CYTOCHROME P450 ISOZYME PROTEIN VERIFIED IN THE SKIN OF SOUTHERN HEMISPHERE HUMPBACK WHALES (*Megaptera novaeangliae*); IMPLICATIONS FOR BIOCHEMICAL BIOMARKER ASSESSMENT

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Abstract

Large mysticete whales represent a unique challenge for chemical risk assessment. Few epidemiological investigations are possible due to the low incidence of adult stranding events. Similarly their often extreme life-history adaptations of prolonged migration and fasting challenge exposure assumptions. Molecular biomarkers offer the potential to complement information yielded through tissue chemical analysis, as well as providing evidence of a molecular response to chemical exposure. In this study we confirm the presence of cytochrome P450 reductase (CPR) and cytochrome P450 isoenzyme 1A1 (CYP1A1) in epidermal tissue of southern hemisphere humpback whales (*Megaptera novaeangliae*). The detection of CYP1A1 in the integument of the humpback whale affords the opportunity for further quantitative non-destructive investigations of enzyme activity as a function of chemical stress.

Keywords: Humpback whale; CYP1A1; Biomarkers; Western blot

Introduction

Persistent Organic Pollutants (POPs) are toxic anthropogenic chemicals and ubiquitous environmental contaminants. They are characterized by extremely long half-lives, bioaccumulation potential, and their capacity for undergoing Long Range Environmental Transport (LRET) hereby appearing in remote locations far from emission sources. POPs are subject to the Stockholm Convention, a legally binding treaty to ratifying nations that aims to reduce and ultimately eliminate sources of these chemicals to the environment (UNEP, 2002). The first generation of POPs originally listed under the convention were lipophilic, chlorinated aromatic compounds, such as organochlorine (OC) pesticides. Frequently semi-volatile, these legacy POPs are known to undergo fractionation along temperature gradients depositing out of the atmosphere at colder temperatures according to their volatility (Wania and Mackay, 1993). In this manner Polar Regions have been shown to act as environmental sinks for these compounds (Wania and Mackay, 1996).

Although the adverse effects of OCs have been evidenced in controlled laboratory experiments on smaller mammals (e.g. Arzuaga et al., 2009; Ross et al., 1997; Song et al., 2010) elucidating the direct toxicological impact of contaminants in wildlife presents a greater challenge. Long-lived cetaceans are often at the greatest risk of accumulating toxic levels of OCs and there is growing evidence that OC exposure plays a contributory role in a suite of adverse health effects including suppression of immune function (e.g. Lahvis et al., 1995), endocrine disruption (e.g. Brouwer et al., 1989; Reijnders, 1986), and cancer induction (e.g. Martineau et al., 2002).

The 2010 International Whaling Commission (IWC) steering group on contaminants emphasized the need for directing research effort towards linking cetacean chemical exposure to individual or population level health effects to facilitate robust population based risk-assessments (IWC, 2010). Addressing this challenge must be facilitated through the optimum use of opportunistic stranding data combined with non-destructive investigations on free ranging animals. Stranding data for large mysticetes, such as humpback whales, is extremely limited. Unlike smaller species of cetaceans, particularly odontocetes, that may live-strand en mass (Brabyn et al., 1992; Mazzuca et al., 1999) humpback whales rarely live-strand (Willey et al., 1995). Further, the carcass is often in an advanced state of decomposition and useful necropsies, disease identification, and collection of visceral organs is not feasible (Willey et al., 1995). In view of the slow accumulation of data via stranding events, progress in mysticete toxicology must be underpinned by further advances in non-destructive methods combined with utilization and advancement of the molecular level investigations that such samples afford.

The cytochrome P450 (CYP) enzyme system has evolved to both bioactivate and metabolise endogenous and exogenous compounds (Bock and Kohle, 2009; Lewis et al., 1998). Although CYP enzymes are most prevalent in the liver, studies have detected some CYP isozymes in a number of extra-hepatic tissues, including mammalian skin (Oesch et al., 2007; Yengi et al., 2003). The high specificity and inducibility of certain CYP isozymes for select chemical structures have facilitated their use as biomarkers of exposure and molecular level effects (Sarkar et al., 2006; Troisi and Mason, 1997). CYP1A1 is known to be inducible by planar OC compounds (Oritz De Montellano, 2005; Whitlock, 1999) and has been detected in the skin of all mammals investigated, including a number of cetacean species (Fossi et al., 2008; Hooker et al.,

2008; Oesch et al., 2007; Wilson et al., 2010; Yengi et al., 2003). Specifically, CYP1A1 has been detected via immunohistochemistry in the integument of northern hemisphere humpback whales (Angell et al 2004). CYP1A1 activity in cetacean skin has been correlated with blubber OC concentrations in a number of species, including e.g. fin whales (*Balaenoptera physalus*), striped dolphins (*Stenella coeruleoalba*) and Northern bottlenose whales (*Hyperoodon ampullatus)* (Fossi et al., 1992; Hooker et al., 2008; Marsili et al., 1998). Recent advances in *in vitro* practices have confirmed the dose-dependent inducibility of CYP1A1 in sperm whale (*Physeter macrocephalus*) skin employing b-naphthoflavone (Godard et al., 2004). *In vitro* CYP1A1 activation using OC inducers has further been performed using striped dolphin (*Stenella coeruleoalba*) and bottlenose dolphin (*Tursiops truncatus*) dolphin skin cultured fibroblasts (Fossi et al., 2008). To date no peer-reviewed studies have investigated epidermal CYP activity or OC contaminant burdens of any southern hemisphere populations of humpback whales.

This study investigated the presence of CYP1A1 protein, via western blotting, in the skin of southern hemisphere humpback whales to further facilitate toxicokinetic and toxicodynamic analyses.

2. Methods

2.1 *Sample Collection*

Skin and blubber biopsies were obtained from humpback whales, of Breeding Stock E (as designated by the IWC) at two differing time points during their annual migration between

2008 and 2009. Sampling occurred in Moreton Bay Marine Park, North Stradbroke Island, South East Queensland, Australia (approx 27° 26 S, 153° 34 E; Fig. 1).

All biopsies were obtained from a 6-metre rigid hull outboard vessel with a modified air rifle and flotation biopsy darts (Paxarms Ltd, New Zealand). The biopsy dart tips had dimensions of 2.0 cm length and 0.7 cm diameter. Biopsies were collected from the dorsal region ventral and posterior to the dorsal fin, as recommended by Lambertsen et al. (1994). Skin, for enzyme analysis, was snap frozen within 15 minutes of collection and later stored at -80°C until time of analysis. Skin sub-samples for genetic determination were maintained on ice and later stored in Dimethyl sulfoxide (DMSO, ≥99.9%, Sigma-Aldrich) at -20^{o} C until time of analysis.

2.2 *Sex Determination*

Sex determination of individual animals was carried out at the Australian Marine Mammal Centre (AMMC) using a 5' exonuclease assay of the polymorphisms in the sex-linked Zinc Finger genes (Morin et al., 2005).

2.3 *Study animals*

Five free-swimming humpback whales (3 males and 2 females), from both northward and southward migration sampling events were selected for the current study (Table 1).

2.4 *Preparation of extra-hepatic microsomes*

Isolation of microsomal fractions from humpback whale integument was performed briefly as follows. The skin sample was homogenized by freezing in liquid nitrogen and pulverizing 3-4 times with a ceramic mortar and pestle in a Tris-acetate buffer (pH 7.4) (White et al., 1994). Igepal (10%) was added to the homogenate to aid in the chemical breakdown and the homogenate was centrifuged at 12 000 x *g* for 20 minutes at 4°C, and again at 100 000 × *g* for 60 minutes at 4°C. The pellet was re-suspended in Tris-acetate buffer (pH 7.4) and stored at -80°C until biochemical analysis. Protein concentration was determined using the method of Lowry et al. (1951) with bovine serum albumin (BSA) as the reference standard. The amount of tissue (wet weight) collected by biopsy ranged from 0.27-0.42 g. These quantities were sufficient for determination of CYP expression by western blot analysis with protein yields ranging between 1.55-2.68 mg/g skin.

2.5 *Western Blotting*

NADPH-CYP reductase (CPR) and the isozyme CYP1A1 were characterized by western blotting. Primary polyclonal antibodies for CPR and CYP1A1 respectively were α-rabbit anti-cytochrome P450 reductase (Stressgen Bioreagents) and α-rabbit CYP1A1 antibody (Abcam), with HRP linked goat-α-rabbit secondary polyclonal antibody (Abcam). Molecular weight markers were obtained from Biorad (precision plus protein dual standard). Each well of a NuPAGE 10% Bis-tris gel contained 10 µg of microsomal protein of each sample and were separated at 120 V for 1.5 hours at room temperature before being transferred to a nitrocellulose membrane at 90 V for one hour at 4°C. The membrane was then blocked for 1.5 hours in 1% skim milk in TBS (pH 7.5) and incubated with primary antibody (1:1000) for one hour. The membrane was vigorously rinsed in TBST, before being incubated with secondary antibody for

one hour (1:3500). After further vigorous rinsing in TBST the bands were visualized via chemiluminescent detection (Amersham ECL plus, GE healthcare), on Fuji Medical X-ray Film (Imaging Solutions, QLD). The blots were performed in duplicate with positive controls for CPR and CYP1A1 of NADPH-cytochrome P450 reductase protein of natural rat (Stressgen Bioreagents) and Aroclor 1254 induced rat liver microsomes, (CYP1A1; Moltox).

3. Results and Discussion

The existence of the CYP system in humpback whale skin was verified in a test sample by the immunochemical presence of the CYP reductase system (CPR). Subsequently CYP1A1 was probed for and detected in five individual whales selected for this study (Fig. 2).

CYP1A1 was present in both genders and in individuals on both the northward and southward legs of the migration journey. The protein concentration in the immunoblot of each individual whale was maintained constant (10 μg) hence the thickness of each band is a semi-quantitative indication of the level of induction of CYP1A1. A high level of inter-individual variability was observed in the level in the induction of CYP1A1 between the five individuals. This is anticipated as internal factors, such as genetics, age, and gender, combined with external environmental factors are thought to significantly influence CYP1A expression (Ho et al., 2007; Lin et al., 2003; Smart and Daly, 2000; Sy et al., 2001). As these findings are not coupled with detailed information regarding individual-specific internal or external factors, no comprehensive discussion of the individual expressions can be made. Of notable interest however is the observation that the two female individuals appear to have the lowest levels of CYP1A1 protein (Fig 2; Whale 3 & 5). This observation is congruent with

expected contaminant burdens of females of reproductive age compared to males within same age category. Reproductive females expend large amounts of energy through parturition and lactation thereby effectively transferring lipid energy stores and associated OC burdens to their young (Aguilar and Borrell, 1994; Debier et al., 2003).

To date there is no peer-reviewed literature on OC contaminant burdens of southern hemisphere humpback whales and as previously discussed toxicological and epidemiological data is also lacking. Although lower trophic level mysticetes are less susceptible to accumulation of toxic OC burdens compared to odontocete counterparts, their extreme life-history adaptation of simultaneous fasting, migration and reproduction potentially places them in an elevated risk category through seasonal re-mobilisation of accumulated OC burdens. Quantification of CYP1A1 activity will provide, at minimum, a biologically relevant complementary measure to chemical analysis, and at best an early biological effect of exposure to contaminant inducers, facilitating the scope for improved chemical risk assessments for the species.

Conclusion

This study provides the first evidence of CPR in southern hemisphere humpback whale integument and verifies the presence of CYP1A1 enzyme protein, hereby expanding the repertoire of biochemical assessments possible on the species via non-destructive methods. The CYP1A1 protein was expressed in both genders, including a lactating female, and at both migration time points. The findings represent a quality assurance step in ongoing studies of enzyme activity as a function of subcutaneous blubber OC concentration.

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References

Aguilar, A., Borrell, A., 1994. Reproductive transfer and variation of body load of organochlorine pollutants with age in fin whales (*Balaenoptera physalus*). Archives of Environmental Contamination and Toxicology 27, 546-554.

Angell, C., Wilson, J.Y., Moore, M.J., Stegeman, J.J. 2004. Cytochrome P450 1A1 expression in cetacean integument: Implications for detecting contaminant exposure and effects. Marine Mammal Science 20 (3), 554-566.

Arzuaga, X., Ren, N., Stromberg, A., Black, E.P., Arsenescu, V., Cassis, L.A., Majkova, Z., Toborek, M., Hennig, B., 2009. Induction of gene pattern changes associated with dysfunctional lipid metabolism induced by dietary fat and exposure to a persistent organic pollutant. Toxicological Letters 189, 96-101.

Bock, K.W., Kohle, C., 2009. The mammalian aryl hydrocarbon (Ah) receptor: from mediator of dioxin toxicity towards physiological functions in skin and liver. Biological Chemistry. 390, 1225-1235.

Brabyn, M.W., Mclean, I.G., 1992. Oceanography and coastal topography of herd stranding sites for whales in New Zealand. Journal of Mammalogy. 73 (3), 469-476.

Brouwer, A., Reijnders, P.J.H., Koeman, J.H., 1989. Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin-a and thyroid hormone deficiency in the common seal (*Phoca Vitulina*). Aquatic Toxicology 15, 99-105.

Debier, C., Pomeroy, P.P., Dupont, C., Joiris, C., Comblin, V., Le Boulenge, E., Larondelle, Y., Thome, J.P., 2003. Quantitative dynamics of PCB transfer from mother to pup during lactation in UK grey seals *Halichoerus grypus*. Marine Ecology-Progress Series 247, 237-248.

Fossi, M.C., Casini, S., Bucalossi, D., Marsili, L., 2008. First detection of CYP1A1 and CYP2B induction in mediterranean cetacean skin biopsied and cultured fibroblasts by Western blot analysis. Marine Environmental Research 66, 3-6.

Fossi, M.C., Marsili, L., Leonzio, C., Disciara, G.N., Zanardelli, M., Focardi, S., 1992. The use of nondestructive biomarker in mediterranean cetaceans - Preliminary data on MFO activity in skin biopsy. Marine Pollution Bulletin 24, 459-461.

Godard, C.A.J., Smolowitz, R.M., Wilson, J.Y., Payne, R.S., Stegeman, J.J., 2004. Induction of cetacean cytochrome P4501A1 by beta-naphthoflavone exposure of skin biopsy slices. Toxicological Sciences 80, 268-275.

Ho, S., Chen, C., Satoh, J., Yim, S., Gonzalez, F.J. 2007. Dietary phytochemicals regulate whole body CYP1A1 expression throughout aryl hydrocarbon receptor nuclear translocator dependent system in gut. The Journal of Clinical Investigation 117 (7), 1940-1950.

Hooker, S.K., Metcalfe, T.L., Metcalfe, C.D., Angell, C.M., Wilson, J.Y., Moore, M.J., Whitehead, H., 2008. Changes in persistent contamination concentration and CYP1A1 protein expression in biopsy samples from northern bottlenose whales, *Hyperoodon ampullatus*, following the onset of nearby oil and gas development. Environmental Pollution 152, 205-216.

IWC, 2010. Report of the IWC Pollution 2000+Phase II Workshop.
International Whaling Commission 22-24 February 2010.

Lahvis, G.P., Wells, R.S., Kuehl, D.W., Stewart, J.L., Rhinehart, H.L., Via, C.S., 1995. Decreased lymphocyte responses in free-ranging bottlenose dolphins (*Tursiops truncatus*) are assoicated with increased concentration of PCBs and DDT in peripheral blood. Environmental Health Perspectives 103, 67-72.

Lambertsen, R.H., Baker, C.S., Weinrich, M., Modi, W.S., 1994. An improved whale biopsy system designed for multidisciplinary research, in: Fossi, M.C. (Ed.), Nondestructive biomarkers in vertebrates. Lewis Publishers, pp. 220-244.

Lewis, D.F.V., Eddershaw, P.J., Dickins, M., Tarbit, M.H., Goldfarb, P.S., 1998. Structural determinants of cytochrome P450 substrate specificity, bindign affinity and catalytic rate. Chemico-Biological Interactions 115, 175-199.

Lin, P.P., Hu, S.W., Chang, T.H., 2003. Correlation between gene expression of aryl hydrocarbon receptor (AhR). hydrocarbon receptor nuclear translocator (Arnt), cytochrome P4501A1 (CYP1A1) and 1B1 (CYP1B1), and inducibility of CYP1A1 and CYP1B1 in human lymphocytes. Toxicological Sciences 71, 20-26.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemisty 193, 265-275.

Marsili, L., Fossi, M.C., Notarbartolo diSciara, G., Zanardelli, M., Nani, B., Panigada, S., Focardi, S., 1998. Relationship between organochlorine contaminants and mixed function oxidase activity in skin biopsy specimens of mediterranean fin whales. Chemosphere 37, 1501-1510.

Martineau, D., Lemberger, K., Dallaire, A., Labelle, P., Lipscomb, T.P., Michel, P., Mikaelian, I., 2002. Cancer in wildlife, a case study: Beluga from the St. Lawrence estuary, Quebec,Canada. Environmental Health Perspectives 110, 285-292.

Mazzuca, L., Atkinson, S., Keating, B., Nitta, E., 1999. Cetacean mass strandings in the Hawaiian Archipelago, 1957-1998. Aquatic Mammals 25 (2), 105-114.

Morin, P.A., Nestler, A., Rubio-Cisneros, N.T., Robertson, K.M., Mesnick, S.L., 2005. Interfamilial characterization of a region of the ZFX and ZFY genes facilitates sex determination in cetaceans and other mammals. Molecular Ecology 14, 3275-3286.

Oesch, F., Fabian, E., Oesch-Bartlomowicz, B., Werner, C., Landsiedel, R., 2007. Drug-metabolizing enzymes in the skin of man, rat, and pig. Drug Metabolism Reviews 39, 659-698.

Oritz De Montellano, P.R., 2005. Cytochrome P450 Structure, Mechanism, and Biochemistry. Kluwer Academic/Plenum Publishers, New York.

Reijnders, P.J., 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. Nature 324, 456-457.

Ross, P.S., De Swart, R.L., van der Vliet, H., Willemsen, L., De Klerk, A., Van Amerongen, G., Groen, J., Brouwer, A., Schipholt, I., Morse, D.C., Van Loveren, H., Osterhaus, A.D., Vos, J.G., 1997. Impaired cellular immune response in rats exposed perinatally to Baltic Sea herring oil or 2,3,7,8-TCDD. Archives of Environmental Contamination and Toxicology 71, 563-574.

Sarkar, A., Ray, D., Shrivastava, A.N., Sarkar, S., 2006. Molecular biomarkers: their significance and application in marine pollution monitoring. Ecotoxicology 15, 333-340.

Smart, J., Daly, A.K., 2000. Variation in induced CYP1A1 levels, relationship to CYP1A1, Ah receptor and GSTM1 polymorphisms. Pharmacogenetics 10, 11-24.

Song, C., Kanthasamy, A., Anantharam, V., Sun, F., Kanthasamy, A.G., 2010. Environmental Neurotoxic Pesticide Increases Histone Acetylation to Promote Apoptosis in Dopaminergic

Neuronal Cells: Relevance to Epigenetic Mechanism of Neurodegeneration. Molecular Pharmacology 77, 621-632.

Sy, S.K., Tang, B.K., Pastrakuljic, A., Robert, E.A., Kalow, W., 2001. Detailed characterization of experimentally derived human hepatic CYP1A1 activity and expression using differential inhibition of ethoxyresorufin O-deethylation by fluvoxamine. European Journal of Clinical Pharmacology 57 (5), 377-380.

Troisi, G.M., Mason, C.F., 1997. Cytochromes P450, P420 and Mixed Function Oxidases as Biomarkers of Polychlorinated Biphenyl (PCB) Exposure in Harbour Seals *(Phoca vitulina)*. Chemosphere 35, 1933-1946.

UNEP, 2002. Regionally based assessment of persistant toxic substances, in: Facility, G.E. (Ed.), Mediterranean regional report.

Wania, F., Mackay, D., 1993. Global Fractionation and Cold Condensation of Low Volatility Organochlorine Compounds in Polar-Regions. Ambio 22, 10-18.

Wania, F., Mackay, D., 1996. Tracking the Distribution of Persistant Organic Pollutants. Environmental Science and Technology 30, 390-396.

White, R.D., Hahn, M.E., Lockhart, W.L., Stegeman, J.J., 1994. Catalytic and Immunochemical Characterization of Hepatic-Microsomal Cytochromes P450 in Beluga Whale (Delphinapterus-Leucas). Toxicology and Applied Pharmacology 126, 45-57.

Whitlock, J.P., 1999. Induction of cytochrome P4501A1. Annual Review of Pharmacology and Toxicology 39, 103-125.

Willey, D.N., Asmutis, R.A., Pitchford, T.D., 1995. Stranding and mortality of humpback whales, *Megaptera novaeangliae*, in the mid-Atlantic and southeast United States, 1985-1992. Fishery Bulletin 93, 196-205.

Wilson, J.Y., Moore, M.J., Stegeman, J.J., 2010. Catalytic and immunochemical detection of hepatic and extrahepatic microsomal cytochrome P40 1A1 (CYP1A1) in white-sided dolphin (*Lagenorhynchus acutus*). Aquatic Toxicology 96, 216-224.

Yengi, L.G., Xiang, Q., Pan, J.M., Scantina, J., Kao, J., Ball, S.E., Fruncillo, R., Ferrom, G., Wolf, C.R., 2003. Quantification of cytochrome P450 mRNA levels in human skin. Analytical Biochemistry 316, 103-110.

Figure and Table Captions

Fig. 1. The geographical location of study sampling site; North Stradbroke Island, Queensland, Australia

Table 1. Migration status, sex, estimated age class, and pod composition.

Fig. 2. The above Western Blot (WB) shows the presence of CYP1A1 in the skin of 5 individual, humpback whales. Arrowhead indicates expected molecular size of CYP1A1 (57KDa). *= purified rat liver microsome CYP1A1, which was included on the blots as a positive control.