-mouse antibody for the CYP1A2 protein in the liver of the MacKenzie River beluga was less than that of the anti-rat antibody used in the present study. The presence of a beluga protein similar to rat CYP1A2, nonetheless, can only be suggested by our data and indicates the need for further investigation.

We measured similar EROD activity levels in the CA belugas as was reported in the MacKenzie River beluga collected in 1989 (White et al., 1994). Histological examination revealed that SL4 was the least autolyzed of the dead stranded SL beluga. Although the EROD activity was low, the Ab cross-reactivity of CYP1A proteins in SL4 was most intense of all the animals under study, despite the apparently poorer tissue preservation relative to the CA animals. This suggests that if appropriately preserved adult liver tissue could be obtained, higher expression of CYP1A protein would be observed, consistent with higher levels of AhR agonist exposure in SL beluga than in the CA belugas.

4.2. CYP2B

Isozymes of the CYP2, CYP3 and CYP4 subfamilies are also inducible in mammals (Lewis and Lake, 1997; Lewis, 2000). In polar bear, the expression of CYP2B1 cross-reactive protein was highly correlated with levels of *ortho*-chlorine substituted PCBs and chlordane pesticides (Bandiera et al., 1995; Letcher et al., 1996). However, immunochemical assessment of the expression of CYPs other than CYP1A is rare in cetaceans, and comparisons among individuals from different populations are not known (White et al., 1994, 2000; Boon et al., 2001). CYP2B substrates are usually nonplanar, lipophilic compounds (Lewis et al., 1998). It has been postulated that marine mammals are basically deficient in CYP2B-associated proteins (Goksøyr, 1995). Until recently, there has been little immunochemical evidence of CYP2B proteins in cetaceans. For example, White et al. (1994) did not detect bands using polyclonal and monoclonal anti-rat antibodies against CYP2B, nor with a putative anti-scup CYP2B antibody. Cross-reactive bands were, however, visible using anti-rabbit CYP2B4 and anti-dog CYP2B11 polyclonal antibodies. Little to no CYP2B-like protein expression has been reported for other cetaceans. Goksøyr (1995) found low cross-reactivity in minke whale using the Amersham ECL CYP2B1/2 kit. Boon et al. (2001) did not detect a cross-reactive protein with a monoclonal anti-rat CYP2B1/2 in the liver of sperm whale (*Physeter macrocephalus*). The polyclonal anti-rat CYP2B1 antibody used in the present study specifically recognizes rat CYP2B1 and CYP2B2. Two weakly cross-reactive bands were observed in SL6 and in CA1-10. We conclude from the electrophoretic migration that the two bands represent CYP2B-type proteins.

In this study, major metabolites associated with CYP2B activity in mammals, 16α- and 16β-hydroxytestosterone (Arlotto et al., 1991), were not detected in any of the beluga samples using the testosterone hydroxylase assay. However, androstenedione formation was measurable in the CA animals, and is also known to be catalyzed in part by CYP2B1 in rats (Arlotto et al., 1991). It is also possible that for beluga whale in general, testosterone is a poorer substrate for CYP2B-like enzymes. Evidence supporting the existence of CYP2B-type catalytic activity in SL and CA beluga whales comes from the fact that animals from both populations are capable of metabolizing *ortho*-chlorine substituted PCBs, known to be CYP2B1 substrates in rodents (McFarland and Clarke, 1989), leading to the formation of methylsulfonyl- ($MeSO_2$-) PCBs (Letcher et al., 2000b). However, it is difficult to conclude that $MeSO_2$-PCB and $MeSO_2$-DDE formation is contingent only on CYP2B-like mediation of precursor PCB metabolism. In seals, for example, other CYP isozymes (e.g. CYP3A) are involved in the PCB arene oxide formation from *ortho*-chlorine substituted PCB congeners (Li et al., 2003).

4.3. CYP3A

CYP3A is a constitutive P450 that metabolizes steroid hormones, but it is also associated with the biotransformation of xenobiotics such as the chlorobor-

nanes and PCBs in seals (van Hezik et al., 2001; Li et al., 2003). Immunoblots with anti-P450con identified a single CYP3A-like protein in SL and CA beluga liver microsomes. Recently, Celander et al. (2000) confirmed the presence (particularly in liver) of CYP3A-like enzymes in various tissues of four pilot whales by immunochemical methods. Testosterone hydroxylase activities and Western blotting have also implicated the existence of CYP3A-like enzymes in minke whale (Goksøyr et al., 1988, 1989). The molecular weight of the CYP3A-type protein found in our beluga study, 52 kDa, compares favorably with the immunological results for other cetacean species. 6β-Hydroxytestosterone formation is a marker of CYP3A activity in mammals, and in all CA and SL6 microsomes the major conversion of testosterone was to 6β-hydroxytestosterone. A significant correlation between 6β-hydroxytestosterone and androstenedione formation would suggest that CYP3A mediates, at least in part, the formation of androstenedione in beluga whales.

4.4. CYP2E

CYP2E enzymes metabolize substrates of relatively low molecular weight and diverse structure (Lewis et al., 1998) and are associated with xenobiotic metabolism (Peter et al., 1990). The rabbit anti-human CYP2E1 antibody used in this study is reactive towards CYP2E1 in humans and CYP2E proteins in rat, rabbit and hamster hepatic microsomal fractions. A hepatic CYP2E-like protein detected in the SL and CA animals is in agreement with the previous immunochemical findings in the liver of MacKenzie River delta beluga (White et al., 1994).

4.5. Epoxide hydrolase

Currently, no literature has been published on the existence of EH in either cetaceans or pinnipeds, although Bandiera et al. (1995) immunochemically characterized this enzyme in polar bear, which is at the top of the arctic marine food web. EH is an important enzyme in the formation of hydroxylated (HO) metabolites of halogenated aromatic contaminants such as PCBs (Letcher et al., 2000a). HO-PCBs and HO-polybrominated diphenyl ethers (PBDEs) are generally thought to be formed via CYP-mediated formation of arene epoxide intermediates or possibly via direct aromatic hydroxylation of PCB and PBDE congeners (Letcher et al., 2000a; Hakk and Letcher, 2003). The only known report of HO-PCBs and HO-PBDEs in a cetacean species was in the blood of killer whale (*Orcinus orca*) fed a diet of wild Pacific herring (*Clupea pallasi*) (Bennett et al., 2002). Although a constitutive enzyme, EH exhibits moderate induction by certain anthropogenic compounds, such as Aroclor 1254 in humans (Hassett et al., 1998). The presence of an EH protein similar to that found in rabbit is indicated by the immunoblots of all SL and CA beluga samples. To our knowledge this is the only study on EH in marine mammals other than polar bear (Bandiera et al., 1995); the importance of EHs in xenobiotic metabolism calls for further investigation of these enzymes.

4.6. UDP-glucuronosyltransferase

Limited knowledge exists regarding phase II enzymes in belugas and in marine mammals in general. $MeSO_2$-PCB and -DDE metabolites found in the adipose tissue of SL and western Hudson Bay beluga is chemical marker evidence of phase II glutathione conjugation with *meta–para*-arene oxide intermediates formed via CYP enzyme-mediation from precursor PCBs (Letcher et al., 2000b). Goksøyr et al. (1988) found UDPGT catalytic activities of 640 and 55.4 pmol/mg/min towards 4-nitrophenol and testosterone metabolism, respectively, in minke whale. Wolkers et al. (1998) characterized UDPGT activity in ringed seal (*Phoca hispida*) hepatic microsomes by measuring the glucuronidation activity towards 1-naphthol metabolism. We found very similar 1-naphthylglucuronide formation rates in the CA belugas. The UDPGT activity of the neonate, SL6, was within the range of activities found in the juvenile CA belugas. Western blotting showed a UDPGT-type protein in three of the SL belugas and all of the CA belugas we studied, with electrophoretic migrations comparable to that of a human molecular weight standard. The molecular weights of UDPGT in the samples and the standard are not expected to be identical due to potential differences in glycosylation of the proteins (Gentest).

A number of variables, such as enzyme induction or suppression, nutritional and reproductive status, age,

sex and genetic variation, may account for differences in protein expression and catalytic activity (and thus, contaminant biotransformation capacity) among individual belugas and populations. Another factor may be sample preservation, especially in marine mammal research where it is very difficult to obtain fresh tissues from stranded individuals (Moore and Stegeman, 1996). Given the ∼500 km coastline of the SL estuary where standings of endangered beluga generally occur, and the large distances carcasses must be transported before invasive sampling can be performed, it is extremely rare that enzymatically viable tissues can be obtained. From a sampling perspective, we were thus fortunate to come across SL6, which was found stranded alive, and also to obtain fresh CA tissues. For SL1 to SL5, questionable tissue preservation compromised the immunochemical and catalytic integrity of the phase I and II enzymes in these belugas. Loss of the heme group (as indicated by the absence of total P450) and loss of binding site integrity may partially explain the loss of catalytic activity. Generally less intense bands than those of the CA samples (or undetectable bands in the case of CYP2B) in the immunochemical analysis likely reflects loss of epitopic integrity, resulting in less cross-reactivity with the chosen antibodies. Preliminary examination (based on tissue firmness, i.e. less firm and more "pudding-like" in appearance suggested more degradation) of the SL1 to SL5 livers did not indicate comparatively better preservation of SL4 hepatic tissues, which showed generally darker immunoblot bands and measurable EROD activity. However, SL4 microsomes were an orange–yellow color comparable to the SL6 and CA microsomes, whereas microsomes from the other SL samples were dark green. Additionally, histopathological examination indicated that SL4 hepatic tissues were much less autolysed than tissues from the other dead stranded SL belugas. Despite the tissue degradation, immunochemical analysis still proved useful, regarding qualitative information on phase I and II proteins present in SL1 to SL5.

The acquisition of tissue in the form of skin/blubber biopsies from free-ranging live SL belugas may be a non-lethal alternative to the use of extracted tissues from SL beluga carcasses (Hobbs et al., 2003) for measuring physiological, biochemical, genetic and expression differences between the SL and other beluga populations. Gauthier et al. (1999) recently reported the successful culturing of skin fibroblasts from arctic beluga whale, which are likely to have retained enzymatically viable CYP enzymes. CYP activity has also been measured in skin biopsies of other marine mammals (*Stenella coeruleoabla*, *Balaenoptera physalus*) (Fossi et al., 1999). However, MFO activity was only assessed through EROD, PROD, BROD and AHH assays, all of which are mediated mainly by CYP1A in marine mammals (Watanabe et al., 1989; Letcher et al., 1996; Addison et al., 1998). It remains to be demonstrated whether other xenobiotic-metabolizing enzyme activities in beluga skin or other nondestructively sampled tissues would be a useful alternative to liver in studies of metabolic potential. Here, we have been able to report the presence of phase I and UDPGT enzymes in the main organ involved in detoxification/biotransformation. These results provide the rationale for future investigation into the presence of these enzymes in alternate, nondestructively sampled tissues, and thus for the use of such tissues in biotransformation and biomarker studies in beluga whales.

5. Conclusions

We report on the detailed immunochemical and catalytic profiling of the enzymes mediating xenobiotic-metabolism in beluga whales from more than one distinct population. Immunochemical results indicated that profiles of several major CYP enzymes, EH and UDPGT are similar between animals from the two populations, and for certain enzymes are comparable with previous hepatic enzyme characterization in beluga and other odontocete species. For the first time, proteins homologous to CYP3A, EH and UDPGT were identified in beluga whales. Hydroxytestosterone and 1-naphthylglucuronide formation in adequately preserved tissues from individuals from both populations indicate the presence of CYP3A-type and UDPGT activities, respectively, in addition to CYP1A catalytic activity. Although questionable preservation of most of the SL samples lead to virtually undetectable catalytic activity, immunochemical protein profiling was still possible. We have shown that CA beluga have the potential to act as models for contaminant biotransformation studies on SL beluga, which will help to explain the potential impact of specific organic pollutants on the endangered SL popula-

tion, or in contaminant-exposed animals from other populations. Freshly sampled CA hepatic tissues are currently being used as a model in vitro metabolism system to assess biotransformation of organohalogen contaminants in beluga whales.

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