Detoxification Enzyme Activities (CYP1A1 and GST) in the skin of humpback whales as a Function of organochlorine burdens and Migration Status

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Introduction

Persistent Organic Pollutants (POPs) are ubiquitous and toxic environmental contaminants [1]. Frequently semi-volatile, they have a propensity for long range atmospheric transport to colder Polar Regions [2]. The lipophilicity of organochlorine (OC) POP structures ensures that they are efficiently assimilated into lipid-dependent Polar food webs [3]. Marine mammals are at the greatest risk of accumulating toxic OC burdens due to their longevity, position at the top of the food chain and their significant adipose mass [4].

Southern hemisphere humpback whales *(Megaptera novaeangliae)* were hunted to near extinction early in the twentieth century (ref). The recovering population forms at least seven distinct breeding stocks that migrate between Southern Ocean feeding grounds and their equatorial breeding and calving grounds (IWC, 2006). These are the longest migrations known in any mammal and are associated with up to seven months of fasting [5]. Recently we showed the significant metabolic concentration of OC blubber burdens that occurs during these extreme migrations events [6] and hypothesised that they are associated with significant mobilisation and redistribution of toxic OCs between body tissues as shown in humans [7] and wildlife undergoing rapid weight loss [8, 9]. Baleen whales are commonly attributed a lower chemical risk category due to their lower trophic level [10]. Extreme nutritional fluctuations; be they through behavioural adaptations such as migration, or starvation through habitat loss or degradation, however warrant concerted chemical risk assessments to better understand the elevated risk associated with periods of nutritional stress.

Investigating toxicological threats to large, free-moving cetaceans presents inherent challenges including; lack of demographic data, incomplete knowledge of species physiology and limited access to suitable study materials [11]. *In-vitro* molecular assessments using skin and blubber tissues obtained from biopsies of free-swimming animals has constituted a primary focus for the advancement of marine mammal toxicology over the past decade [12-16]. Substrate inducible biotransformation enzymes have shown some promise as wildlife biomarkers of OC exposure and molecular effects [17]. Cytochrome P450 (CYP) enzymes play a major role in the catalysis of oxidation reactions involving exogenous compounds. The CYP1A forms show high specificity for planar hydrocarbons such as polychlorinated-dibenzo-*p*-dioxins and furans (PCDDs/PCDFs), coplanar polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs)

[18]. CYP1A1 activity is mediated via the Aryl-hydrocarbon (Ah) receptor protein[19], and a common Ah receptor mediated mechanism of action is the basis for the development of the Toxicity Equivalency Factors (TEFs) approach to risk assessment of planar hydrocarbon ligands [20].

Reactive metabolic intermediates formed through "Phase I" detoxification (e.g. CYP450 action) [19], may be further metabolised by "Phase II" enzymes which act to further enhance the polarity of the metabolite through e.g. conjugation with endogenous co-factors. Glutathione-s-transferase, catalyzes the conjugation of nucleophilic Glutathione (GSH) with nonpolar compounds that contain an electrophilic carbon, nitrogen or sulphur atom [21]. In contrast to the higher specificity of the CYP450 system, GST induction is generally accepted to be a generic, evolutionarily conserved response to oxidative stress [21].

Previously we verified the presence of an active CYP450 system and the presence of CYP1A1 enzyme proteins in the skin of humpback whales [22], paving the way for further quantitative investigation. In this study we further investigate the presence and activity of GST in humpback whale epidermal tissue and explore the relationship between both epidermal GST and CYP1A1 activity in relation to adjacent blubber OC burdens and the migration status of individual animals.

Methods

Sample Collection

Skin and blubber biopsies were obtained from the southern hemisphere humpback whale (*Megaptera novaeangliae)* breeding stock E1 [23] on their annual migration along the east Australian coastline. Animals were targeted at two time points on the migration whilst passing North Stradbroke Island, Queensland; 1) northward migration (June/July; post-summer feeding) and 2) southward (September/October; end of fasting). Biopsies were obtained with a Paxarms™ air rifle and flotation darts with stainless steel cutting heads (2.0 cm L x 0.7 cm D). Immediately upon sample retrieval the skin fraction was separated from the blubber tissue and placed in a liquid nitrogen dewar before storage at -80°C until enzyme extraction. The blubber fraction was maintained on ice before storage at -20°C until chemical extraction.

Tissue Extraction

Enzymal fractions were extracted from the whale epidermal tissue (0.1-0.3 g) by pulverising the frozen sample with a ceramic mortar and pestle in a 0.1 M Tris-acetate buffer (pH 7.4) [24]. The sample and homogenate were kept cool throughout extraction through gradual addition of liquid nitrogen. 200 μL Igepal (10%) (Sigma Aldrich) was added to the homogenate and the sample centrifuged at 12000 *g* for 20 minutes at 4°C. The supernatant, the cytosolic fraction, was pippetted off and stored at -80°C until application in GST western blot and activity assays. The pellet, the microsomal fraction, was resuspended in Tris-acetate buffer (pH 7.4) and stored at -80°C until CYP1A1 activity analysis. Protein concentrations were determined using the method of Lowry et al. (1951) [25] with bovine serum albumin as the reference standard.

CYP 1A1 Activity

7-ethoxyresorufin-O-deethylase (EROD) binding was used as a measure of CYP1A1 activity in epidermal microsomal fraction. Activity assays were run spectrophotometrically according to Kennedy et al. (1994)[26]. EROD activities are expressed as pmol resorufin / min / mg protein. CYP1A1 activity was measured in 25 animals; including 20 males, 4 females and a single calf-of-the-year (Table 1).

GST Western Blotting

Epidermal cytosol, without the microsomal fraction, was probed for GST protein by western blotting with a goat polyclonal GST primary antibody (Abcam) generated against xx animal GST and anti-goat IgG-HRP secondary antibody (Sigma-Aldrich). Bands were detected by the ECL kit as per manufacturer instructions (GE Healthcare). GST protein was verified by western blotting in six animals including …(Figure 1), before proceeding to the activity assay. A positive control of xxx (purchased from) was used in the western blot and a band corresponding to the GST predicted size of ~25-30 kDa was detected in the control and all whale samples tested.
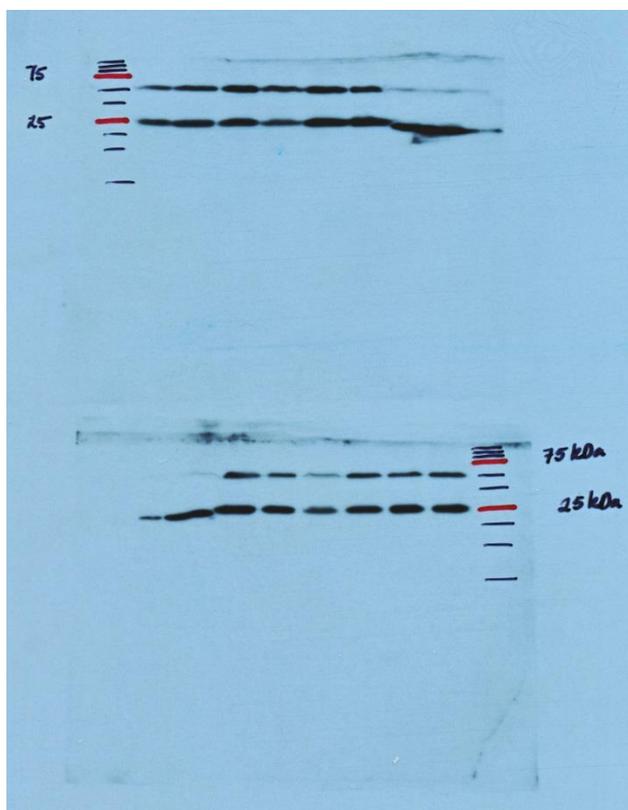

Figure 1: GST WB

GST Activity

GST activity was measured spectrophotometrically through the production of the GST-CDNB conjugation product via the method of Habig et al. (1974)[27]. GST activity was measured in 43 animals; including (males females data to come) of which 29 had associated POP concentrations data and 21 CYP1A1 activity data (Table 1). Results are expressed on a…..basis

Student t-test (p=0.05) was used to explore differences between migration and gender cohorts. Pearson product coefficient was used to investigate relationships between enzymal activities and contaminant groups and toxicity equivalency factors (TEF).

Table 1: Individuals included in the current study presented along with epidermal EROD and GST activities (pmol/min/mg protein)and contaminant concentrations (ng/g lipid)

Early-Migration Males

Animal ID	1N08	3NO8	4N08	7N08	8NO8	13N08	14N08	15NO	16NO	18NO	19N08
EROD Activity		15.2	14.4		14.4		16.2				32.9
GST Activity	5.6	6.3		14.7	6.9	2.0		8.7	6.2	8.0	10.6
HCB		30.6	42.5	62.0	17.6	132.8	22.0		137.4	91.6	28.4
ΣDDT		11.2	14.4	26.3	6.8	41.5	7.7		57.7	23.7	11.6
Σ_{32}PCB		1.7	2.2	4.8	4.3	7.5	1.1		11.7	3.9	1.8
Σchlordanes		3.3	4.0	8.8	3.5	18.4	2.1		21.0	9.2	2.9
Σtoxaphenes		7.2	8.5	14.2	7.4	42.9	5.7		29.2	22.4	9.2
TEF (8 PCBs)		0.06	0.07	0.08	0.12	0.22	0.05		0.15	0.08	0.06

Animal ID	21NO	24NO	25NO	26N08	28NO	1N09	2N09	7NO9	8NO9	10NO	12NO
EROD Activity	17.1		23.5		18.8	19.7		20.6	19.7	20.7	17.5
GST Activity	12.0	5.7	5.6	4.5	6.2	5.6	9.6	8.1	5.8	5.4	4.2
HCB	38.2	19.6			70.3	59.2	66.7	96.6	8.6	22.0	29.0
ΣDDT	13.1	6.1			2.8	64.2	28.4	27.2	13.1	19.4	23.4
Σ_{32}PCB	2.5	0.7			12.8	47.4	5.4	9.2	2.3	4.0	4.0
Σchlordanes	4.2	2.3				189.4	17.0	62.01	21.7	35.8	64.4
Σtoxaphenes	8.8	5.09				36.2	35.2	28.9	54.0	29.9	82.9
TEF (8 PCBs)	0.06	0.03			0	0.47	0.82	0.24	0.17	0.2	0.19

Early Migration Females

Animal ID	5N09	9NO9	11N08
EROD Activity			11.3
GST Activity	6.0	7.7	
HCB			
ΣDDT			
Σ_{32}PCB			
Σchlordanes			
Σtoxaphenes			
TEF (8 PCBs)			

Late Migration Males

Animal ID	1SO7	13SO8	14SO8	7S09	9SO9	10S09	12SO9	13SO9	16SO9	21SO9
EROD Activity		19.2		19.5	12.2	13.0	19.7	13.8		16.5
GST Activity	4.3	2.5	2.4	7.6	3.2		8.5	2.7	6.6	4.0
HCB		60.3			149.5	192.1	87.4			54.2
ΣDDT		28.3			75.3	95.2	50.6	5.8		25.7
Σ_{32}PCB		5.3			11.4	0.2	0.8			1.2
Σchlordanes		9.2								
Σtoxaphenes		13.5								
TEF (8 PCBs)		0.1			0.54	0.5	1.03			0.24

Late Migration Females

Animal ID	12SO8	15S08	3S09	4SO9	5SO9	11S09	15S09
EROD Activity		16.0		11.0		13.7	
GST Activity	2.2		9.6	5.6	4.9	6.9	6.8
HCB							
ΣDDT							

Σ_{32}PCB	
Σchlordanes	
Σtoxaphenes	
TEF (8 PCBs)	
Calf	
Animal ID	**2S09**
EROD Activity	16.6
GST Activity	15.3
HCB	110.
ΣDDT	27.7
Σ_{32}PCB	14.1
Σchlordanes	49.3
Σtoxaphenes	65.8
TEF (8 PCBs)	0.3

*ΣDDT (o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT, p,p'DDT); ΣPCB (IUPAC no: 18, 28, 31, 33, 37, 47, 52, 66, 74, 99, 101, 105, 114, 118, 122, 123, 128, 138, 141, 149, 153, 156, 157, 167, 170, 180, 183, 187, 189, 194, 206, 209); ΣChlordanes (Heptachlor-exo-epoxide, Heptachlor-endo-epoxide, Heptachlor-endo-epoxide, cis-Chlordane, Oxy-chlordane, Chlordene, Heptachlor, trans-Nonachlor, cis-Nonachlor); Σtoxaphenes (Tox-26,-32,-40+41,-42a, -44, -50, -62)

Results & Discussion

Measured enzymal activities did not show any strong correlation with eachother or specific compound groups (Table 2). The only strongly correlating (r=0.52) parameters were GST activity with PCB_8 TEF values. Whilst the small number of female samples available prevented comprehensive assessment of gender and female migrational cohort differences, no trends were apparent from this study. Similarly, CYP1A1 activity did not differ significantly between northward and southward migrating male cohorts whereas GST activity was significantly depressed in southward compared to northward migration males (p=0.03).

Table 2: Correlations (*r*) between measured enzymal activities and contaminant measured values and TEFs. *r*-values (+ or –ive) of 0-0.09 (no correlation), 0.1-0.3 (small correlation), 0.3-0.5 (moderate correlation) and 0.5-1.0 (strong correlation

	EROD	HCB	ΣDDT	Σ32PCB	ΣChlordanes	ΣToxaphenes	TEF
GST	0.44	-0.21	-0.18	-0.16	-0.21	-0.39	0.52
EROD		-0.35	-0.24	0.06	0.07	0.03	-0.08

EROD and GST Activity

An evident coupling of the selected phase I and phase II detoxification enzymes may be expected if an individual was suffering a significant toxic insult via the Ah receptor. The persistent organic pollutant (POP) profiles of the E1 breeding population of southern hemisphere humpback whales and their principal prey item, Antarctic krill (*Euphausia superba),* have recently been comprehensively described [6, 28]. It was found that typical AhR binding compounds such as coplanar PCBs and dioxins/furans do not feature prominently in the Southern Ocean food web OC chemical profile and they have therefore not been targeted in ongoing chemical analyses of higher trophic level predators such as humpback whales. The correlation between CYP1A1 and GST activity in the current study was found to be only moderate (r=0.44). This indistinct signal may be explained by both low burdens of OC CYP1A1 ligands as well as the significant concurrent roles of both enzymes in production and detoxification of endogenous compounds (ref).

Enzyme Activity in response to measured POPs

Environmental contaminants do not occur in isolation, but as complex mixtures [29]. Whilst typical OC CYP1A1 ligands are not expected to feature prominently in the chemical profiles of the study animals, elevated levels of one lipophilic OC compound may indicate elevated levels of other compounds bearing similar physico-chemical properties. EROD activity detected in epidermal tissues showed only weak or moderate, and at times even negative, relationships with quantified groups of OC contaminant in the adjacent blubber tissue (Table 2). Even TEF values calculated on eight quantified co-planar PCB congeners revealed no significant relationship with this enzyme activity (r=-0.08). Similarly epidermal GST activity was consistently weakly or moderately negatively correlated with contaminant groups detected in adjascent blubber OC burdens, althrough strongly correlated with blubber PCB TEFs (r=0.52). In the absence of a corresponding evidenced CYP1A1 relationship, and in light of the low TEFs measured, this observation is unlikely to indicate a dependent relationship. It is feasible that levels of circulating OC Ahr inducing substrates were simply too low to play a discernible role in the induction of these enzyme and secondly, any OC xenobiotic related enzyme activity was overshadowed by its other biological other functions.

Epidermal enzyme activity may reflects circulating levels of OCs more closely than blubber levels as the interface between the two tissues is well vascularization (ref). If outer blubber layers (i.e. sampled

sections), are not reflective of the entire blubber depth, due to weight-loss induced mobilisation, re-distribution and metabolic concentration, we may expect there to be an uncoupling in the enzyme response. It has however been shown that there is little stratification in humpback whales (Waugh et al…). Further, studies in other species has shown that blubber concentrations of OCs accurately reflect blood levels in other cetaceans [30] suggesting the cause lies with the relative contributory role of Ahr inducers compared to the other biological roles of the enzyme.

Enzyme Activity and Fasting

In contrast to the absence of a significant difference in the expression of EROD activity between northward and southward migrating cohorts, an interesting observation was made for GST activities. Our results showed that GST activity was significantly depressed in southward migrating male whales (p=0.03). This finding is contradictory to what we would expect based upon lipophilic OC metabolic concentration and mobilisation which we expect to be greatest at the final stages of migration when energy reserves are lowest. This contradictory finding points to additional influences on GST function throughout the migration event. Studies in estivating toads has shown that a significant fall in GST occurs during dormancy [31]. The authors attribute this observed decrease in GST levels due to cessation of feeding which, coupled with metabolic suppression, reduces the need for processing of xenobiotics and other metabolic end-products that result in the generation of reactive oxygen species (ROS). A second component may be the reduced availability of the GSH co-factor required for binding of metabolic end-products. GSH levels in the liver are closely related to dietary intake of the component amino acid, cysteine. It has been observed that a marked suppression of the availability of GSH occurs upon prologed starvation of rats [32]. Little is known about the physiological adaptation of baleen whales to extended periods of fasting. These results indicate a need to better understand the detoxification capabilities of whales, not only against anthropogenic xenobiotics, but also against the cummulative effects of ROS stress during prolonged fasting.

Conclusion

These results do not lend evidence to support the role of CYP1A1 and GST as effective biomarkers of OC contamination in southern hemisphere humpback whales. This in part can be attributed to the lower importance of typical CYP1A1 inducing OCs in the Southern Ocean food web.

Through this study we observed a suppression of GST activity in late migration animals. The significance of this finding and the role of this enzyme in further health assessment and understanding the fasting metabolism of migrating humpback whales requires further investigation.

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