CYTOCHROME P450 1A AS A BIOMARKER OF CONTAMINANT EXPOSURE IN FREE-RANGING MARINE MAMMALS

by

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ABSTRACT

Marine mammals are exposed to high concentrations of organochlorine contaminants that have been linked to adverse health effects. Hepatic cytochrome P450 1A (CYP1A) is a widely used biomarker of organochlorine exposure. CYP1A catalyzes the biotransformation of xenobiotic compounds and is specifically induced by planar aromatic and halogenated hydrocarbons. CYP1A analysis has traditionally involved harvesting liver samples from dead animals. However, because of legal and ethical constraints, the use of liver for biomarker studies in free-ranging marine mammals has become increasingly unacceptable. The objective of this study was to determine whether CYP1A in skin biopsies, obtained using minimally-invasive techniques, could be used as a biomarker of organochlorine exposure in wild harbour seals and killer whales. This study consisted of three groups: (1) 20 free-ranging harbour seal pups were captured in southern British Columbia (BC) and temporarily housed in captivity. Skin-blubber and liver biopsies were collected, and three seals were orally treated with ß-naphthoflavone (BNF), a known CYP1A inducer. (2) Skin-blubber biopsies were collected in the field from 42 seals (pups and adults) in BC and Washington State. (3) Skin-blubber biopsies were collected from 13 free-ranging killer whales in BC. CYP1A enzyme activity and protein levels were quantified in both liver and skin biopsies from seals using the ethoxyresorufin *O*-deethylase (EROD) and immunoblot assays, respectively. Cutaneous CYP1A expression was near the detection limit and did not correlate with hepatic CYP1A. However, in both tissues, CYP1A protein levels were induced by BNF treatment, and CYP1A expression increased during three weeks in captivity. Hepatic

CYP1A expression correlated with blubber contaminant levels, and cutaneous CYP1A protein levels were higher in pups from Washington State than in pups from BC. EROD activity was not detected in killer whale skin, though a possible CYP1A protein band was detected by immunoblot analysis. In this study, CYP1A was quantified in small liver and skin biopsies obtained from free-ranging marine mammals using minimally-invasive methods. However, further studies are needed to validate the use of CYP1A as a biomarker of organochlorine exposure in marine mammal skin.

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AHH	aryl hydrocarbon hydroxylase
AhR	aryl hydrocarbon receptor
AP	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate, *p*-toluidine salt
BNF	ß-naphthoflavone
BPMO	benzo[a]pyrene monooxygenase
DDT	dichlorodiphenyltrichloroethane
DMSO	dimethylsulfoxide
EROD	ethoxyresorufin *O*-deethylase
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
LOD	limit of detection
LOQ	limit of quantitation
MC	methylcholanthrene
NADPH	nicotindiamide adenine dinucleotide phosphate tetrasodium salt
NBT	nitro blue tetrazolium
OC	organochlorine
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
POP	persistent organic pollutant
PVDF	polyvinyldenfluoride
RCQ	relative contour quantity
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
TEF	toxic equivalency factor
TEMED	N,N,N′N′-tetramethylenediamine
TEQ	toxic equivalent

APPENDICES

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1. INTRODUCTION

1.1 Persistent Organic Pollutants

Anthropogenic chemical compounds are present in the air, water, sediments, and in the tissues of humans and wildlife worldwide (AMAP, 1998). Large-scale production of organochlorine (OC) compounds such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), and other chlorinated pesticides began during the 1940's and 1950's, and many of these compounds are still in use today (AMAP, 1998). PCBs are one of the most ubiquitous groups of anthropogenic compounds. Due to their chemical stability, PCBs were synthesized for use in heat transfer mixtures, hydraulic fluids, and as electrical insulators. Production, importation, and most non-electrical uses of PCBs were banned in Canada in 1977; however, some PCBs remain in use or in storage (Pierce *et al.*, 1998).

Other OC compounds routinely detected in the environment include polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). PCDDs and PCDFs are by-products of incomplete combustion processes involving chlorine and phenolic compounds. PCDDs and PCDFs are also by-products of herbicide manufacture (Pierce *et al.*, 1998). Pulp mills used to be a major source of PCDDs in Canada, but changes to the bleaching process and bans on the use of pentachlorophenol as a wood preservative have greatly decreased PCDD output (Hagen *et al.*, 1997; Pierce *et al.*, 1998). Municipal incinerators and old landfill sites are the major sources of PCDDs and PCDFs today (Pierce *et al.*, 1998).

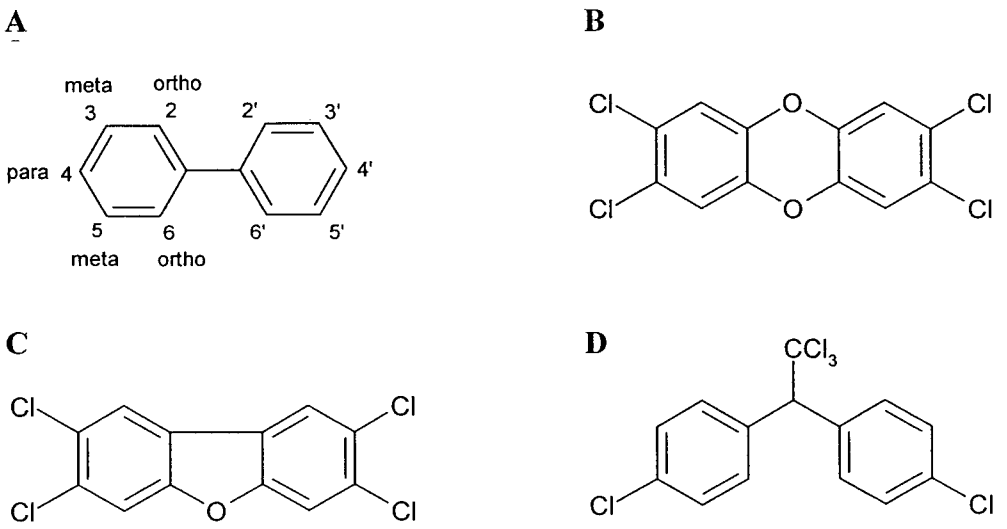


Figure 1.1: Organochlorine chemical structures: (A) PCB showing the designations for substituted chlorine atoms. (B) 2,3,7,8-tetrachlorodibenzodioxin (TCDD). (C) 2,3,7,8-tetrachlorodibenzofuran (TCDF). (D) dichlorodiphenyltrichloroethane (DDT).

Organochlorines belong to a larger group of chemicals, collectively called Persistent Organic Pollutants (POPs). In 2001, the United Nations adopted the Stockholm Convention, which aims to reduce or eliminate twelve of the most toxic POPs, including PCBs, PCDDs, PCDFs, DDT, and other pesticides worldwide. Although industrialized countries such as Canada and the United States have banned or restricted the use of all twelve POPs targeted by the Stockholm Convention (Schmidt, 1999), some of these compounds are still produced or used in other parts of the world (Walker, 2000). Additionally, unregulated compounds, such as polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), and newer POPs are being released into the environment (Meerts *et al.*, 2000). Unlike PCBs and DDT, the environmental effects of these compounds are poorly understood.

1.1.1 POPs in the marine environment

The world's oceans are particularly susceptible to large inputs of industrial and agricultural pollutants. Pollutants enter the marine environment as a result of run-off, leakage, dumping, or atmospheric transport (AMAP, 1998). Areas immediately surrounding point sources such as pulp mills can be directly contaminated, whereas areas remote from human activity are contaminated indirectly by sediment transport, and more significantly, by atmospheric transport and deposition. PCDFs associated with sediments can be transported by ocean currents over distances of greater than 30 kilometers (Macdonald *et al.*, 1992), and organochlorines are transported thousands of kilometers by circulating air masses in the atmosphere. Atmospheric transport partially explains the continued presence of organochlorines in regions where these compounds are no longer produced, and it is largely responsible for the occurrence of OCs in remote regions such as the Arctic (AMAP, 1998; Muir *et al.*, 1999). After initial deposition to the Earth's surface, OC compounds may re-enter the atmosphere by volatilization or resuspension of dust or snow by wind. This cycle of transport and deposition may occur multiple times (Macdonald *et al.*, 2000).

The main factor responsible for the persistence of OCs in the marine environment is their relatively high resistance to chemical and biological degradation. Organochlorines can remain buried in sediments for long periods of time and can be re-exposed by human activity or bottom-dwelling organisms, or by other natural factors such as tides. Additionally, hydrophobic OCs accumulate in the lipid stores of organisms and are subsequently biomagnified through aquatic food webs. Biomagnification is the

successive increase in concentration of a chemical with increasing trophic level (i.e. as contaminants are passed up the food chain) (Muir *et al.*, 1999).

1.1.2 POPs in marine mammals

As top predators in the marine food chain with relatively large energy requirements, marine mammals are particularly susceptible to the accumulation of lipophilic chemicals in their blubber (Muir *et al.*, 1999).

The first scientific reports of contaminants in marine mammals were published in 1966. By the end of the 1960s, PCBs, dieldrin, and DDT and its metabolites had been documented in the tissues of several pinniped (seal, sea lion, walrus) and cetacean (whale, dolphin, porpoise) species (O'Shea and Tanabe, 1999). After regulations were implemented for PCB use and production in North America and Europe in the 1970s, PCB concentrations decreased in several environmental compartments, including marine mammals. For example, in Eastern Canada, mean total DDT concentrations declined in adult female grey seal (*Halichoerus grypus*) tissue from 13.2 to 4.0 mg/kg (lipid weight basis) between 1974 and 1982 (Addison *et al.*, 1984a). PCB concentrations did not change in grey seals and decreased slightly in harp seals (*Pagophilus groenlandicus*) over the same period (Addison *et al.*, 1984a). Concentrations of DDT, PCBs, and hexachlorobenzene (HCB) also decreased in blubber of western Arctic ringed seals (*Phoca hispida*) between 1971 and 1991 (Addison and Smith, 1998). In general, organochlorine concentrations in marine mammals leveled off during the late 1980s and early 1990s (Addison and Smith, 1998; Ross and Troisi, 2001). This stabilization is

attributed to leakage of existing PCB stores, continued use of DDT in some regions of the world, and environmental cycling (Walker, 2000; Ross and Troisi, 2001).

PCBs and DDT continue to dominate the contaminant profiles of marine mammals in most locations, and high levels of OC pollutants continue to be measured in marine mammal species worldwide (Ross and Troisi, 2001). PCB and DDT in a number of marine mammal species are shown in Table 1.1. Recently, killer whale (*Orcinus orca*) populations in British Columbia were identified as being among the most PCB-contaminated marine mammals in the world (Ross *et al.*, 2000). PCB concentrations in killer whales often exceed those of other highly contaminated cetacean populations, such as the St. Lawrence beluga whales (*Delphinapterus leucas*), which researchers suspect have suffered reproductive and immunological effects as a result of PCB exposure (Béland *et al.*, 1993; De Guise *et al.*, 1998) (refer to Table 1.1).

The contaminant levels shown in Table 1.1 were measured in blubber and are expressed on a lipid weight basis. However, several factors such as different methods of PCB quantification (eg. number and identity of congeners measured), the use of different tissues for PCB analysis (eg. blubber, liver, blood), natural confounding factors (eg. age, sex, condition), and different ways of expressing contaminant levels (eg. per gram wet weight or lipid weight), often preclude direct comparison of PCB concentrations between studies.

Table 1.1: Organochlorine levels in blubber of pinnipeds and cetaceans from various locations

Species	Location	Year	Sex/Age	Σ PCB	Σ DDT	Ref.
Pinnipeds						
Largha seal	Japan	1996	M/various	2.3	-	1
Ribbon seal	Japan	1996	M/various	1.1	-	1
Ringed seal	Russia	1995	M/adult	4.2	3.6	2
Harbour seal	Southern Norway	1998/99	Both/various	4.8-56	1-5	3
Harbour seal	Sweden	1988	M/adult	28-82	7-10	4
Harbour seal	St. Lawrence River, Canada	1994-96	M/adult	54	9	5
Harbour seal	St. Lawrence River	1994-96	M/pup	18	5	5
Harbour seal	Strait of Georgia, B.C.	1996-97	Both/pups	2.4	-	6
Harbour seal	Puget Sound, Washington	1996-97	Both/pup	18	-	6
Cetaceans						
N. Resident killer whale	British Columbia, Canada	1993-96	M/various	37.4	-	7
S. Resident killer whale	British Columbia	1993-96	M/various	146.3	-	7
Transient killer whale	British Columbia	1993-96	M/various	251.2	-	7
Beluga whale	St. Lawrence River	1987-90	M/various	8-412	3-389	8
Sperm whale	Spain	1979-80	M/various	10	5	9

Values are presented as the arithmetic mean or range, expressed as mg/kg lipid in blubber.
[1] Chiba *et al.*, 2002; [2] Nakata *et al.*, 1998, [3] Ruus *et al.*, 2002; [4] Blomkvist *et al.*, 1992; [5] Bernt *et al.*, 1999; [6] Ross *et al.* (submitted); [7] Ross *et al.*, 2000; [8] Muir *et al.*, 1996; [9] Aguilar, 1983.

1.1.3 Toxicity of organochlorines

Organochlorines are known to produce several toxic responses in living organisms. Toxic responses observed in laboratory animals and wildlife species include immunosuppressive effects, reproductive and developmental toxicity, carcinogenesis, endocrine disruption, and neurotoxicity (Colborn *et al.*, 1993; Safe, 1994). In marine mammals, OCs have been implicated in reproductive, developmental, and immunological deficiencies (Ross *et al.*, 1996). PCBs have been associated with decreased reproductive success in pinnipeds (DeLong *et al.*, 1973; Helle *et al.*, 1976a,b; Reijnders, 1986). In California sea lions (*Zalophus californianus californianus*), PCB and DDT concentrations in blubber were 6.6 and 8.0 times higher in females that gave birth to premature pups compared to those that gave birth to full-term pups (DeLong *et al.*, 1973). Although these results are suggestive of an association between PCB blubber levels and reproductive success, they may be partly artifactual. Females that successfully reproduce have lower OC levels than those that abort because OCs are off-loaded during lactation. In Baltic ringed seals (*Phoca hispida baltica*), pathological changes of the uterus were associated with higher contaminant levels (Helle *et al.*, 1976a,b). In one study, higher levels of PCBs and DDT were found in non-pregnant females compared to pregnant seals, and half of non-pregnant seals had enlarged uteri and scars in the uterine wall, suggesting that abortion or resorption had occurred following implantation (Helle *et al.*, 1976a).

Béland *et al* (1993) found a number of abnormalities in beluga whales in the St. Lawrence River that may have been associated with high contaminant levels. These whales had a high prevalence of tumours and lesions to the digestive system and other

glandular structures. No such lesions were found in Arctic belugas, which had PCB and DDT concentrations that were 18 and 12 times lower than St. Lawrence whales (Béland *et al.*, 1993). In samples from the same contaminated population, environmentally relevant concentrations of organochlorines were shown to reduce lymphocyte proliferation *in vitro* (De Guise *et al.*, 1998).

Impaired immunological function was observed in captive harbour seals (*Phoca vitulina)* with chronic exposure to environmental contaminants accumulated through the diet. A number of immunological effects, including diminished natural killer cell activity and T-cell function, were observed in seals fed a diet of herring from a relatively contaminated region (De Swart *et al.*, 1994; Ross *et al.*, 1996). An immunotoxic threshold of 17 mg/kg lipid weight was established for PCBs in these captive harbour seals. As shown in Table 1.1, blubber PCB concentrations exceed this threshold in a number of marine mammal species.

1.1.3.1 The Aryl hydrocarbon receptor

Many of the biochemical and toxic effects of PCBs, PCDDs, and PCDFs are mediated by binding to the aryl hydrocarbon receptor (AhR). The resulting receptor-ligand complex acts as a nuclear transcriptional enhancer for gene expression of metabolic enzymes, such as cytochrome P4501A (Safe, 1994; Whitlock and Denison, 1995). The unligated receptor consists of two 90 kDa heat-shock protein molecules (hsp90) and other proteins. Binding of a ligand to the receptor results in dissociation of hsp90 and association with an AhR nuclear translocator protein (ARNT). Activation of transcription of TCDD-responsive genes occurs via a dioxin-responsive enhancer (DRE) sequence (Hahn, 1998).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has the highest known affinity for AhR binding and is considered to be the most potent compound among PCBs, PCDDs, and PCDFs (Safe, 1994). A mechanistic link between TCDD-AhR binding and toxicity has been shown using AhR knockout mice (Fernandez-Salguero *et al.*, 1996). No significant toxic or pathologic effects were observed in AhR knockout mice at a dose of TCDD ten-fold higher than that shown to cause pathological changes in the liver and thymus of wild-type mice (Fernandez-Salguero *et al.*, 1996).

Aryl hydrocarbon receptors have been detected in all vertebrate classes (fish, reptiles, amphibians, birds, mammals) and have been cloned in a number of vertebrate species. In marine mammals, full length AhR cDNA sequences have been reported for beluga whales, harbour seals, and Baikal seals (*Phoca sibirica)* (Jensen and Hahn, 2001; Kim and Hahn, 2002; Kim *et al.*, 2002). Harbour seal and beluga whale AhR proteins are 82% identical in overall amino acid sequence (Kim and Hahn, 2002). It has been shown that the AhR in beluga whales and harbour seals exhibit specific binding of [^{3}H]TCDD and bind [^{3}H]TCDD with an affinity at least as high as mouse AhR (Jensen and Hahn, 2001; Kim and Hahn, 2002). This information suggests that the AhR is highly conserved among vertebrates and that dioxin-mediated toxicities also occur in marine mammals.

Toxic equivalency factors (TEFs) compare the ability of coplanar PCBs and 2,3,7,8-substituted PCDDs and PCDFs to bind to the Ah receptor and to elicit AhR-mediated toxic responses relative to 2,3,7,8-TCDD. 2,3,7,8-TCDD has been assigned a TEF of 1.0 (Van den Berg *et al.*, 1998), and TEFs have been assigned to other planar PCB, PCDD, and PCDF congeners for mammals, birds, and fish, based on their potency relative to 2,3,7,8-TCDD (Table 1.2). These TEFs have been established by the World

Health Organization based on dose-response studies in the literature (Van den Berg *et al.*, 1998). For mammals, TEF values are largely based on *in vivo* studies in rodents (Van den Berg *et al.*, 1998). Where TEF values have been determined for several different AhR-mediated responses (eg. enzyme induction, carcinogenicity, reproductive toxicity), a mean TEF is calculated and an overall TEF is assigned (Safe, 1994). The contribution of an individual compound to the overall toxicity of a contaminant mixture is calculated by multiplying the concentration of the compound in an environmental sample (eg. water or tissue) by its TEF. The resulting value is the toxic equivalent (TEQ). Toxic equivalents for individual congeners are added to give an overall TEQ for a contaminant mixture. The equation for calculating the TEQ of a chemical mixture is as follows, where n = number of congeners:

$$TEQ=\sum [PCDD_i \times TEF_i]_n + \sum ([PCDF_i \times TEF_i])_n + \sum ([PCB_i \times TEF_i])_n$$

There are many other halogenated compounds besides PCDDs, PCDFs, and coplanar PCBs that meet the criteria for inclusion in the TEF concept; however, there is currently insufficient data to be able to assign TEFs to those compounds (Van den Berg *et al.*, 1998). TEFs also do not account for non-AhR-mediated toxicities or possible synergistic or antagonistic interactions between congeners.

Table 1.2: Toxic Equivalency Factors (TEFs). The most recent World Health Organization TEFs for mammals, as published by Van den Berg *et al.* (1998). International Union of Applied Chemistry (IUPAC) numbers are included for PCBs.

Organochlorine	Structure (PCB IUPAC #)	Mammalian TEFs
PCDDs	2,3,7,8-tetraCDD	1.0
	1,2,3,7,8-pentaCDD	1.0
	1,2,3,4,7,8-hexaCDD	0.1
	1,2,3,6,7,8-hexaCDD	0.1
	1,2,3,7,8,9-hexaCDD	0.1
	1,2,3,4,6,7,8-heptaCDD	0.01
	octaCDD	0.0001
PCDFs	2,3,7,8-tetraCDF	0.1
	2,3,4,7,8-pentaCDF	0.5
	1,2,3,7,8-pentaCDF	0.05
	1,2,3,4,7,8-hexaCDF	0.1
	2,3,4,6,7,8-hexaCDF	0.1
	1,2,3,6,7,8-hexaCDF	0.1
	1,2,3,7,8,9-hexaCDF	0.1
	1,2,3,4,6,7,8-heptaCDF	0.01
	1,2,3,4,7,8,9-heptaCDF	0.01
	octaCDF	0.0001
Non-*ortho* PCBs	3,3′,4,4′-tetraCB (77)	0.0001
	3,4,4′,5-tetraCB (81)	0.0001
	3,3′,4,4′,5-pentaCB (126)	0.1
	3,3′,4,4′,5,5′-hexaCB (169)	0.01
Mono-*ortho* PCBs	2,3,3′,4,4′-pentaCB (105)	0.0001
	2,3,4,4′,5-pentaCB (114)	0.0005
	2,3′,4,4′,5-pentaCB (118)	0.0001
	2′,3,4,4′,5-pentaCB (123)	0.0001
	2,3,3′,4,4′,5-hexaCB (156)	0.0005
	2,3,3′,4,4′,5′-hexaCB (157)	0.0005
	2,3′,4,4′,5,5′,-hexaCB (167)	0.00001
	2,3,3′,4,4′,5,5′-heptaCB (189)	0.0001

1.1.3.2 Metabolism of organochlorines

Marine mammals are able to metabolize certain organochlorine compounds. In pinnipeds and cetaceans, PCB congeners that attain a planar configuration are more readily metabolized (Tanabe *et al.*, 1988; Boon *et al.*, 1997). The first indication that marine mammals selectively metabolize OC congeners came from studies in which PCB patterns were observed to differ between marine mammals and their prey (fish). Boon *et al.* (1997) concluded that differences in congener patterns between predator and prey can be explained by the availability of PCBs from food and their subsequent biotransformation by metabolic enzymes (the cytochrome P450 enzyme system, discussed in section 1.2.1) (Boon *et al.*, 1987; Boon *et al.*, 1994). Boon *et al.* (1997) also observed concentration-dependent changes in PCB patterns within various marine mammals species. This observation indicated that metabolic enzymes were induced by increased PCB concentrations, resulting in increased metabolism of certain congeners in these animals (Boon *et al.*, 1997). Another important finding was that PCB congener patterns differed between marine mammals and terrestrial animals that feed at the top of the food chain, suggesting that the metabolic capacity for specific congeners varies between species (Tanabe *et al.*, 1988). Lower metabolic enzyme activities observed in marine mammals compared to terrestrial mammals, for example, may lead to higher bioaccumulation of certain PCB congeners in marine mammals (Tanabe *et al.*, 1988).

Studies with captive harbour seals have provided more direct evidence for metabolism of PCDDs and PCDFs in this species. During a 15-day fasting experiment, body burdens of 2,3,7,8-TCDD and 2,3,7,8-TCDF were less than 10% of the estimated cumulative intake of these compounds from herring. The half-life of 2,3,7,8-TCDD was

estimated to be less than one week, as blood concentrations decreased from 2.4 ng/kg lipid to less than half that after a week of fasting, presumably due to metabolism and excretion of this contaminant (De Swart *et al.*, 1995). However, in free-ranging pinniped populations, relatively high levels of PCDDs and PCDFs have been found (eg. in British Columbia), likely reflecting continued contamination of prey from point sources such as pulp mills and urban environments (Ross and Troisi, 2001). For example, mean total PCDD and PCDF concentrations in blubber of nursing harbour seal pups from British Columbia were 187 and 20 ng/kg lipid respectively (Simms *et al.*, 2000); whereas, in grey seals from the east coast of Canada, PCDD and PCDF concentrations were 12 and 8 ng/kg lipid, respectively, in mothers, and 7 and 2 ng/kg, respectively, in pups (Addison *et al.*, 1999).

There is also evidence to suggest that killer whales metabolize dioxin-like compounds. Ross *et al.* (2000) found that PCDDs and PCDFs were present at low levels in blubber compared to PCBs (eg. mean total PCB concentration was 37 mg/kg lipid in male northern residents; mean total combined PCDD and PCDF concentrations in males and females were 1050 ng/kg and 258 ng/kg lipid, respectively), and there were no differences in TEQ concentrations for these compounds between immature and adult animals or between adult males and adult females.

1.2 Biomarkers

Biomarkers are biological responses to one or more chemicals that give a measure of exposure and sometimes of toxic effect (Peakall, 1994). In contrast to the classical approach to environmental toxicology, in which contaminants measured in environmental compartments (i.e. water and sediments) are related to adverse effects observed in experimentally treated laboratory animals (Peakall, 1994), biomarkers can provide a measurement of response to contaminant exposure that is biologically relevant in the species of interest.

Characteristics of an ideal biomarker include the following (WHO, 1993; Oikari *et al.*, 1993):

- reflects interaction of the host biological system with the chemical(s) of interest;
- has known (high) specificity and sensitivity to the interaction;
- is common to individuals in a population and across species, and is of defined variability within the normal, non-exposed group of interest;
- has a validated method that is simple, inexpensive, and reproducible, and measurements have defined and appropriate accuracy and precision; and
- is non-lethal and minimally-invasive

Biomarkers may be biochemical, physiological, histological, morphological, or behavioural. Commonly studied biomarkers include:

- induction of metallothionein in response to cadmium exposure (Addison, 1996);
- inhibition of acetylcholinesterase by exposure to organophosphates and carbamates (Addison, 1996);

- disruption of vitamin A homeostasis (Simms *et al.*, 2000b);
- disruption of the thyroid hormone system (Brouwer, *et al.*, 1989) ; and
- induction of monooxygenases in response to organochlorine and PAH exposure (Addison, 1996)

In this study, we will look at cytochrome P450 1A (CYP1A), a monooxygenase, as a biomarker of organochlorine exposure.

1.2.1 Cytochrome P450 1A

1.2.1.1 Background

CYP1A belongs to a superfamily of monooxygenases that catalyze the metabolism of lipophilic endogenous and xenobiotic compounds to more water-soluble products. Due to its wide range of substrates, the CYP enzyme system is one of the most important xenobiotic metabolizing systems in vertebrates (Schenkman, 1999). A substrate binds to the heme-containing CYP enzyme, and the enzyme then accepts an electron from NADPH via NADPH-cytochrome P450 reductase. Oxygen binds to the reduced hemoprotein, forming the oxycytochrome P450, and then accepts a second electron from the reductase, or from ferrous cytochrome b_5. Protonation and cleavage of the O-O bond produces a molecule of water, and the remaining oxygen atom that is bound to iron is transferred to the substrate. The oxidized substrate is subsequently released from the CYP enzyme (Schenkmann, 1999; Bandiera, 2001).

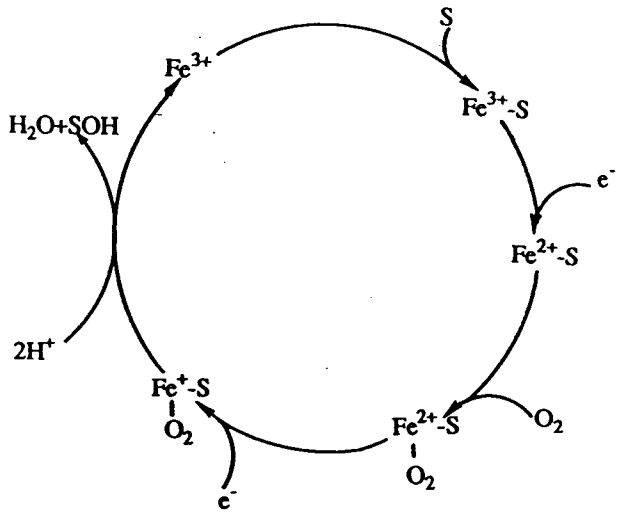


Figure 1.2: The CYP cycle, where S = substrate (Substrate + NADPH + H^+ + O_2 → Substrate-O + $NADP^+$ + H_2O) (from Lewis, 1996).

Although the physiological role of CYP1A is to detoxify potentially harmful compounds, there is evidence to suggest that high CYP1A activity in itself can lead to adverse health effects (Barouki and Morel, 2001), and a mechanistic link has been established between CYP1A induction and toxicity (Poland and Knutson, 1982; Safe 1984). Two mechanisms by which CYP1A can lead to toxicity are the production of toxic metabolites (eg. hydroxylated and methylsulfonyl PCBs) (Brouwer *et al.*, 1989; Bergman *et al.*, 1997; Letcher *et al.*, 2000) and the production of reactive oxygen species (ROS) (Palace *et al.*, 1996). Hydroxylated PCBs can interfere with endocrine transport systems in laboratory animals, such as by competing with thyroxine for the binding site on the thyroxine-transporting protein in the circulation (Bergman *et al.*, 1997). Such a mechanism is also suspected in contaminant-associated disruption of vitamin A and thyroid hormone homeostasis in captive and free-ranging harbour seals (Simms *et al.*,

2000b; Brouwer *et al.*, 1989; De Swart *et al.*, 1994). Methylsulfonyl-PCBs have been shown to interfere with enzyme activity and endocrine-related processes in rats and mice (Letcher *et al.*, 2000). In addition, induction of CYP activity has been shown to result in the proliferation of oxyradicals, which can cause cellular damage, such as the breakdown of lipid membranes (Palace *et al.*, 1996).

1.2.1.2 CYP1A as a biomarker of organochlorine exposure

CYP1A induction is probably the most widely used biomarker of organochlorine exposure, as it is specific for a limited group of organic chemicals, and its mechanism of response to contaminant exposure is relatively well understood (Addison, 1996) (refer to Figure 1.3). Synthesis of certain CYP enzymes is known to be induced by exposure to certain classes of environmental contaminants, and CYP1A induction has been used as a biomarker of exposure to dioxin-like chemicals (the planar PCCDs, PCDFs, and PCBs) in fish, birds, and mammals (Rattner *et al.*, 1989; Bosveld and Van den Berg, 1994). The CYP1A enzymes are regulated by the Ah receptor and catalyze the metabolism of planar aromatic and chlorinated hydrocarbons such as non-*ortho*- and mono-*ortho*-substituted PCBs and benzo[a]pyrene (Van den Berg *et al.*, 1998).

CYP1A induction is typically measured in terms of enzyme activity, protein concentration, or mRNA concentration (Table 1.3). CYP catalytic activity can be measured using several different mixed function oxidase (MFO) enzyme systems. The MFOs typically used to measure CYP1A activity are ethoxyresorufin *O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH), also referred to as benzo[a]pyrene hydroxylase or benzo[a]pyrene monooxygenase (BPMO).

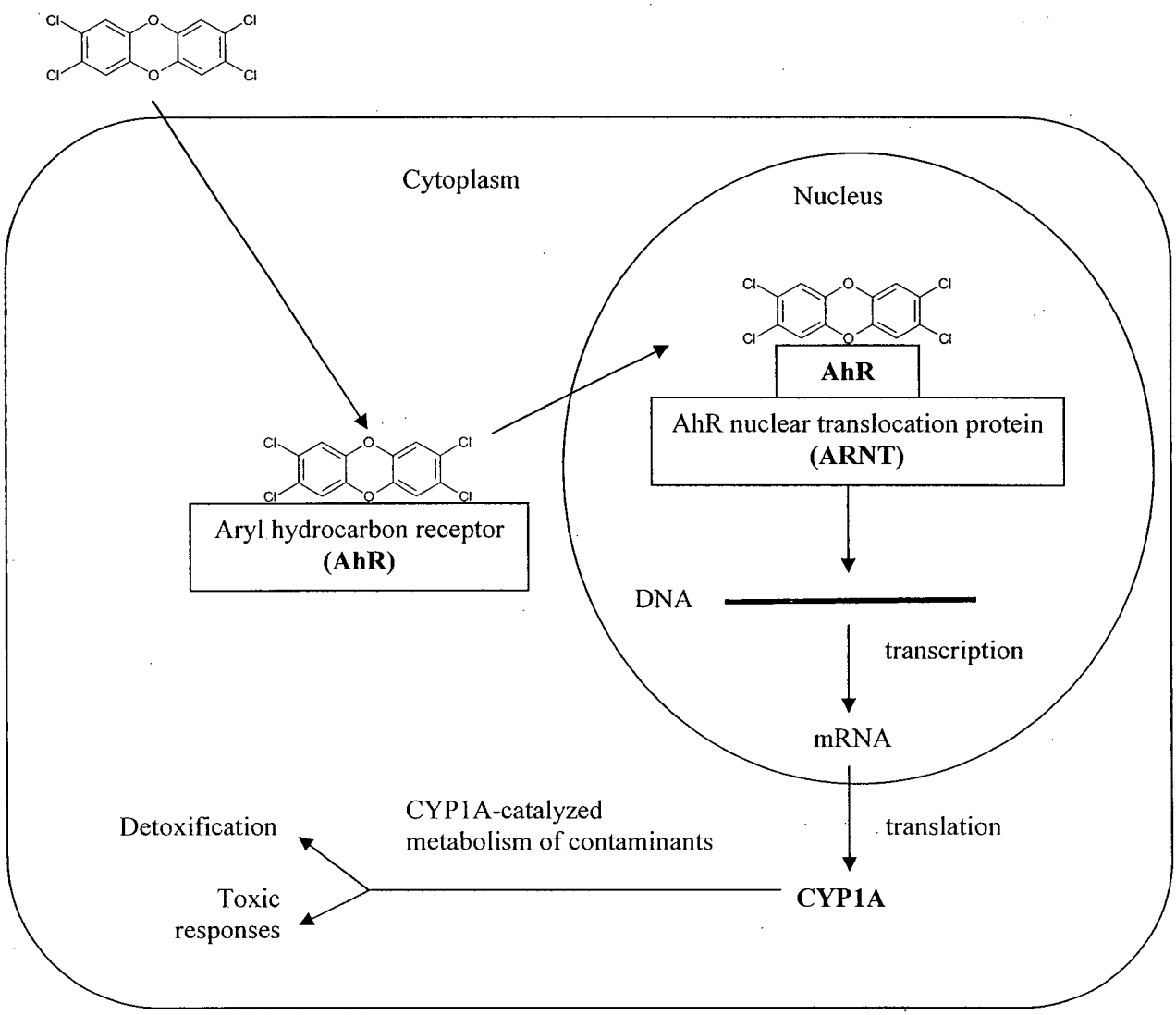


Figure 1.3: Simplified mechanism of CYP1A induction. Signal transduction by dioxin-like ligands is mediated by the Ah receptor, which forms a transcription factor complex with ARNT. This heterodimer binds to specific DNA sequences called dioxin responsive elements (DRE), resulting in transcription of the CYP1A gene and synthesis of CYP1A protein (Whyte *et al.*, 2000).

Table 1.3: Summary of the most common methods used to measure CYP1A induction

Measurement	Method
CYP1A protein concentration	Immunoblotting
	Immunohistochemistry
CYP1A enzyme activity	Ethoxyresorufin *O*-deethylase (EROD)
	Aryl hydrocarbon hydroxlyase (AHH)
CYP1A mRNA concentration	Northern blotting
	Reverse transcription polymerase chain reaction (RT-PCR)

Hepatic CYP1A catalytic activity and CYP1A protein levels have been measured both in pinnipeds (Goksoyr *et al.*, 1995; Troisi and Mason, 1997; Mattson *et al.*, 1998; Nyman *et al.*, 2000) and cetaceans (Watanabe *et al.*, 1989; Goksoyr *et al.*, 1995), and several studies have found correlations between catalytic activity, CYP1A levels, and contaminant burdens (Goksoyr *et al.*, 1995; Troisi and Mason, 1997; Mattson *et al.*, 1998; Nyman *et al.*, 2000). Correlations between contaminant levels and CYP1A expression suggest that CYP1A induction occurs in these animals.

Mattson *et al.* (1998) compared CYP1A activities and apoprotein levels in Baltic ringed seals from an area with high organochlorine pollution (n=17) versus seals from a relatively unpolluted area (n=19). EROD activities were three times greater in seals from the heavily polluted region than in seals from the less polluted region (~800 pmol/mg protein/min versus ~250 pmol/mg/min). Polluted and reference regions were chosen based on contaminant data from previous studies. In another study, a five-fold difference in hepatic EROD activity was observed in grey seals (~1200 versus 250 pmol/mg/min) and a three-fold difference in ringed seals (~1500 versus 500 pmol/mg/min) between

heavily polluted and reference areas (Nyman *et al.*, 2000). In the same study, EROD activity and CYP1A1 protein levels were positively correlated in liver. This correlation has also been observed in other seal species, including harbour and hooded seals (Goksoyr *et al.*, 1995). Troisi and Mason (1997) investigated cytochrome P450 enzymes and mixed function oxidase activity as biomarkers of PCB exposure in harbour seal pups that had died of bacterial infections and physical injuries. Hepatic EROD activity and total PCB blubber levels were positively correlated for these animals (n=5), though the poor health condition of these seals may have been a confounding factor.

It is often not possible to make direct inter-study comparisons of CYP1A expression in marine mammals. Different protocols for measuring CYP1A levels exist, and these have not been standardized among laboratories. Immunoblotting results are dependent on the antibodies used and the methods used to quantify protein bands. CYP1A enzyme activities cannot be easily compared between studies if different enzyme assays are used (eg. EROD vs. AHH), or if activities are reported in different units. Additionally, comparison of CYP1A expression in marine mammals is often problematic because factors such as species, age, sex, and health status (condition) also need to be considered.

1.2.1.3 CYP1A in skin

Due to its relatively large size and high monooxygenase activity (Rattner *et al.*, 1989), the liver has been the focus of most CYP induction studies in both laboratory animals and wildlife. Liver samples used in the marine mammal studies cited above were obtained from stranded animals, from animals killed during commercial or subsistence harvests, or from animals killed for scientific purposes. Killing wild animals in order to

obtain tissue samples is generally unacceptable from an ethical standpoint, and is often illegal. Samples from stranded animals may not provide accurate information due to degradation of tissues after death, or the fact that deceased animals comprised a non-normal cross-section of the population (i.e. old, diseased). For these reasons, the development of non-lethal (minimally-invasive) tissue sampling techniques is essential for biomarker research. For marine mammals, skin and blubber are the only tissues that can be readily obtained from live, free-ranging animals.

To date, CYP1A expression in marine mammal skin and its usefulness as a biomarker of organochlorine exposure has not been well described. Fossi *et al.* (1997) and Marsili *et al.* (1998) reported detection of CYP1A induction in skin biopsies of southern sea lions (*Otaria flavescens*) and Mediterranean fin whales (*Balaenoptera physalus*), respectively. Fossi *et al.* compared epidermal CYP-mediated benzo[a]pyrene monooxygenase (BPMO) activity in free-ranging male sea lions from a region contaminated by oil and organic and chemical materials, and a control region. BPMO activity was approximately three to four times higher in skin samples from the heavily polluted area (n=12) compared to those from the control site (n=4). In one stranded sea lion, BPMO activity in skin was found to be one-fifth of that in liver (Fossi *et al.*, 1997). Unfortunately, enzyme activity was measured as BPMO activity and expressed as fluorescence units/hour/g tissue, which precluded a comparison of activity values with most other studies. Marsili *et al.* (1998) reported a positive correlation between epidermal BPMO activity and blubber DDT and PCB concentrations in male fin whales (n=14). However, the ages of the individual male fin whales were unknown. Age may

be a confounding factor in this study, as OC concentrations in blubber generally increase in males with age.

Cutaneous induction of CYP1A after topical application of known CYP inducers has been demonstrated in laboratory rodents (Ichikawa, 1989; Khan *et al.*,1992; Jugert, 1994; Agarwal *et al.*, 1994). However, our study represents the first such attempt *in vivo* in a marine mammal species. In river otters (*Lontra canadensis*) treated with crude oil, CYP1A1 expression measured by immunohistochemical staining increased approximately four-fold in skin biopsies (Ben-David *et al.*, 2001). Godard *et al.* (2002) reported induction of CYP1A in sperm whale (*Physeter macrocephalus*) skin biopsies using immunohistochemistry following *in vitro* BNF treatment. These data have yet to be published.

1.2.1.4 Species specificity of CYP1A

Certain forms of CYP enzymes are species-specific (Gonzalez, 1989). However, cloning and expression studies have shown that some CYP genes are conserved across species, and the major microsomal CYP gene families (1, 2, 3, and 4) are found in all vertebrates studied, from fish to mammals (Stegeman and Livingstone, 1998). Some CYP families, such as CYP2, are quite diverse, and it is difficult to determine homologous gene lineages between vertebrate species. Others, such as the CYP1 family, are well-conserved among species, and antibodies prepared against purified CYP isozymes in one vertebrate species can be used to identify and characterize CYPs in other vertebrate species (Ronis *et al.*, 1989a,b; Bandiera *et al.*, 1995; Lin *et al.*, 1998). Additionally, the induction of CYP1A by xenobiotic compounds appears to represent a common response among vertebrates (Stegeman and Livingstone, 1998).

In mammals, the CYP1A subfamily consists of two isozymes, CYP1A1 and CYP1A2 (Nebert *et al.*, 1991). These two enzymes have been identified in rats, mice, rabbits, humans, and some marine mammals, but there is uncertainty as to whether the two forms exist in all mammals. Wolkers *et al.* (1998) observed cross-hybridization of only one CYP1A mRNA band in ringed seal liver with human CYP1A cDNA probes, and one CYP1A protein band cross-reacted with an anti-rat CYP1A antibody on immunoblots. Goksøyr *et al.* (1992) detected two CYP1A protein bands in both harp (*Phoca groenlandica*) and hooded seal (*Cystophora cristata*) liver using an anti-cod CYP1A1 antibody. However, Nyman *et al.* (2000) were unable to distinguish between CYP1A1 and CYP1A2 protein bands in ringed and grey seal liver. Chiba *et al.* (2002) detected two hepatic CYP1A protein bands in largha seals (*Phoca largha*) and ribbon seals (*Phoca fasciata)*, using a polyclonal anti-rat CYP1A1 antibody. In whales, White *et al.* (1994) reported a single hepatic CYP1A band in beluga using a polyclonal anti-mouse CYP1A1 antibody, Goksøyr *et al.* (1995) detected two CYP1A bands in minke whale (*Balaenoptera acutorostrata*) liver using anti-cod CYP1A antibodies, and Boon *et al.* (2001) reported cross-reactivity of anti-mouse and anti-rat antibodies with a single hepatic CYP1A protein band in sperm whale. Given the results cited above, it is likely that both CYP1A forms (1A1 and 1A2) exist in marine mammals, but that the specificity of CYP1A probes and the sensitivity of the techniques used may not be adequate to detect both forms in all cases.

The first CYP1A protein sequences in marine mammals have only recently been identified (Teramitsu *et al.*, 2000; Tilley *et al.*, 2002). Antibodies for marine mammal CYP proteins are therefore not yet commercially available, and immunodetection of

marine mammal CYP1A remains dependent on cross-reactivity with anti-CYP antibodies from other animal species. To date, hepatic CYP1A1 cDNA fragments from minke whale, dall's porpoise (*Phocoenoides dalli*), largha seal, ribbon seal, grey seal, and harp seal, and CYP1A2 cDNA fragments from steller sea lion (*Eumetopias jubatus*), grey seal, and harp seal have been cloned (Termamitsu *et al.*, 2000; Tilley *et al.*, 2002). CYP1A1 sequences were >99% identical between grey and harp seals and identical between largha and ribbon seals, but seals and cetaceans belong to separate genetic lineages, with gene sequences in seals being most similar to those of dogs (Termamitsu *et al.*, 2000; Tilley *et al.*, 2002).

1.2.1.5 Natural factors affecting CYP1A expression

Although contaminants have been shown to influence CYP1A expression, numerous physiological factors, such as age, diet, reproductive status, and health status also influence levels and activities of CYP enzymes (Mattson *et al.*, 1998). Age can influence CYP1A expression if genes coding for CYP1A enzymes are under developmental control. Developmental control of hepatic CYP1A expression has been demonstrated in rats, rabbits, fish, and humans (Mukhtar and Bickers, 1983; Strom *et al.*, 1992; Rich *et al.*, 1993; Sonnier and Cresteil, 1998; Sarasquete *et al.*, 2001). In general, hepatic CYP1A is absent or occurs at low levels in fetal and neonatal animals and increases with age.

In marine mammals, limited information is available on the developmental regulation of CYP expression. In minke whales, hepatic EROD activity was about 80% lower in fetal samples than in juveniles and adults, but turnover of ethoxyresorufin (activity/nmol P450) was found to be higher in fetal samples than in most of the adult

samples (Goksøyr, 1986). In grey seals, liver EROD activity was not detectable in seals less than one day old; EROD activity increased with age up to 5-12 days post-partum and was lower than that of adults (Addison and Brodie, 1984b). In harbour seals, lower EROD activity was observed in newborn pups and fetuses than in adults (Addison *et al.*, 1986). Mattson *et al.* (1998) found that hepatic EROD activity in Baltic ringed seals showed a weak positive correlation with age.

A variety of other physiological factors have been shown to affect CYP expression. Dietary constituents such as carotenoids and flavenoids have been shown to induce CYP1A enzymes in rodents (Gradelet *et al.*, 1996; Breinholt *et al.*, 1999). Health status may also affect CYP1A expression, as hepatic CYP activities are affected (generally suppressed) by infection and by various inflammatory stimuli (Morgan, 1997). These factors will be addressed more fully in the discussion section.

In adult marine mammals, CYP1A expression may be influenced by the length of time an animal has had to accumulate lipophilic contaminants (i.e. age). However, accumulation of contaminants is also affected by sex and reproductive status. In marine mammals, tissue concentrations of contaminants increase with age in males, but reproductive females offload contaminants to their offspring through lactation (Ross and Troisi, 2001).

Conflicting results between studies and a lack of data in marine mammals often makes interpretation of CYP1A expression difficult and speculative. An association of contaminant levels with effects is often precluded in marine mammal studies due to a lack of control over confounding factors, particularly age and condition. It is, therefore, extremely important to eliminate as many potential confounding factors as possible (eg.

age, sex, condition, reproductive status) when evaluating CYP1A expression as a response to contaminant exposure.

1.3 Study Overview

1.3.1 Description of study species

1.3.1.1 Harbour seals (Phoca vitulina)

Harbour seals are widely distributed in temperate coastal waters of the Northern Hemisphere. In southern British Columbia and Washington State, harbour seals are non-migratory and often inhabit shallow areas where sandbars and beaches are uncovered at low tide. They are generally solitary but loosely aggregate at haul-out sites while resting. Since 1970 harbour seals have been protected in British Columbia, and numbers increased from an estimated 10,000 animals in 1970 to 80-90,000 in 1988. The current harbour seal population in B.C. is estimated at 108,000 (Olesiuk, 1999). There are approximately 15,000 harbour seals in the inland waters of Washington State (Jeffries *et al.*, in press). Hake and herring dominate the harbour seal diet in B.C. and Washington State, but other fish, molluscs, and crustaceans are also consumed (Olesiuk, 1993). Females bear one pup per year with a peak pupping season in July or August, depending on location. Lactation lasts for four to six weeks, after which time the pup is abandoned by its mother. At birth, the average weight of seals is about 11 kg, and this weight is doubled by weaning time (Cottrell *et al*, 2002). Most females mature at three to four years of age, and most males by five years of age. The average weight of fully grown females is 65 kg and males average 87 kg (Bigg, 1969).

1.3.1.2 Killer whales (Orcinus orca)

Two sympatric populations of killer whales, residents and transients, frequent the coastal waters of British Columbia. Resident killer whales feed on fish, principally salmonids. Transients feed almost exclusively on pinnipeds and cetaceans (Ford *et al.*, 1998). Male killer whales live an estimated fifty to sixty years and females eighty to ninety years. Females bear one calf every three to five years (Ford *et al.*, 1998). The resident population consists of two distinct subpopulations referred to as the northern and southern residents. The northern and southern resident subpopulations are currently estimated at 200 and 81 individuals, respectively (Ellis, pers. comm.) The transient population is estimated at 220 individuals (Ellis, pers. comm). The southern resident population has declined from a peak of 99 individuals, and there is concern about the possible causes of this decline. The Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has identified three major threats to southern resident killer whales: boat traffic (noise and physical interference), a diminishing food supply, and high levels of toxic contaminants.

1.3.2 Challenges associated with wildlife toxicology studies

In contrast to working with laboratory animals in a controlled environment, there are considerable challenges associated with studying free-ranging wildlife species. Obtaining samples from marine mammals is costly and requires a great deal of time, effort and logistical support. Sampling is also dependent on uncontrollable factors such as weather, ocean conditions, and the behaviour of the animals. Because of these factors, it may not always be possible to obtain the desired sample size or samples from a desired

cohort. Additionally, many marine mammals are protected under Canadian law, and therefore, must be handled under the scrutiny of Animal Care and Scientific Permitting bodies. The development and application of minimally-invasive sampling approaches is, therefore, necessary in order to minimize the impact that research has on these animals.

Adequate preservation of tissue samples in the field is also important. Supplies, such as liquid nitrogen, must be taken into the field, and extra care must be taken to maintain relatively sterile conditions when handling samples. The amount of information that can be obtained from these animals in the field is also limited. Body measurements such as length and weight can be determined for live-captured harbour seals, but specific ages can only be determined if a tooth is removed, and aging cannot be done until post-sampling.

After sampling is completed, tissue samples must be used efficiently. Samples are often divided up for several different studies, and due to the small amount of tissue available, it is not always possible to use study samples for validation and optimization experiments. It may be necessary to use archived samples from dead animals instead. This is not ideal, as there may be differences between archived samples and fresh samples (eg. archived samples may not have been preserved immediately after death of the animal or degradation of tissue components may have occurred during prolonged storage).

Toxicological studies in which the aim is to identify a cause-effect relationship between contaminant exposure and a biological response (biomarkers) are especially challenging when working with marine mammals. In general, the animals' environment cannot be controlled (eg. diet, sources of environmental contaminant exposure), and

therefore, it is more difficult to identify specific agents responsible for the observed biological responses. Captive studies of wild marine mammals are expensive and logistically challenging. Permits must be obtained, and individuals trained in the care of these animals must be employed. In order to evaluate a particular biomarker, it is helpful, if not necessary, to have baseline data for this response in the species of interest and such information is usually not available for marine mammals. Additionally, it is more difficult to obtain positive controls for toxicological studies in marine mammals, as it is usually not acceptable to treat these animals with toxic compounds.

Toxicological studies in free-ranging marine mammals are extremely valuable to understanding the effects of contaminant exposure in these animals. Such studies provide 'real-world' relevance (eg. exposure to complex contaminant mixtures) that laboratory animal studies lack (Ross, 2000). Figure 1.4 summarizes the various approaches that contribute to our understanding of contaminant effects in marine mammals.

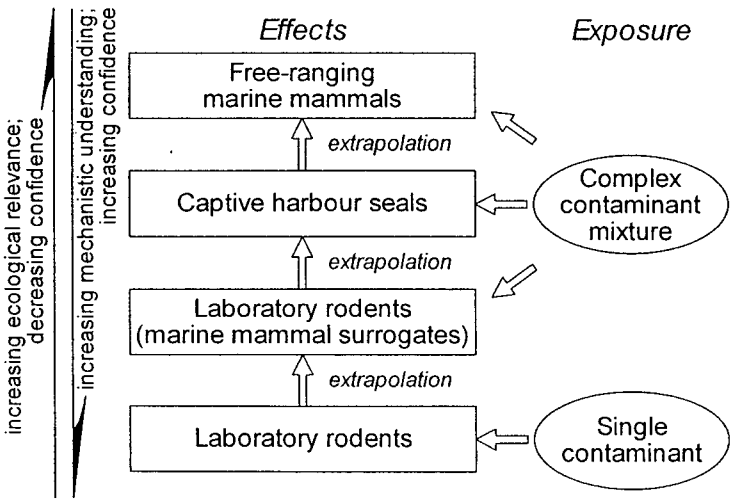


Figure 1.4: A 'weight of evidence' approach is required to assess the risk of toxic injury in free-ranging marine mammals. This involves extrapolation between single chemical laboratory rodent studies, surrogate studies using rodents as seals, captive feeding studies of seals fed complex contaminant mixtures, and observational and minimally-invasive biomarker studies of free-ranging marine mammal populations (from Ross, 2000).

1.3.3 Overall goals of the present study

The importance of ecotoxicological studies in marine mammals is twofold. First, information from such studies may provide information about the health of marine mammal populations. Causal links between contaminants and events such as virus-related mass mortalities of marine mammals may be established, or alternatively ruled out. Second, toxicological information obtained from marine mammals can provide an indication of the overall health of the marine environment.

The objectives of this study were:

- to develop techniques to measure CYP1A protein levels and enzyme activity in harbour seal and killer whale skin biopsies
- to determine if CYP1A could be used as a biomarker of organochlorine exposure in harbour seals and killer whales

Our study will focus on the development of minimally-invasive sampling techniques for free-ranging marine mammals. No CYP studies have been published on marine mammals in British Columbia, and to our knowledge, no studies have been published on CYP1A in skin of harbour seals or killer whales from any location. Only one report exists of CYP1A expression in killer whales (CYP1A enzyme activity was detected in liver of killer whales from Japanese waters) (Watanabe *et al.*, 1989). We used the harbour seal as a model for method development in marine mammals. Relative to other marine mammal species, harbour seals are locally abundant and easy to sample, and they are the subject of ongoing toxicological studies in our laboratory (Simms *et al.*, 2000a,b; Mos and Ross, 2002).

1.4 Research Hypothesis

Skin provides a reliable measure of systemic (hepatic) CYP1A in harbour seals and can be used as an effective biomarker of organochlorine exposure in harbour seals and killer whales.

1.5 Experimental Hypotheses

1) CYP1A can be measured in both liver and skin biopsies from harbour seals and in skin biopsies from killer whales.
2) CYP1A induction in harbour seal skin is positively correlated with CYP1A induction in liver.
3) CYP1A induction in skin is positively correlated with organochlorine concentrations in blubber.

1.6 Specific Objectives

- to establish protocols for homogenizing small liver and skin samples obtained by biopsy from harbour seals;
- to measure ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A protein levels in microsample homogenates of liver and skin from harbour seals, using a plate reader assay and immunoblotting, respectively;
- to measure CYP1A induction in liver and skin of ß-naphthoflavone (BNF; a CYP1A inducer)-treated harbour seals;
- to assess whether CYP1A expression in harbour seal skin correlates with that in liver;
- to measure EROD activity and CYP1A protein levels in skin biopsies from killer whales using techniques validated in harbour seal skin;
- to determine if CYP1A measurements in liver of harbour seals, and in skin of harbour seals and killer whales, correlate with organochlorine concentrations in blubber.

2. MATERIALS AND METHODS

2.1 Overview

Three biomarker studies were conducted in marine mammals:

(1) a study of captive harbour seals with analysis of liver and skin-blubber biopsies;

(2) a study of free-ranging harbour seals with analysis of skin-blubber biopsies;

(3) a study of free-ranging killer whales with analysis of skin-blubber biopsies.

Tissue biopsies from harbour seals and killer whales were analyzed for CYP1A enzyme activity and CYP1A protein concentration using the ethoxyresorufin *0*-deethylase (EROD) assay and immunoblotting, respectively. Experiments were carried out to optimize protein extraction from tissue biopsies, while minimizing the amount of tissue used, and to validate and optimize the EROD and immunoblot assays for our marine mammal tissue samples (described in section 2.11). The primary methodological goal was to measure CYP1A in small tissue samples collected using minimally-invasive techniques.

2.2 Chemicals

The contents of buffers and reagents used in this study are listed in Table 2.1. Chemicals and reagents used in this study were obtained from the following sources:

Aldrich Chemical Company Inc. (Milwaukee, WI, USA)

Resorufin

BDH Chemicals (Toronto, Ontario, Canada):

Sodium chloride (KCl); sodium hydroxide (NaOH); sodium carbonate (Na_2CO_3); disodium hydrogen orthophosphate (Na_2HPO_4); potassium dihydrogen orthophosphate (KH_2PO_4); magnesium chloride ($MgCl_2 \cdot 6H_2O$); dimethylsulfoxide (DMSO), cupric sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$); isopropanol

Bio-Rad (Richmond, CA, USA):

Bromphenol blue

BIOSOURCE International (Camarillo, CA, USA)

Goat $(Fab')_2$ anti-rabbit immunoglobulins, alkaline phosphatase conjugated

Fisher Scientific (Fair Lawn, NJ, USA):

Trizma base (Tris); acrylamide; N,N′-methylene-bis-acrylamide (Bis), N,N,N′,N′-tetramethylenediamine (TEMED); ammonium persulphate (AP); sodium dodecyl sulphate (SDS); glycine; ß-mercaptoethanol; reagent grade methanol; HPLC grade methanol; sodium chloride (NaCl); bovine serum albumin (BSA); nitro blue tetrazolium (NBT); 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (BCIP); glycerin; Sodium/potassium (Na/K) tartrate tetrahydrate

Gibco

Goat serum

ICN Biochemicals, Inc. (Cleveland,, OH, USA):

N, N-dimethylformamide (DMF)

MERCK

Coomassie blue R250

Oxford Biomedical Research Inc. (distributed by Cedarlane Laboratories, ON, Canada)

Polyclonal goat-anti rabbit 1A1 and 1A2 IgG

Pacific (Vancouver, BC, Canada):

Skim milk powder

SIGMA Chemical Co. (St. Louis, MO, USA):

Ethylenediaminetetra-acetate disodium salt (EDTA); pyronin Y; polyoxyethylene sorbitan monolaurate (Tween 20); 1-butanol; N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES); dimethylsulfoxide (DMSO); ß-naphthoflavone (BNF); nicotindiamide adenine dinucleotide phosphate tetrasodium salt (NADPH); ethoxyresorufin

Dr. S.M. Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada):

Purified rat cytochrome P450 1A1; purified rat cytochrome P450 1A2; liver microsomes prepared from 3-MC treated adult male Long Evans rats; rabbit anti-rat cytochrome P450 1A1 IgG; rabbit anti-rat cytochrome P450 1A2 serum; rabbit anti-trout cytochrome P450 1A1 IgG prepared against a 15 amino acid peptide of the trout CYP1A1 sequence; control rabbit IgG; control rabbit serum

Dr. P. Thomas (Rutgers, The University of New Jersey, Piscataway, NJ, USA):

Monoclonal mouse anti-rat cytochrome P450 1A1 IgGs

Table 2.1: Buffers and reagents used in this study

Buffer or reagent	Contents
Tris-HCl buffer (for homogenizing tissues)	50 mM Tris-HCl, pH 7.4 at room temperature (r.t.); 150 mM KCl; 2 mM EDTA
Lowry assay reagents	(a) 2% $NaCO_3$ anhydrous in 0.1 M NaOH (b) 2% Na/K tartrate tetrahydrate (c) 1% $CuSO_4 \cdot 5H_2O$
Sample dilution buffer	62.5 mM Tris-HCl, pH 6.8 at r.t.; 10.8% glycerol; 0.001% bromphenol blue; 1% SDS; 5% ß-mercaptoethanol
Separating gel	0.375 M Tris-HCl, pH 8.8 at r.t.; 0.1% SDS; 7.5% acrylamide bis; 0.042% AP; 0.03% TEMED
Stacking gel	0.125 M Tris-HCl, pH 6.8 at r.t.; 0.1% SDS; 3% acrilyamide bis; 0.08% AP; 0.05% TEMED
Separating gel wash buffer	0.68 M Tris-HCl, pH 8.8 at r.t.; 0.18% SDS
Stacking gel wash buffer	0.23 M Tris-HCl, pH 6.8 at r.t.; 0.18% SDS
Electrophoresis buffer	50 mM glycine; 0.38 M trizma base; 0.2% SDS
Transfer buffer	0.13 M glycine; 25 mM trizma base, 0.1% SDS
Membrane blocking buffer	50 mM NaCl; 10 mM Tris-HCl, pH 7.4 at r.t.; 1 mM EDTA, pH 7.4 at r.t.; 5% skim milk powder
Modified PBS (10x)	1.3 M NaCl; 26 mM KCl; 81 mM Na_2HPO_4; 15 mM KH_2PO_4; 2 mM EDTA
Antibody dilution buffer	0.05% Tween 20; 2% BSA; 5% goat serum in modified PBS
Membrane wash buffer	0.05% Tween 20 in modified PBS
Gel staining solutions	(a) Fixative: 50% methanol; 10% glacial acetic acid (b) Stain: 50% methanol; 10% glacial acetic acid (c) Destain I: 50% methanol; 10% glacial acetic acid (d) Destain II: 5% methanol; 7% glacial acetic acid
Substrate solution	0.03% NBT; 0.015% BCIP; 0.05 mM $MgCl_2$ in 0.1 M Tris-HCl, pH 9.5 at r.t.

r.t. = room temperature

2.3 Sample Collection

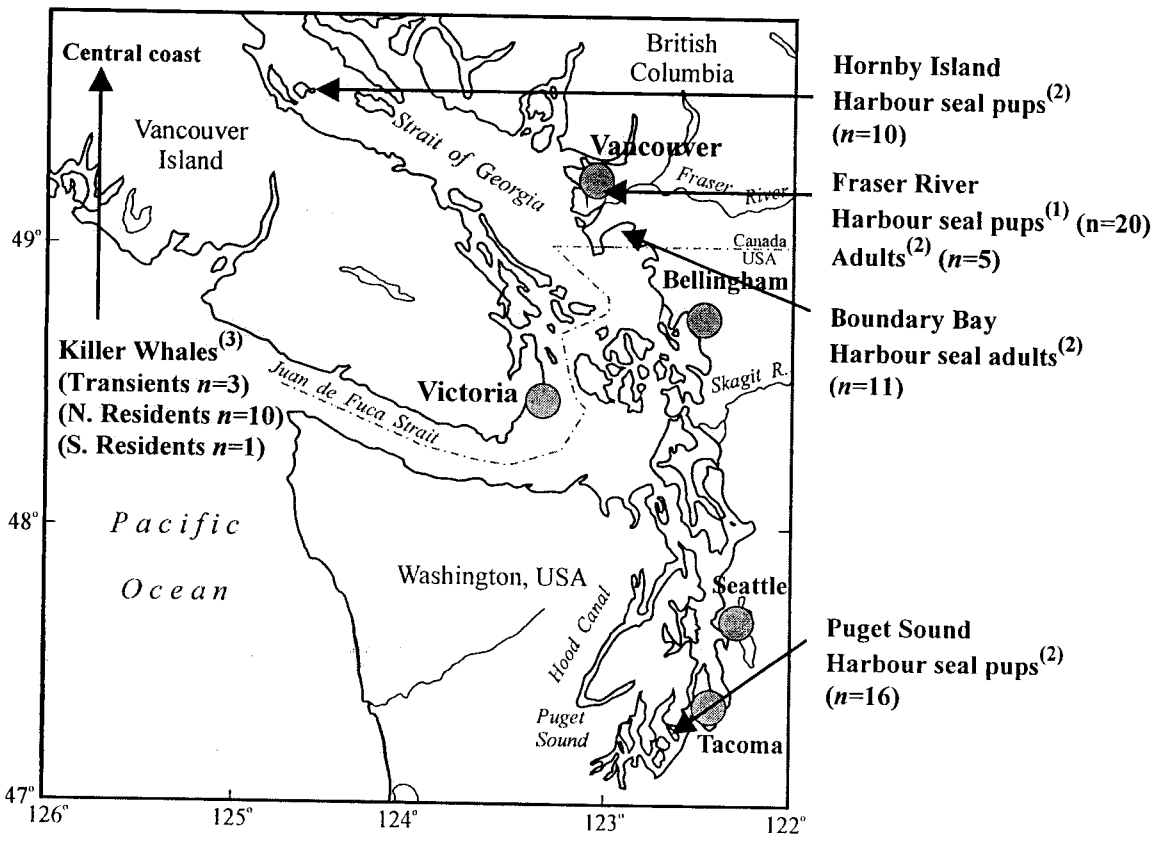


Figure 2.1: Marine mammal sampling sites in British Columbia and Washington State. Superscript numbers indicate study groups: (1) captive harbour seal study; (2) study of free-ranging harbour seals; (3) study of free-ranging killer whales.

2.3.1 Study 1: Captive harbour seals (Fraser River pups)

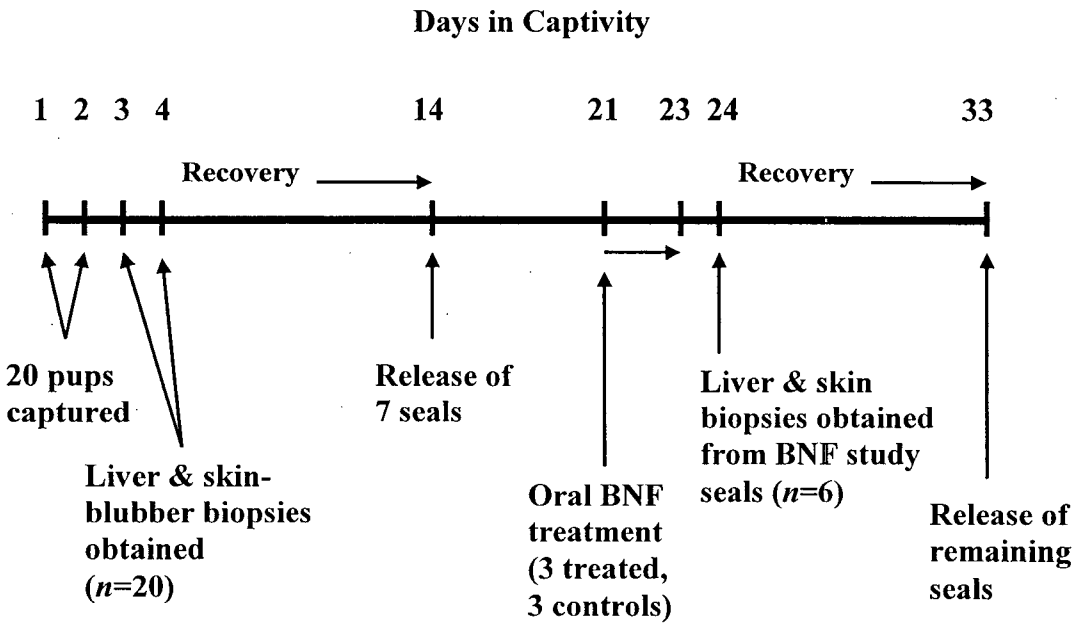


Figure 2.2: Study 1: Captive harbour seals. Twenty harbour seal pups were captured from the Fraser River estuary, BC, temporarily held in captivity, and then released. Liver and skin-blubber biopsies were taken from all twenty seals one or two days post-capture. Six seals were used for an *in vivo* induction study, and liver and skin biopsies were taken a second time from these animals. Seals were treated with 50 mg/kg BNF for three days. Skin biopsies were taken from the hind flipper rather than from the pelvic region during the induction study.

2.3.1.1 Animal capture and care

Twenty 3-6 week-old harbour seal (*Phoca vitulina*) pups were live-captured from the middle arm of the Fraser River estuary, British Columbia (49°10'N, 123° 11'W) on August 17-18, 2000. Beached seals were caught with a specialized seine net as they entered the water (Redden Net Company, Vancouver, Canada) (Jeffries *et al.*, 1993), and the ages of the pups were estimated by body weight, as described elsewhere (Cottrell *et al.*, 2001). Seals were transported to the Department of Fisheries and Oceans Cultus

Lake Salmon Research Laboratory in canine transport cages and were kept in four outdoor freshwater pools (5 m diameter x 1.7 m deep) with 3 m diameter haul-out islands. Flow-through freshwater was maintained at 12°C. Seals were fasted for two days upon arrival and then subjected to liver biopsy surgery under general anaesthetic. Following surgery, seals were fed herring twice daily, supplemented with multivitamins and sodium chloride twice weekly. Because seals were not yet consuming fish in the wild, they were initially fed herring by hand. Seals were weighed at capture and then again every four to five days during their time in captivity. Seals were captured under Fisheries and Oceans Canada Animal Care Committee and Scientific Use Permits issued to P.S. Ross and were cared for by a professional animal care technician (J. Sicree) from the Marine Mammal Center, Sausalito, CA, under the supervision of veterinarians (D. Huff, M. McAdie, and D. Clegg). Seals were held in captivity for either two or five weeks and were subsequently released at the capture site. Sample size reflected logistical and housing constraints, the relative success of previous sampling efforts, and the requirements for the different biological endpoints being estimated.

2.3.1.2 Tissue biopsies

Liver and skin/blubber biopsies were obtained by veterinary staff two days post-capture, while animals were under general anaesthetic. Ten minutes prior to induction of anaesthesia, seals were given an intramuscular injection of 0.02 mg/kg atropine sulphate in the gluteal muscles to maintain constant heart rate. Anaesthesia was induced with 4-5% isofluorine gas, using an open-ended 2 L plastic beverage bottle as a mask. Seals were then intubated with a lubricated endotracheal tube (5 mm internal diameter) fitted with an inflatable cusp. Oxygen flow rate was 2 L oxygen per minute. Biopsy sites were

shaved with clippers and scrubbed with betadine, hibitane, and isopropyl alcohol. Liver biopsies were taken through a 1 cm incision using a 14 gauge x 10 cm EZ Core biopsy needle (Products Groups International, Inc., Lyons, CO, USA). Skin and blubber samples were taken from the side of the body anterior to the pelvis using an 8 mm biopsy punch (Acuderm, Ft. Lauderdale, FL, USA). Xylocaine topical anaesthetic was sprayed on the skin/blubber biopsy site post-biopsy. Liver biopsies were rinsed in phosphate-buffered saline (PBS) solution, weighed, and placed in cryovials in liquid nitrogen. Skin/blubber biopsies were wrapped in aluminum foil, placed in cryovials and stored in liquid nitrogen. Seals were kept indoors overnight in dry, sterilized fiberglass pools to recover following tissue sampling.

2.3.1.3 ß-Naphthoflavone treatment

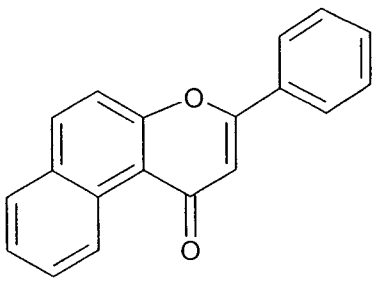


Figure 2.3: Chemical structure of ß-naphthoflavone (BNF)

Beta-naphthoflavone (BNF), a CYP1A inducer, was used in this study as a positive control for CYP1A expression and was orally administered to three seals at twenty-three days post-capture, while three additional seals served as negative controls. Gelatin capsules containing pure BNF were prepared and administered in herring.

Control seals received only herring. Seals were dosed with BNF at 50 mg/kg body weight/day for three days. This dose regime was based on dosages reported in the literature for laboratory animals (Boobis *et al.*, 1977; Vyas *et al.*, 1983). All seals were fed an equal number of fish per day, and feedings were done at the same time each day. Seals that were not yet feeding on their own were chosen for the treatment group and were hand-fed to ensure that they received the full dosage of BNF. Control seals were free-feeding. All six seals were males. Liver and skin biopsies were obtained approximately 24 hours after the last BNF dose while seals were under general anaesthetic.

2.3.2 Study 2: Free-ranging harbour seals

Skin biopsies were obtained from young and adult harbour seals in the field from several sites around Hornby Island, BC (August 8-9), the Fraser River estuary, BC (August 14), Boundary Bay, BC (August 22), and from Puget Sound, Washington (September 18-19) (see Table 2.2 for sample summary). Seals were captured using the same technique described in section 2.3.1.1 at the Fraser River, Boundary Bay, and Puget Sound sites. Due to the rocky terrain around Hornby Island, seals were captured by hand or using a 60 cm diameter fish landing net (Cottrell *et al.*, 2002). Seals were manually restrained, weighed, and the biopsy site was shaved using clippers or a straight-edge razor and was cleaned as described above (section 2.3.1.2). Lidocaine (0.7 cc) was injected intradermally to anaesthetize the biopsy site. Two skin/blubber biopsies were taken from the side of the body anterior to the pelvis using an 8 mm biopsy punch (Acuderm). Xylocaine anaesthetic spray was used post-biopsy. Biopsies were wrapped

in foil, placed in cryovials, and immediately frozen in liquid nitrogen. Seals were released immediately at the site of capture after sampling was completed.

For the purposes of this study, adult harbour seals from the Fraser River and Boundary Bay were considered as a single population. These groups are combined in subsequent sections and are referred to as 'adults'.

2.3.3 Study 3: Free-ranging killer whales

Skin/blubber biopsies were obtained from three transient and ten northern resident killer whales (*Orcinus orca*) by G.M. Ellis[1], J.K.B. Ford[1], and L. Barret-Lennard[2] ([1]Fisheries and Oceans Canada, [2]Vancouver Aquarium) during the summer of 2000. Biopsies were obtained from live, free-ranging whales using a light-weight pneumatic dart system described elsewhere (Barrett-Lennard *et al.*, 1996). A skin/blubber biopsy was also obtained from an orphaned southern resident killer whale using an 8 mm biopsy punch during its relocation from Washington State to British Columbia waters in July, 2002. All biopsies were immediately stored in liquid nitrogen. The sex and approximate age of almost all resident killer whales and a large proportion of the transient killer whales that frequent BC coastal waters are known due to a photo-identification study that has been ongoing since 1973 (Ford *et al.*, 1994; Ford and Ellis, 1999).

Table 2.2: Summary of marine mammal samples used in this study

Species	Year	Sampling site	Age class	Sex ratio (M:F)	Body weight range† (kg)	Skin samples	Liver samples	Contaminant analysis of blubber
(1) Harbour seal	2000	Fraser River	Pups	15:5	17.2-30.8	20 + 6 (3 BNF-treated + 3 controls)*	12 + 6 (3 BNF-treated + 3 controls)*	complete
(2) Harbour seal	2001	Fraser River	Adults	1:4	48.5-111	5	N/A	no
Harbour seal	2001	Boundary Bay	Adults	6:5	50-104	11	N/A	no
Harbour seal	2001	Hornby Island	Pups	6:4	19-34	10	N/A	complete
Harbour seal	2001	Puget Sound	Pups	8:8	19.75-29	16	N/A	no
(3) Killer whale (N. Residents)	2000	B.C.	Various	6:4	N/A	10	N/A	complete
Killer whale (Transients)	2000	B.C.	Various	3:0	N/A	3	N/A	complete
Killer whale (S. Residents)	2002	B.C.	~ 2.5 years	Female	N/A	1	N/A	no

*All seals in BNF study were males. These 6 seals were from the group of 20 seals originally caught (biopsies were taken a second time from these seals for the BNF study).
†Weight at capture
Refer to Appendix I for specific data for each animal sampled

2.4 Tissue Preparation

Liver and skin biopsies were transferred from liquid nitrogen to aluminum weigh boats and were kept frozen on a dry ice/isopropanol slurry during sub-sampling. If a sample size was sufficiently large, a piece of unthawed tissue was archived in liquid nitrogen. Tissue samples were weighed and homogenized, as described in detail below (sections 2.4.1, 2.4.2). Liver and skin homogenates were spun for 20 minutes at 9000 x g (5°C). Post-mitochondrial supernatant or S9 fraction was separated from pellets using a Hamilton syringe, and pellets were discarded. The S9 fractions were stored at -80°C. Microsomal fractions were not prepared due to small tissue sample sizes. Although microsomal fractions are more concentrated than S9 fractions with respect to CYP enzymes, we would not have been able to recover a volume of microsomes sufficient to conduct our experiments.

2.4.1 Liver

Liver biopsies were homogenized in 1.5 ml polypropylene mini-centrifuge tubes (SIGMA Chemical Co., St. Louis, MO, USA) with ice cold 50 mM Tris-HCl buffer (150 mM KCl, 2 mM EDTA, pH 7.4 at 4°C) using a hand-held pellet pestle mixer (Kontes, 1.5 ml). Buffer was added in an approximate 2:1 ratio (v/w), and samples were homogenized for a constant amount of time (5 bursts for 10 seconds each with 10 seconds between bursts). Samples were kept in an ice bath during homogenization.

2.4.2 Skin

Blubber was removed from skin biopsies using a straight-edge razor blade. The skin-blubber interface was defined by a change in tissue consistency (blubber is softer) and the visible difference in colour between the two tissues. As much hair as possible

was removed from harbour seal skin where samples had been obtained from unshaven biopsy sites (Study 1). Frozen skin samples were sliced into fine sections using a straight edge razor. Harbour seal skin biopsies for the oral BNF study were homogenized with Tris-HCl buffer in an 8:1 ratio (v/w), and untreated skin biopsies from 2000 were homogenized using a 4:1 v/w ratio. A higher v/w ratio was used for BNF study samples because these biopsies were smaller, and the homogenizing generator had a minimum volume requirement. Skin biopsies from the BNF study were smaller (thinner) because they were taken from the flipper; whereas, all other skin samples were taken from the side of the body. For homogenization of captive harbour seal skin samples, half of each sample was kept frozen in a mortar with liquid nitrogen, while the other half was placed in a 5 ml polypropylene test tube (BD Falcon Canada, Oakville, ON) in an ice bath. The first half of the sample was homogenized in the test tube for 3 cycles (10 seconds homogenize/45 seconds rest on ice), and any large pieces of tissue remaining were then refrozen with liquid nitrogen and homogenized for another 2 cycles. The other half of the sample was then added to the test tube, homogenized for 1 cycle, refrozen, homogenized for 3 cycles, refrozen, and homogenized for a final 10 seconds. A relatively large piece of tissue remained after homogenization. A Polytron tissue homogenizer with a stainless steel generator (7 mm diameter) was used to homogenize skin samples from 2000. Sonication and grinding of skin in liquid nitrogen with a mortar and pestle were also tried, but these methods were not successful.

Killer whale and harbour seal skin samples from 2001 were homogenized using a PowerGen 125 tissue homogenizer with a stainless steel saw-tooth generator (Fisher Scientific, Pittsburgh, PA). This generator allowed for smaller volumes than the

generator used previously (250 µl vs. 500 µl minimum) and, therefore, allowed for homogenization of smaller pieces of tissue. A 4-6:1 buffer volume: tissue wet weight ratio was used, depending on the size of the tissue sample. Samples were homogenized for 15 cycles (10 seconds homogenize/20 seconds rest), with addition of the second half of the biopsy after the fifth cycle. The test tube was kept in an ice bath throughout the procedure.

2.5 Total Protein Determination

Total protein content of S9 fractions was measured by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used to generate a standard curve. All samples were measured in duplicate at an absorbance of 695 nm, using a Spectronic 20 spectrophotometer (Thermo Spectronic Bausch & Lomb Analytical, Cambridge, England). A quadratic polynomial curve fit equation was used to calculate the total protein content of samples using Microsoft® Excel 97 (Microsoft Corp., Redmond, WA).

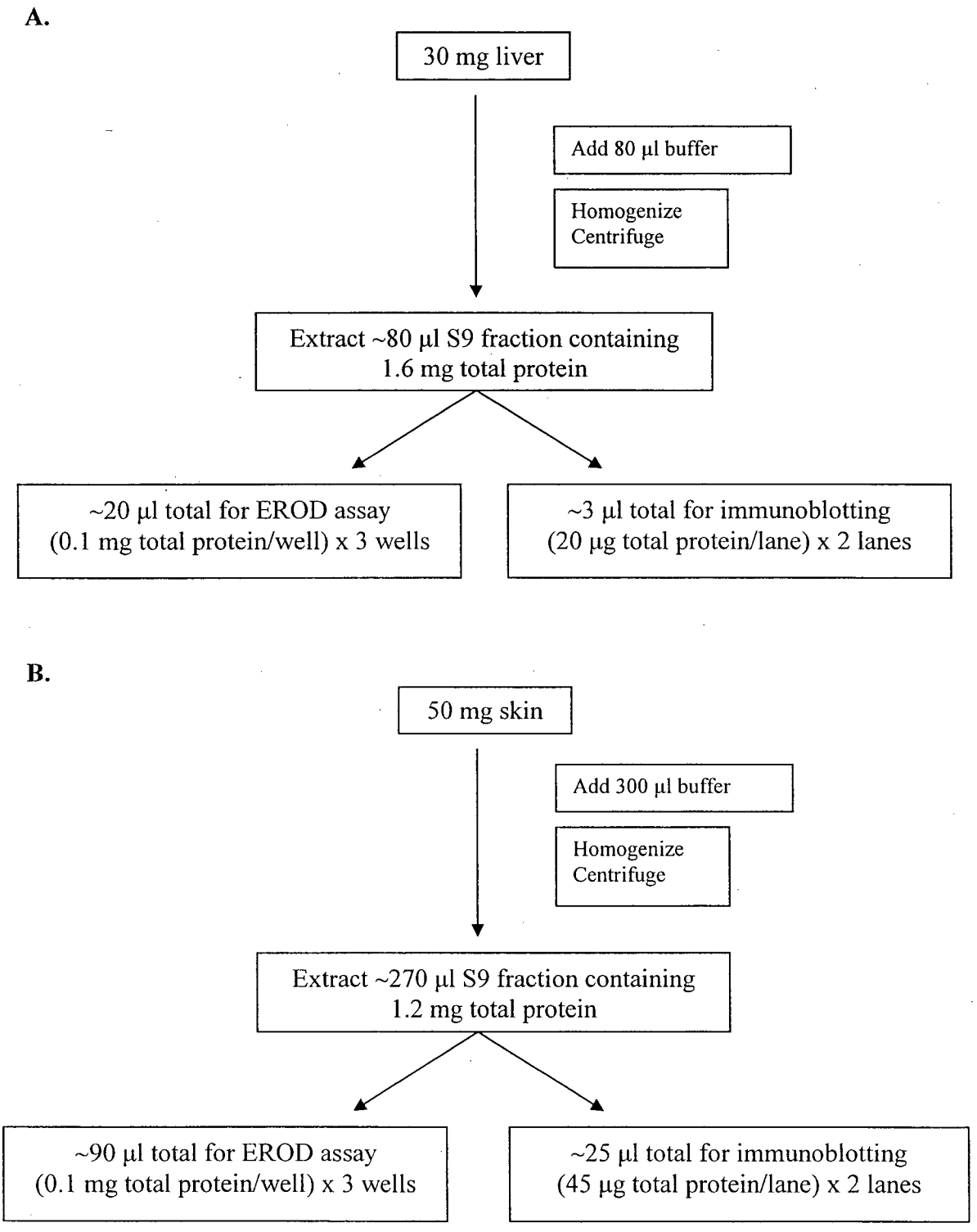


Figure 2.4: Schematic for preparation of S9 fractions from seal liver (A) and skin (B).

Table 2.3: Mean total protein yields obtained from liver and skin biopsies

Study	Sample	Approximate initial biopsy mass (ww) (mg)	Mean amount of tissue homogenized (ww) (mg)	Mean total protein content of S9 fraction (mg)	Mean ratio total protein (mg): homogenized tissue wet weight (mg) (%)
(1)	Fraser River liver	65.9 ± 25.5[†]	34.8 ± 6.7	2.0 ± 0.5	5.6 ± 0.9
	Fraser River skin	155.5 ± 50.4[‡]	121.5 ± 23.7	2.2 ± 0.6	1.8 ± 0.4
(2)	Hornby Island skin	-	62.7 ± 18.1	1.9 ± 0.6	3.0 ± 0.6
	Puget Sound skin	-	58.8 ± 20.0	1.3 ± 0.6	2.2 ± 0.7
	Adult skin	-	58.9 ± 10.4	1.4 ± 0.3	2.3 ± 0.6
(3)	Killer whale skin	100-200	43.1 ± 8.9	1.5 ± 0.4	3.6 ± 0.4

Values are presented as the mean ± SD
ww = wet weight
[†]Liver mass is the mean total tissue mass obtained, rather than mean biopsy mass, as multiple biopsies were taken from each seal to obtain an adequate sample. [‡]Skin biopsy mass data is not available for each harbour seal group. Whole biopsies were not weighed during subsampling to prevent thawing (part of the biopsy was archived). The mean wet weight shown here is calculated from biopsies from six seals used for optimization experiments.

2.6 Enzyme Activity

Ethoxyresorufin *0*-deethylase (EROD) was measured based on the methods of Burke and Mayer (1974) and Kennedy and Jones (1994) using 96-well flat-bottom microtiter plates (Microfluor 2 Black, ThermoLabsystems, Franklin, MA). Fluorescence was measured using a Fluoroskan *Ascent* FL fluorescence plate reader (Labsystems, Helsinki, Finland). Excitation and emission filter wavelengths were 544 nm (half band width = 15 ± 2) and 584 nm (half band width = 16 ± 2), respectively, and a gain of 1.0 was used. Skin and liver S9 fractions, which were initially prepared in Tris-HCl buffer, were thawed, and dilutions in 50 mM Tris-HCl buffer (pH 7.4) were made as necessary. EROD assays were conducted under low, yellow light conditions to minimize the chance of degradation of resorufin and ethoxyresorufin, although studies have shown that photolysis should not be a problem under fluorescent room lighting (Pohl and Fouts, 1980). Samples were loaded in triplicate whenever possible. Reagents and volumes for the respective standards and samples were added as shown in Table 2.4 (total volume=200 µl/well).

Contents of wells were mixed manually with a multi-channel pipette after addition of HEPES buffer and again after addition of S9 fractions. Plates were placed in the plate reader and incubated at 37°C with shaking. After a pre-incubation time of 5 minutes, a fluorescence reading was taken. This was the time zero or blank reading. NADPH was then dispensed automatically to sample wells, and after 10 seconds of shaking, fluorescence readings were taken every minute for 30 minutes (liver) or for 60 minutes (skin). The amount of resorufin formed was determined from a standard curve of resorufin concentration versus fluorescence intensity. EROD activity was calculated

by dividing the amount of resorufin formed by the amount of total protein loaded/well and by the reaction time (pmoles/mg/min). A reaction time that produced a resorufin concentration above the LOQ for the assay and that fell on the linear portion of the resorufin versus reaction time curve was used.

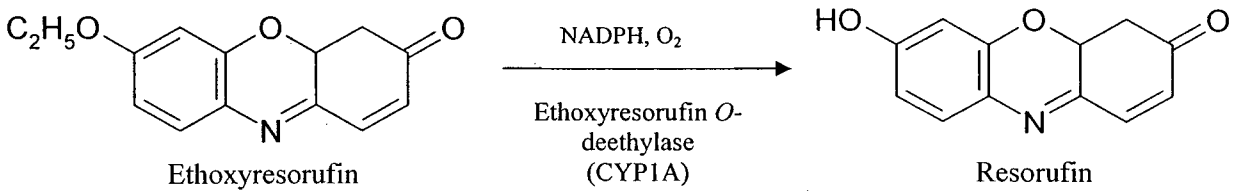


Figure 2.5 : EROD reaction

Table 2.4: Reagents and volumes used in microtiter plates for the EROD assay

Reagent	Standard wells	Sample wells (liver)	Sample wells (skin)
Resorufin in DMSO	10 µl (blank: 0 µl)	none	none
25 uM ethoxyresorufin in DMSO	10 µl	10 µl	10 µl
0.1 M HEPES buffer, 5 mM $MgCl_2$, pH 7.8	180 ul (blank: 190 ul)	160 µl	140 µl
S9 fraction	none	10 µl	30 µl
2.5 mM NADPH in HEPES buffer	none	20 µl	20 µl
Total volume	200 µl	200 µl	200 µl

2.6.1 Determination of resorufin stock concentration

The purity of resorufin can be highly variable (Stagg and Addison, 1995), and this can result in inaccuracies in reported concentrations of resorufin standard solutions. Pure resorufin has an absorption maximum of 572 nm at pH 8.0, with an extinction coefficient of 73 $mM^{-1}cm^{-1}$ (Klotz *et al.*, 1984). By measuring the absorbance of the resorufin stock solution diluted in phosphate buffer, pH 8.0 at 572 nm, and using the formula (absorbance = extinction coefficient x pathlength x concentration), an accurate resorufin concentration can be calculated. This correction was made for the resorufin stock solution used in this study.

2.6.2 Antibody inhibition of EROD activity

To determine whether EROD activity represented a specific measure of CYP1A enzyme activity in harbour seal liver and skin, antibody inhibition experiments were conducted. The EROD assay was performed as described above, except that S9 fractions were preincubated with purified rabbit anti-rat CYP1A1 IgG, rabbit anti-rat CYP1A2 serum, or control rabbit IgG or serum prior to addition of substrate. Samples were preincubated with antibody for 10 minutes at room temperature. Antibody concentrations were chosen based on the amount of total S9 protein loaded per well and antibody was added to wells in a constant volume of phosphate-buffered saline, pH 7.4. Because the IgG concentration in rabbit anti-rat CYP1A2 serum was not known, an estimated concentration of 10 mg IgG/ml was assumed. This was the typical IgG concentration of serum reported in the literature (Harlow and Lane, 1988; Goding, J.W., 1986). It was not possible to use multiple antibody concentrations for all experiments due to insufficient quantities of samples.

The following samples were used in antibody inhibition experiments. A pooled liver sample from BNF-treated seals (n=2) and a pooled liver sample from control (non-BNF-treated) seals (n=3) were incubated with rabbit anti-rat CYP1A1 IgG at three different concentrations. A pooled liver sample from BNF-treated animals (n=3) was also incubated with rabbit anti-rat CYP1A2 serum at two different IgG concentrations. A pooled skin sample from control animals (n=3) was incubated with anti-CYP1A1 IgG at a single IgG concentration. A liver sample from one seal pup that was not part of the study group (captured near Sidney, B.C.) was incubated with rabbit anti-rat CYP1A2 serum at three different concentrations. A pooled skin sample (n=8) was incubated with rabbit anti-rat CYP1A2 serum at three different concentrations.

2.6.3 BNF inhibition

The EROD assay was performed as described in section 2.6, except that different concentrations of BNF dissolved in DMSO were added to S9 fractions prior to the addition of substrate. Final BNF concentrations were chosen based on the final substrate (ethoxyresorufin) concentration (four times higher to twenty times lower than the final substrate concentration). A liver sample from a BNF-treated harbour seal was used for this experiment.

2.7 SDS-PAGE and Immunoblots

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (1970), using a Bio-Rad Mini-PROTEAN® 3 Cell (Bio-Rad Laboratories, Hercules, CA). A 0.75 mm discontinous SDS-polyacrylamide gel was used (3% stacking gel, 7.5% separating gel). Samples were diluted in sample dilution buffer (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 0.001%

bromphenol blue; 1% SDS; 5% ß-mercaptoethanol), boiled for 3 minutes, and loaded at 20 µl/well (see Table 2.5 for total protein concentrations). Electrophoresis was carried out at constant current (30 mA) with cooling for approximately one hour. Proteins resolved by SDS-PAGE were transferred electrophoretically to 7 cm x 9 cm Immobilon-P polyvinyldenfluoride (PVDF) membranes (Millipore Corporation, Bedford, MA) using a modification of the procedure described by Towbin *et al.* (1979). A Bio-Rad Mini Trans-Blot® electrophoretic transfer cell was used, and transfers were run at constant voltage (100 V) for one hour with cooling. After the transfer, gels were fixed for 1 hour in a solution containing 25% isopropanol and 10% acetic acid to remove SDS, stained for 1 hour in a 0.05% Coomassie blue solution, and destained for approximately 3 hours in a solution containing 10% isopropanol, 10% acetic acid. Gels were dried in a slab gel drier with vacuum aspiration for approximately 2 hours. Membranes were left overnight in blocking buffer (10 mM Tris-HCl, pH 7.4; 50 mM NaCl; 1mM EDTA; 5% skim milk powder) at 4°C. The next day, membranes were incubated with polyclonal rabbit anti-rat CYP1A2 serum (1:500 dilution) in antibody dilution buffer (0.05% Tween 20, 2% BSA, 5% goat serum in modified PBS) for 2 hours at 37°C with shaking, followed by three 5-10 minute incubations with wash buffer (0.05% Tween 20 in modified PBS). Membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution) for 2 hours at 37°C with shaking, followed again by three incubations with wash buffer. Immunoreactive proteins were detected colorimetrically by reaction with a substrate solution containing 0.01% NBT, 0.05% BCIP, and 0.05 mM $MgCl_2$ in 0.1M Tris-HCl buffer, pH 9.5 at room temperature. The reaction was stopped with the addition of distilled water when protein bands were sufficiently stained.

2.8 Quantitation of Immunoblots

Staining intensities of protein bands were quantified with a pdi 320 oe scanning densitometer using Quantity One® version 4.2.0 software (Bio-Rad Laboratories, Hercules, CA). Staining intensity was measured as a contour quantity (CQ), calculated by the software program as optical density x contour area (OD x mm^2). Staining intensities of protein bands were then normalized to a purified rat hepatic CYP1A1 standard that was included on each gel. Immunoblot data are shown as relative contour quantities (RCQ)/mg total protein. RCQs for liver and skin samples were calculated using internal standards of 0.1 pmoles rat CYP1A1/lane and 0.01 pmoles rat CYP1A1/lane, respectively. Average RCQs for CYP1A protein were based on duplicate or triplicate determinations for each sample. When both upper and lower CYP1A protein bands were quantifiable, these values were added together. CYP1A protein levels determined this way represent total CYP1A protein (i.e. CYP1A1 and CYP1A2).

Table 2.5: Amount of total protein used for the EROD and immunoblot assays

Sample	EROD assay (mg protein/well)	Immunoblots (µg protein/lane)
Oral BNF study harbour seal liver (treated)	0.01, 0.025, 0.05	2
Oral BNF study harbour seal liver (controls)	0.1, 0.05	10
Oral BNF study harbour seal skin (treated)	0.075	10
Oral BNF study harbour seal skin (controls)	0.075	25
Fraser River harbour seal pup liver	0.1	20
Fraser River harbour seal pup skin	0.12	20-60 †
Puget Sound harbour seal pup skin	0.1	↓
Adult harbour seal skin	0.1	
Hornby Island harbour seal pup skin	0.12	
Killer whale skin	0.12	

† when possible, 60 µg total protein/lane was used. Many skin samples did not have sufficient total protein content to load 60 µg/lane. For these samples, the maximum amount of total protein was loaded by diluting S9 fractions with sample dilution buffer in a 1:1 ratio.

2.9 Contaminant Analysis

Blubber biopsies from seal pups from the Fraser River and Hornby Island and from killer whales were analyzed by the Regional Dioxin Laboratory (Institute of Ocean Sciences, Sidney, B.C.) for congener-specific PCBs, PCDDs, and PCDFs using high resolution gas chromatography/high resolution mass spectrometry. Total Toxic Equivalents (TEQs) were calculated using the most recent toxic equivalency factors (TEFs) defined by Van den Berg *et al.*, 1998.

2.10 Statistical Analysis

Correlation analyses and t-tests were performed using Microsoft® Excel. Unpaired two-tail t-tests were used for comparisons of means, except for analysis of the change in CYP1A expression over time, in which a paired t-test was used. The Grubbs' test was used to identify outliers (GraphPad Software, Inc., San Diego, CA). Normality tests were not conducted due to small sample sizes (InStat version 3.00, GraphPad Software, Inc.), and parametric tests were used. A p value < 0.05 was considered statistically significant for all analyses.

2.11 Assay Validation and Optimization

Intra- and inter-assay experiments were conducted to determine the limit of detection (LOD) and limit of quantitation (LOQ) for the EROD and immunoblot assays. Because we adapted previously published methods and wanted to use as little tissue as possible, additional experiments were conducted to determine the optimal conditions for analysis of our tissue samples.

2.11.1 EROD Assay

2.11.1.1 Intra-assay variation

Intra-assay variability of the EROD assay was measured in a single assay using six resorufin standard concentrations, each analyzed in triplicate. Coefficients of variation (CV) were <15% at all standard resorufin concentrations (Table 2.6).

2.11.1.2 Inter-assay variation

Multiple between-day standard curves were obtained with five different resorufin concentrations, each analyzed in triplicate. The limit of detection (LOD) for the EROD assay was defined as the lowest standard concentration having a fluorescence reading above background, where background was the fluorescence measured in wells containing HEPES buffer and ethoxyresorufin. The limit of quantitation (LOQ) was defined as the standard concentration at which fluorescence readings were consistently above background with a coefficient of variation (CV) <15%. All standard concentrations tested had CV's <15%. At 0.65 pmoles resorufin/well, fluorescence readings were not consistently above background, but at 1.3 pmoles resorufin/well, readings were consistently above background. Based on these results, the LOD was determined to be between 0.65 and 1.3 pmoles resorufin/well. The LOQ was determined to be 1.3 pmoles resorufin/well (Table 2.7). This LOQ value was used for EROD activity in harbour seal samples.

Table 2.6: Intra-assay variation of fluorescence readings for EROD assay standards

Resorufin standard (pmoles/well)	Average fluorescence	CV (%)
0	7.4 ± 0.2	2.2
0.65	7.4 ± 0.4	5.0
1.3	8.9 ± 0.2	2.8
2.6	11.1 ± 0.3	2.4
5.2	17.5 ± 0.9	5.3
10.4	26.5 ± 0.6	2.4
20.8	46.0 ± 1.5	3.4

Values are presented as the mean ± SD of triplicate fluorescence readings

Table 2.7: Inter-assay variation of fluorescence readings for EROD assay standards

Resorufin standard (pmoles/well)	Mean fluorescence	CV (%)
0	5.9 ± 0.4[a]	6.7
0.65	6.9 ± 0.5[b]	7.7
1.3	8.7 ± 0.4[b]	5.0
2.6	10.9 ± 0.6[a]	5.5
5.2	17.3 ± 1.2[a]	7.0
10.4	27.7 ± 1.2[a]	4.8
20.8	47.7 ± 2.2[a]	4.7

Values are presented as the mean ± SD of triplicate fluorescence readings
[a]calculated for six different assays
[b]calculated for three different assays

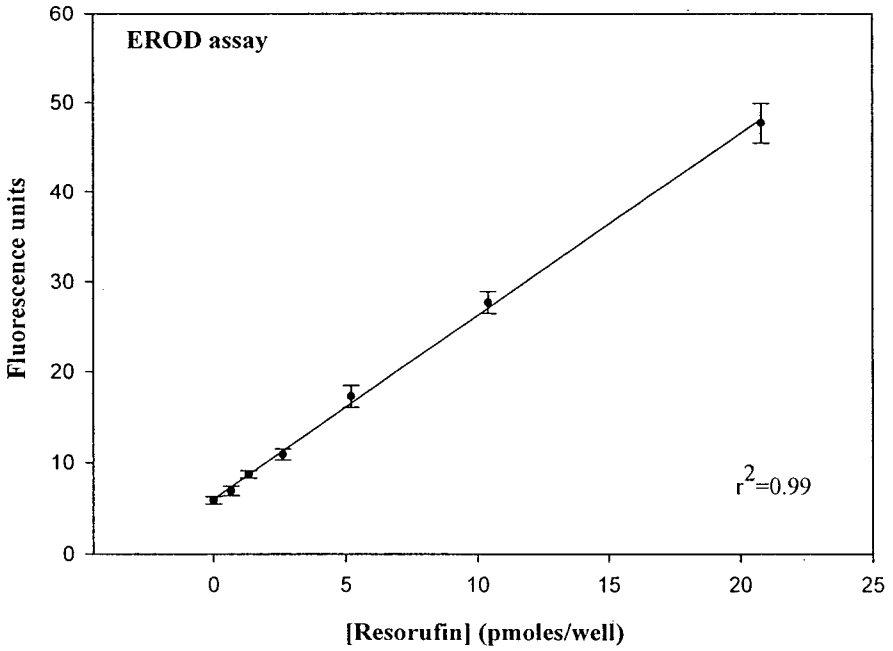


Figure 2.6: Inter-assay calibration curve for the EROD assay. Fluorescence readings were measured in triplicate on six different days for each resorufin standard concentration, with the exception of 0.65 and 1.3 pmole/well concentrations, which were measured on three different days.

2.11.1.3 Substrate concentration for the EROD assay

The effects of substrate concentration on product formation were investigated for harbour seal liver. EROD activity was measured in S9 fractions from four seals using a range of substrate concentrations. The seals used were PV00101 (pup from near Sidney, B.C.), PV01-50 (adult female from the Fraser River estuary), PV0018 (pup from Fraser River estuary), and PV98102 (pup from near Sidney, B.C.). Other than PV0018, samples from these seals were only used in optimization experiments, but not as part of the overall study. The effect of substrate concentration on resorufin formation is shown in Figure 2.7. The results suggested that a substrate concentration of 25 μM was optimal for seal liver. At concentrations above 25 μM, resorufin formation decreased.

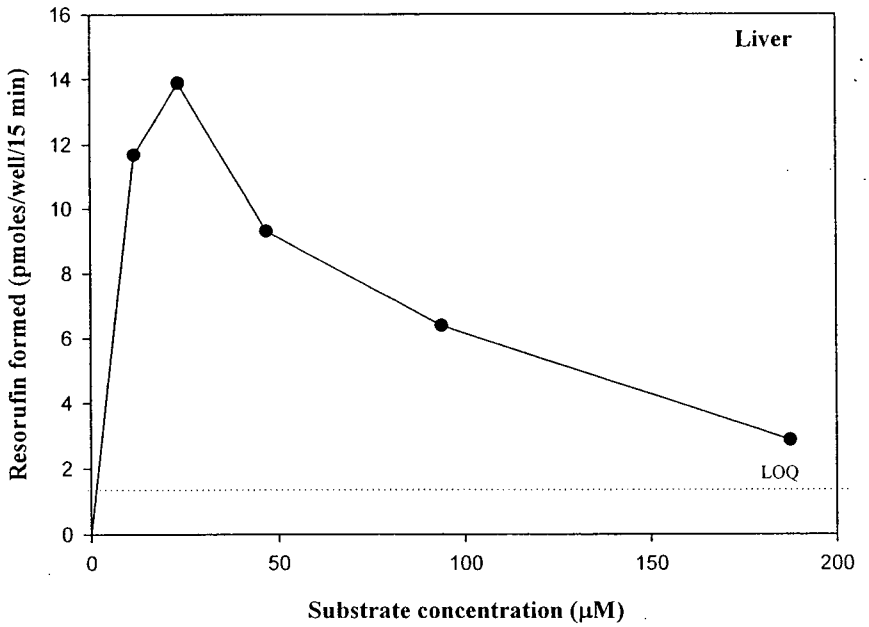


Figure 2.7: Effect of substrate concentration on resorufin formation in harbour seal liver. Liver S9 fraction was from one harbour seal pup (PV00101). Data points are the averages of triplicate fluorescence readings.

2.11.1.4 Total protein concentration for the EROD assay

The effect of total protein concentration on product formation was measured in liver of the same four seals listed in section 2.11.1.3. There was a relatively linear relationship between resorufin production and total protein concentration up to a concentration of 0.5 mg/well. A representative plot is shown in Figure 2.8. A protein concentration that falls on the linear portion of the curve should be used for EROD assays. Based on these results, 0.1 mg total protein/well was chosen as the loading concentration for liver samples.

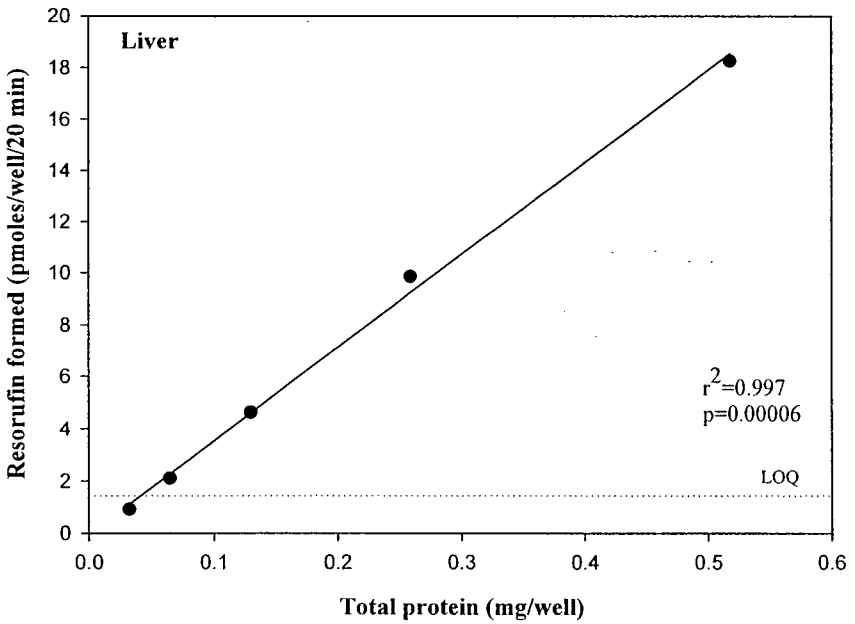


Figure 2.8: Effect of total protein concentration on resorufin formation in harbour seal liver. Liver S9 fraction was from one harbour seal pup (PV00101). Data points are the averages of triplicate fluorescence readings.

Serial dilutions of skin S9 fractions from two harbour seals, PV01-11 (pup from Fraser River) and PV01-34 (adult from Boundary Bay), were used to look at the effect of protein concentration on product formation in skin homogenates. Resorufin concentration increased linearly with protein concentration and then reached a plateau (Figure 2.9). It should be noted, however, that readings for the three lowest protein concentrations were below the limit of detection. Based on these results, a minimum of 0.1 mg total protein/well was chosen as the loading concentration for skin samples.

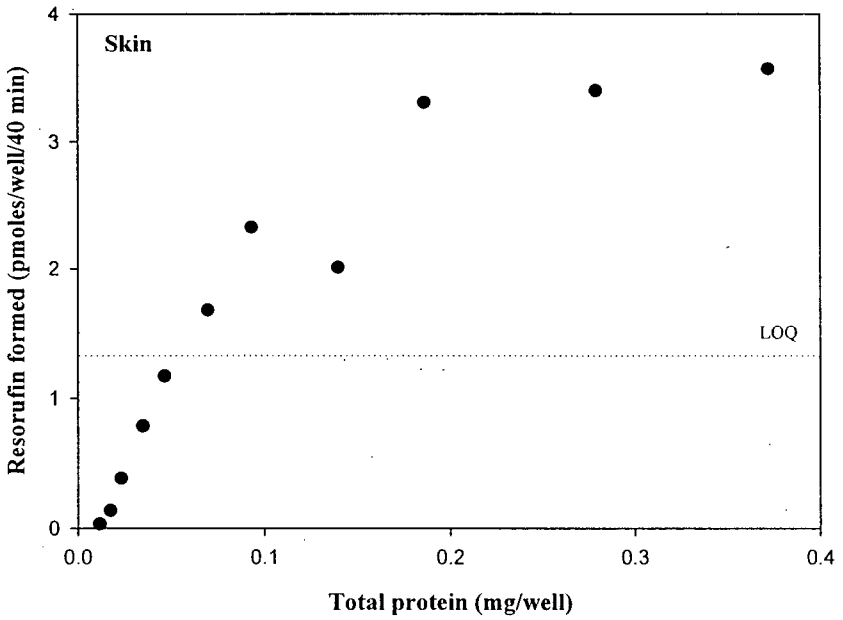


Figure 2.9: Effect of total protein concentration on resorufin formation in harbour seal skin. Skin S9 fraction was from one harbour seal pup (PV01-11). Data points are the averages of triplicate fluorescence readings. 'Data points' that were below the LOQ are shown only for comparative purposes.

2.11.1.5 NADPH concentration for the EROD assay

The concentration of NADPH, a cofactor in the EROD reaction, was also varied to determine if it affected EROD activity in harbour seal liver. Increasing the initial NADPH concentration above 2.5 mM did not have an effect on EROD activity, indicating that NADPH was in excess at this concentration. An initial concentration of 2.5 mM was used for subsequent EROD assays. This concentration is comparable to concentrations used in other EROD assay protocols.

2.11.1.6 Effect of skin biopsy size on total protein yield and EROD activity

To determine whether the amount of tissue homogenized and the buffer dilution factor had an effect on total protein yield and/or EROD activity, different amounts of skin from two harbour seals, PV01-26 (pup from Boundary Bay) and PV01-32 (adult from Boundary Bay), were homogenized in a fixed volume of buffer. The effect of biopsy size on product formation was variable (Figure 2.10). Different pieces of skin likely contained variable amounts of CYP1A, and this factor likely produced some of the scatter observed. Figure 2.11 shows data from the same two seals together with data from PV01-11 and PV01-34. Total protein yield increased linearly as the amount of tissue homogenized was increased (Figure 2.11).

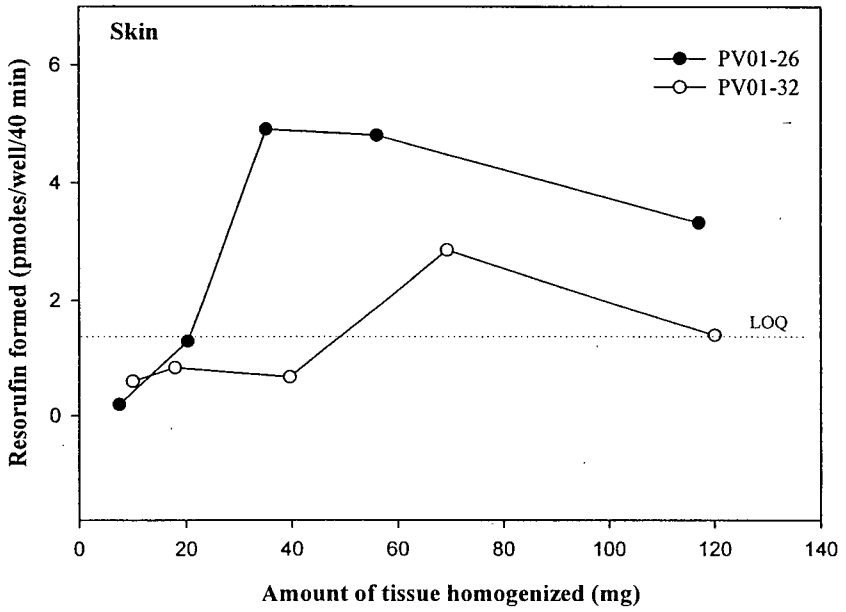


Figure 2.10: Effect of biopsy size and buffer dilution factor on resorufin formation in harbour seal skin.

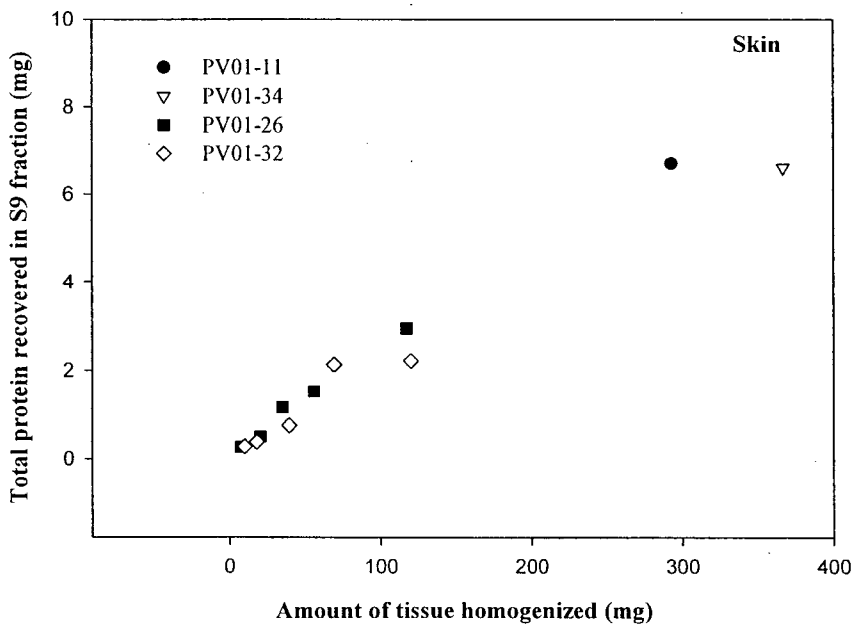
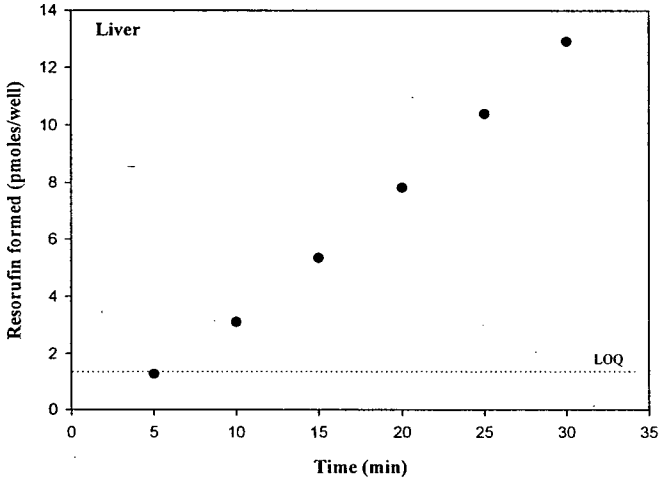


Figure 2.11: Effect of biopsy size on total protein yield for harbour seal skin.

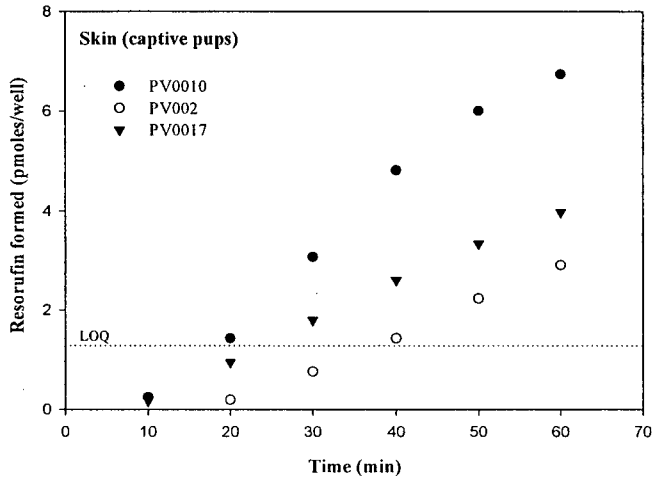
2.11.1.7 Reaction time

Resorufin formation was linear with respect to time for both harbour seal liver and skin (Figure 2.12). EROD activity was calculated using data points from the linear portion of the product vs. time curve for each sample. EROD activity was calculated using a reaction time of 40 minutes for skin samples from Fraser River pups versus 20 minutes reaction time for skin samples from the other populations (Figure 2.12C), as the reaction profile was noticeably different for the Fraser River pups (Figure 2.12B).

A



B



C

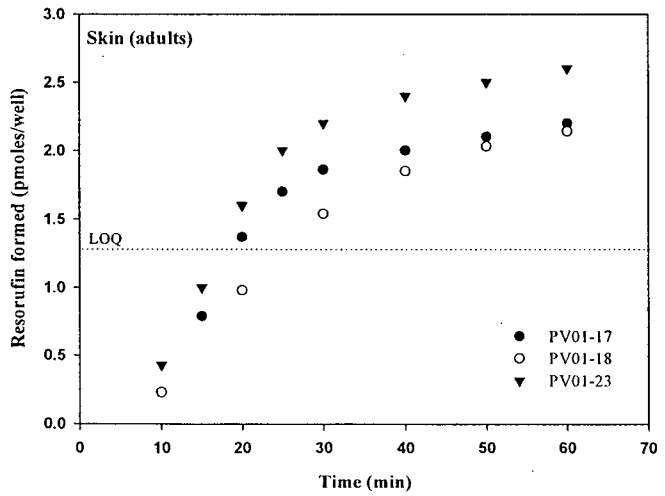


Figure 2.12: Resorufin formation over time in harbour seal liver (A) and skin (B,C). Reaction profiles for three different seals are shown in (B) and (C).

2.11.2 Immunoblot Assay

2.11.2.1 General protocol for the immunoblot assay

Experiments involving minor modifications of the Laemmli (1970) and Towbin (1979) protocols were conducted to optimize the detection of CYP1A bands in harbour seal samples. Both a large gel system (Hoeffer) and mini-gel system (Bio-Rad) were used. Although protein bands were detected by both systems, the mini-gel protocol appeared to be more sensitive. This may have been due to the membranes that were used with the different systems (nitrocellulose for large gels vs. PVDF for mini-gels), as PVDF membranes have a higher binding capacity for proteins (170-200 $\mu g/cm^2$ vs. 80-100 $\mu g/cm^2$) (Bio-Rad Blotting Guide). However, PVDF membranes may also have been responsible for the higher degree of non-specific protein staining obtained with mini-gels.

We varied the composition of the electrophoresis, transfer, and sample dilution buffers using mini-gels in an attempt to increase band resolution and staining intensity for the skin samples. Varying the buffer composition had no effect.

Chemiluminescence was also tested in this study. A few harbour seal and killer whale skin samples were run on large gels, transferred to nitrocellulose membranes, and visualized using chemiluminescence. Chemiluminescence was expected to be more sensitive for detecting proteins on immunoblots than the colorimetric technique used in this study; however, it did not improve our ability to detect CYP1A in harbour seal or killer whale skin and was not further pursued.

2.11.2.2 Total protein concentration for the immunoblot assay

We varied the amount of total protein loaded per lane for harbour seal S9 fractions in order to obtain an optimal response. In general, skin samples (with the

exception of BNF study samples) for both harbour seals and killer whales were loaded at the highest total protein concentration possible by diluting S9 fractions with sample dilution buffer in a 1:1 ratio, up to a maximum of 60 µg total protein/lane. This was necessary because the total protein content of skin S9 fractions was low relative to liver (approximately four times lower), and protein bands were often faint or undetectable for skin samples. Sample loading concentrations are summarized in Table 2.5.

2.11.2.3 Cross-reactivity of antibodies with harbour seal CYP1A

To investigate the specificity and sensitivity of different antibodies with putative harbour seal CYP1A proteins, four different primary antibodies (rabbit anti-trout CYP1A1 IgG, rabbit anti-rat CYP1A2 serum, mouse anti-rat CYP1A1 IgG, goat anti-rabbit 1A1/1A2 IgG) were tested with harbour seal liver S9 and microsomal fractions. Mouse anti-rat CYP1A1 monoclonal IgG and rabbit anti-rat CYP1A2 polyclonal serum were most cross-reactive. The rabbit anti-rat CYP1A2 serum was used for the immunoblot analyses of harbour seal samples. Killer whale skin samples were probed with rabbit anti-rat CYP1A2 serum and rabbit anti-trout CYP1A1 IgG, and similar results were obtained with these two antibodies. Rabbit anti-rat CYP1A2 serum was used for subsequent assays of killer whale skin.

2.11.2.4 Protein transer from gel to membrane

To determine if proteins in the molecular weight range of interest (~ 55,000 Da) were being transferred from gels to membranes, four gels were stained with Coomassie blue after the transfer step. Protein bands on stained gels were compared with bands on immunoblots by measuring the distance that bands had migrated from the top of the gel or membrane respectively. Although the protein bands that were due to non-specific

staining were not transferred completely from gels to membranes, it appeared that the putative CYP1A proteins completely transferred.

2.11.2.5 Intra-assay variation

Three purified rat CYP1A1 concentrations were loaded in five lanes on two gels. Only three concentrations could be assayed due to the limited number of wells in the mini-gels. The 0.01 pmole standard was used as the internal standard to calculate relative contour quantity (RCQ) values for the 0.005 and 0.025 pmole standards. The coefficient of variation was < 20% for both of these concentrations (Table 2.8).

Table 2.8: Intra-assay[a] variation of relative contour quantities for immunoblot standards

[Rat CYP1A1 standard] (pmoles/lane)	Mean relative contour quantity (RCQ)	CV (%)
0.005	0.6 ± 0.1	12.2
0.01	1.0	-
0.025	2.0 ± 0.4	19.0

Values are presented as the mean ± SD
[a] Each standard was loaded in 5 lanes. Only three standard concentrations could be assayed due to the limited number of lanes available for loading.
The 0.01 pmol/lane standard was used as the internal standard for intra-assay RCQ calculations.

2.11.2.6 Inter-assay variation

Standard curves consisting of five different purified rat CYP1A1 concentrations were obtained from assays conducted on five different days (two curves/day). The coefficient of variation for all concentrations was approximately 20% (Table 2.9). The inter-assay calibration curve is shown in Figure 2.13.

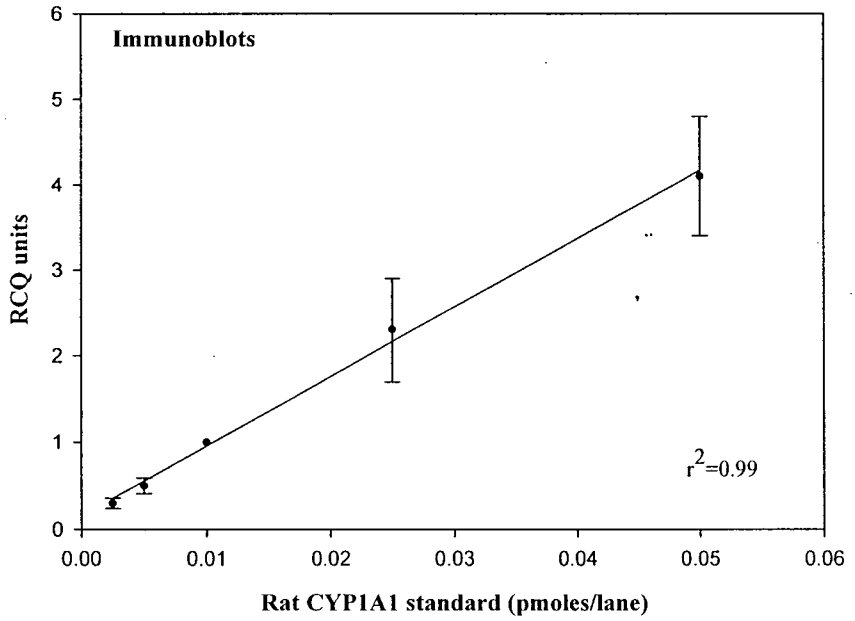
Table 2.9: Inter-assay[a] variability of relative contour quantity (RCQ) values for immunoblot standards

[Rat CYP1A1 standard] (pmoles/lane)	Mean relative contour quantity (RCQ)	CV (%)
0.0025	0.3 ± 0.06	20.9
0.005	0.5 ± 0.09	18.1
0.01	1.0	-
0.025	2.3 ± 0.6	24.4
0.05	4.1 ± 0.7	16.5

Vales are presented as the mean ± SD

[a] Standard curves were determined on five different days (2 curves/day).

The 0.01 pmol/lane standard was used as the internal standard for intra-assay RCQ calculations. The inter-assay CV for the 0.01 pmole/lane standard, using 0.05 pmoles/lane as the internal standard was 17.8%.



Error bars = SD

Figure 2.13: Inter-assay calibration curve for immunoblots. RCQ = relative contour quantity of each CYP1A1 band relative to that of the internal standard (0.01 pmole rat standard).

Purified harbour seal CYP1A is not yet available, therefore rat CYP1A was used as the internal standard for all sample CYP1A protein bands. Because the standards were from a different species than the study samples, the standards could not be used to obtain an absolute measure of CYP1A in harbour seal S9 fractions. In order to determine if harbour seal protein bands were quantifiable, RCQ values of sample bands were compared to those of rat standards. As 0.0025 pmoles was the lowest rat standard concentration analyzed, a value could not be reported for the limit of detection (LOD). The limit of quantitation (LOQ) was defined as the mean inter-assay relative contour quantity (RCQ) value obtained for the 0.0025 pmole rat CYP1A1 standard (RCQ=0.3), where 0.01 pmoles rat CYP1A/lane was the internal standard.

3. RESULTS

3.1 Study 1: Captive Harbour Seal Study

3.1.1 Induction of CYP1A by in vivo BNF treatment

Liver and skin biopsies were obtained from six of the captive harbour seal pups for an induction study (3 BNF-treated, 3 controls). Two CYP1A protein bands having approximately the same mobility as a purified CYP1A1 rat standard were detected by immunoblot analysis. Protein bands are shown in Figures 3.1 (liver) and 3.2 (skin). CYP1A protein bands for liver and skin of BNF-treated harbour seals are shown on the same immunoblot in Figure 3.3. These protein bands had approximately the same mobility as CYP1A in liver microsomes from a 3-MC-treated rat. EROD activity was quantified in all samples, except for a skin sample from one BNF-treated seal (below LOQ).

3.1.1.1 Immunoblot analysis

Oral BNF treatment caused induction of CYP1A protein levels in both liver and skin of harbour seals (Figures 3.1-3.4). Hepatic CYP1A protein levels were approximately five times higher in treated seals than in controls, as assessed by combining upper and lower bands (p=0.0001). Individually, both upper (p=0.00003) and lower (p=0.003) CYP1A bands were also significantly induced in treated seals compared to control seals. A t-test could not be performed for skin as data were obtained for only one control skin sample. However, cutaneous CYP1A protein levels were approximately sixteen times higher in BNF-treated seals (n=3) than in one control seal.

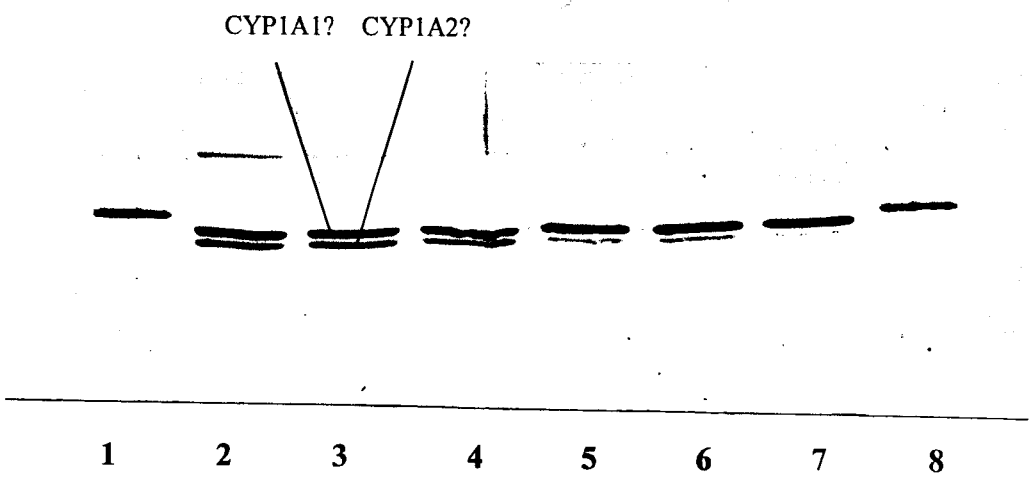


Figure 3.1: Immunoblot of BNF-treated and control harbour seal pup liver samples probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lanes 1 and 8 are purified rat CYP1A1 standards, applied at 0.1 pmoles and 0.05 pmoles per lane respectively. Lanes 2-4 are liver S9 fractions from control (untreated) seals PV001, 07, and 16, each loaded at 10 μg per lane. Lanes 5-7 are liver S9 fractions from BNF-treated seals PV0012, 14, and 15, each loaded at 2 μg per lane. The membrane was developed with substrate solution for 2 minutes.

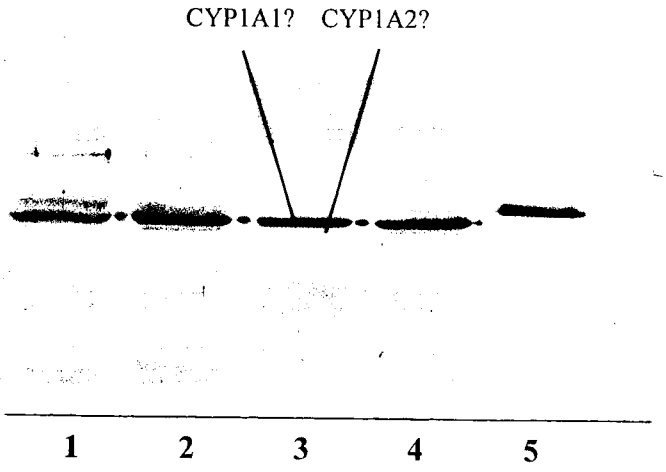


Figure 3.2: Immunoblot of BNF-treated and control harbour seal pup skin samples probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lane 1 is a skin S9 fraction from control (untreated) seal PV0016. Lanes 2-4 are skin S9 fractions from BNF-treated seals PV0012, 14, and 15, respectively. Lane 5 is a purified rat CYP1A1 standard, applied at 0.01 pmoles per lane. Control and treated S9 fractions were applied at final concentrations of 25 μg and10 μg total protein, respectively. The membrane was developed with substrate solution for 3 minutes.

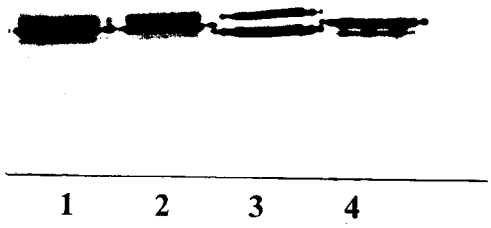


Figure 3.3: Immunoblot showing both BNF-treated liver and skin samples probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lanes 1 and 2 are skin S9 fractions from BNF-treated seals PV0012 and 14, applied at a final concentration of 10 ug total protein per lane. Lane 3 is a 3-MC-treated rat liver microsomal sample, loaded at 0.07 μg per lane. Lane 4 is a liver S9 fraction from BNF-treated seal PV0012, loaded at 2.5 μg per lane. The membrane was developed with substrate solution for 5 minutes.

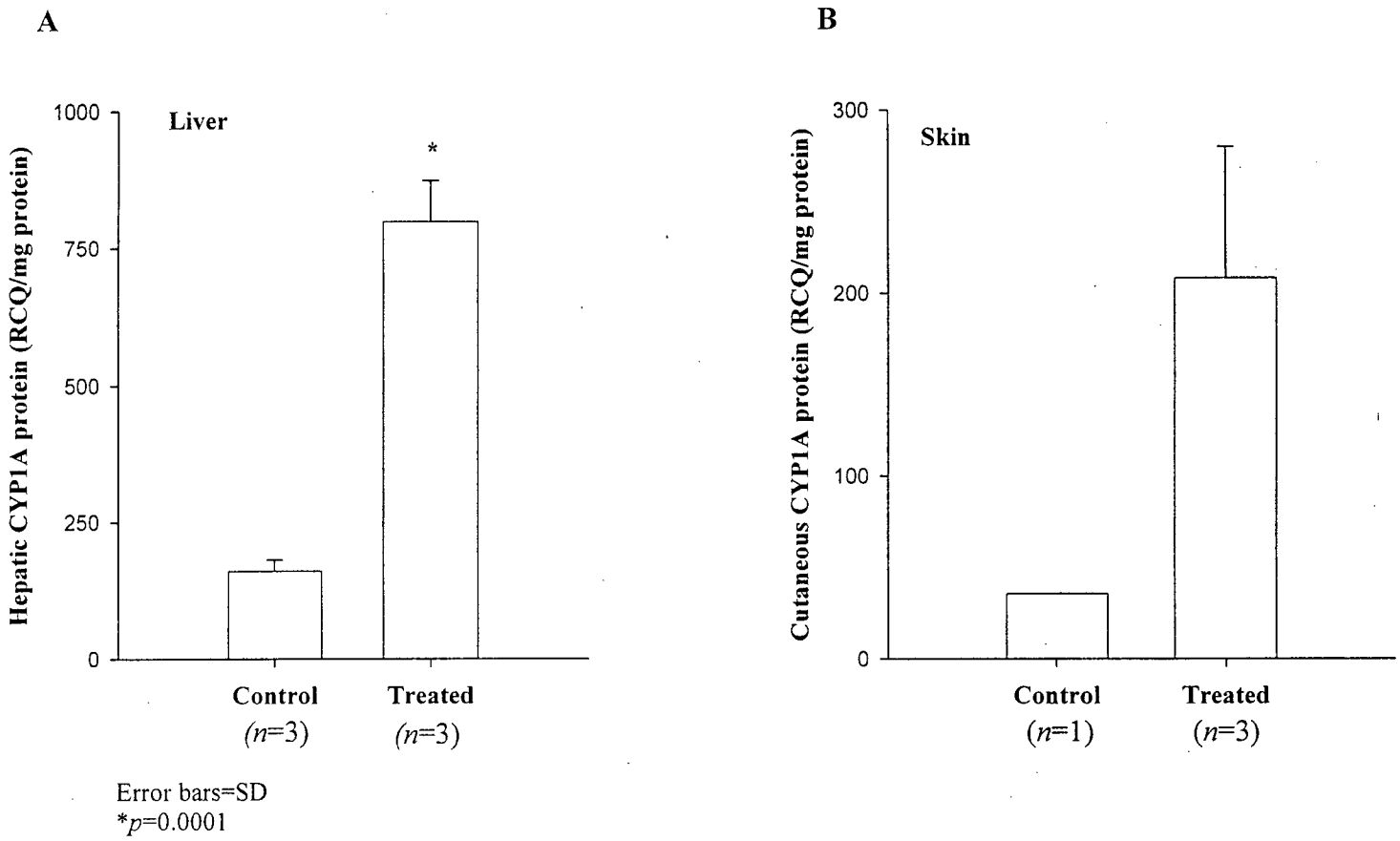


Figure 3.4: Effect of oral BNF treatment on CYP1A protein levels in liver (A) and skin (B) of captive harbour seals. RCQ values for liver and skin are the relative staining intensities of sample bands normalized to the band intensity of 0.1 pmole and 0.01 pmole rat CYP1A1 standards, respectively.

3.1.1.2 EROD activity

Oral BNF treatment did not cause an increase in EROD activity in either liver or skin relative to control seal tissues (Figure 3.5).

3.1.1.3 BNF inhibition of EROD activity

Although BNF is a known *inducer* of CYP1A, BNF treatment has been found to *inhibit* EROD activity (Haasch *et al.*, 1993). An experiment was carried out to determine whether *in vitro* BNF treatment could inhibit EROD activity in harbour seal liver, as has been shown previously in other species (Haasch *et al.*, 1993). EROD activity was inhibited *in vitro* at all BNF concentrations tested. The lowest concentration of BNF (0.0625 μM) caused more than 80% inhibition of EROD activity at a reaction time of 20 minutes (Figure 3.6).

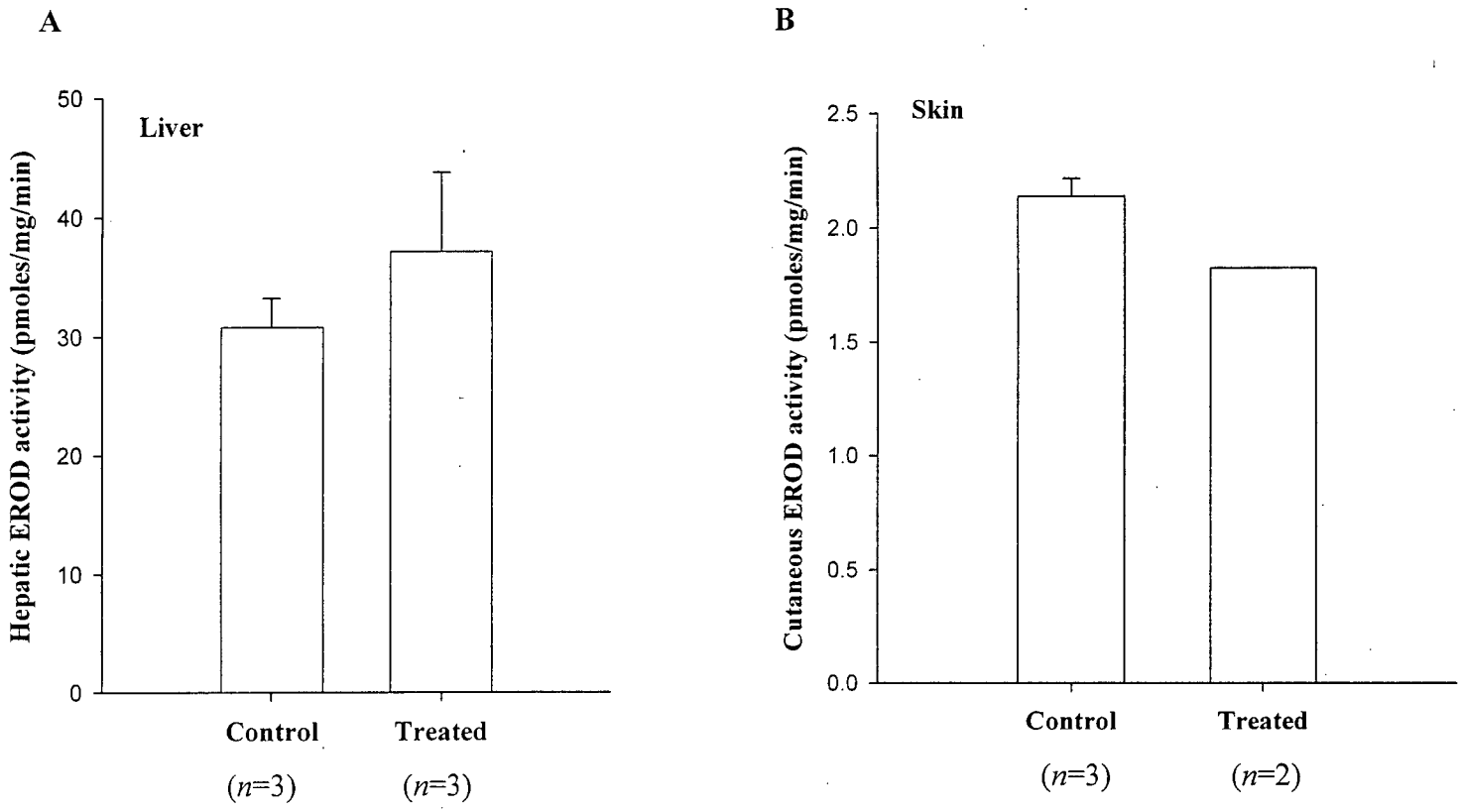


Figure 3.5: Effect of oral BNF treatment on EROD activity in liver (A) and skin (B) of captive harbour seals.

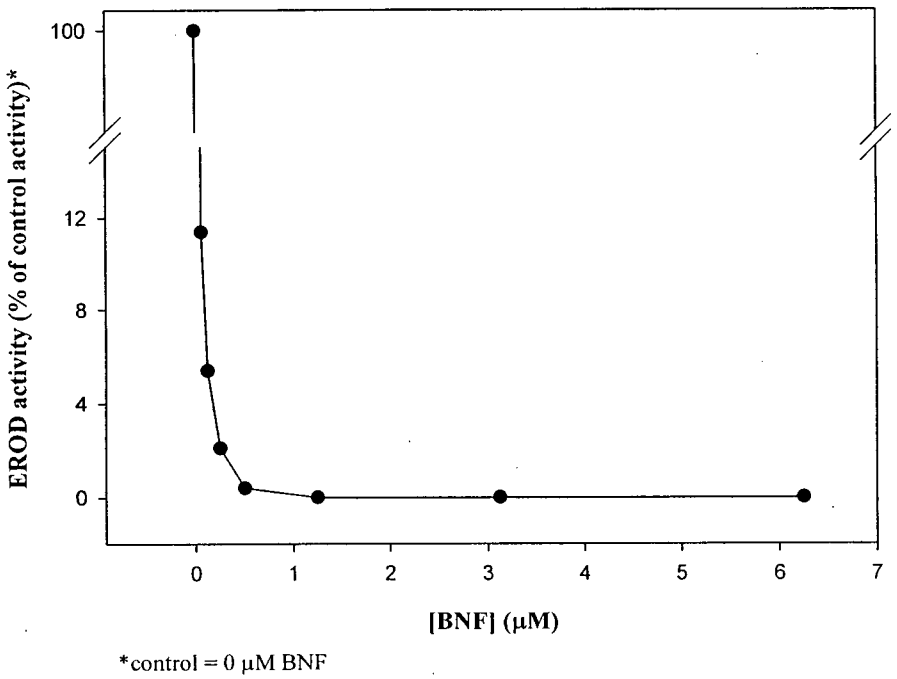


Figure 3.6: Inhibition of hepatic EROD activity by *in vitro* BNF treatment. A liver S9 fraction from one BNF-treated seal (PV0015) was preincubated with BNF dissolved in DMSO and loaded at 0.01 mg total protein/well. EROD activity was measured using a reaction time of 20 minutes.

3.1.2 CYP1A expression in tissue biopsies obtained two days post-capture

3.1.2.1 Liver

Liver biopsies were analyzed for twelve of twenty harbour seal pups captured. Liver from the remaining seals was either insufficient for CYP1A analysis or was used for parallel biomarker studies (vitamin A). EROD activity was detected in all liver biopsy samples, and activity in all but two samples was quantifiable.

For most liver biopsy samples, two CYP1A bands were detected on immunoblots (Figure 3.7). When both an upper and lower band were quantifiable, these values were added together. However, for some blots it was only possible to quantify one band (i.e. only one band stained strongly on immunoblots). Average values (relative contour quantities) for CYP1A protein were based on duplicates or triplicates for each sample, except for PV0012, PV0017, and PV0018. Relative contour quantities (RCQs) for liver samples were calculated using an internal standard of 0.1 pmoles rat CYP1A1/lane. This concentration was more appropriate for the staining intensity of protein bands in liver.

Table 3.1: CYP1A expression in hepatic S9 fractions from Fraser River harbour seal pups

Sample	Average CYP1A protein levels (RCQ/mg total protein)[a]	EROD activity (pmoles/mg/min)[b]
PV004	16.3	1.15
PV007	18.2	< LOQ
PV009	31.3	3.90
PV0011	18.9	1.68
PV0012	16.1	1.80
PV0013	19.9	2.31
PV0014	16.3	2.28
PV0016	19.6	2.97
PV0017	29.5	2.60
PV0018	8.6	< LOQ
PV0019	24.9	4.36
PV0020	20.9	3.23

LOQ (EROD) = 0.65 pmoles/mg/min
LOQ (immunoblots) = 0.3/mg total protein loaded
[a] RCQ (relative contour quantity) is the staining intensity of sample bands normalized to a 0.1 pmole/lane rat CYP1A1 standard.
[b] EROD activity was calculated at 20 minutes reaction time for all samples.

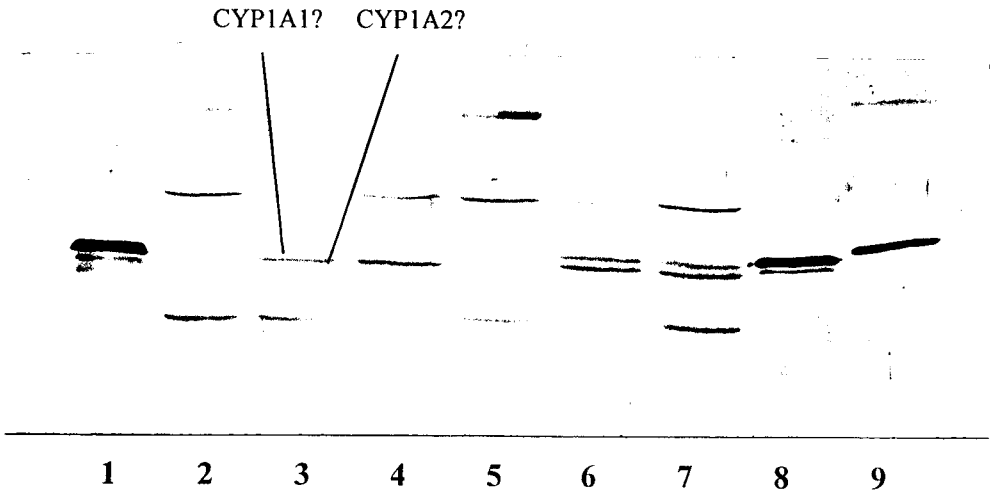
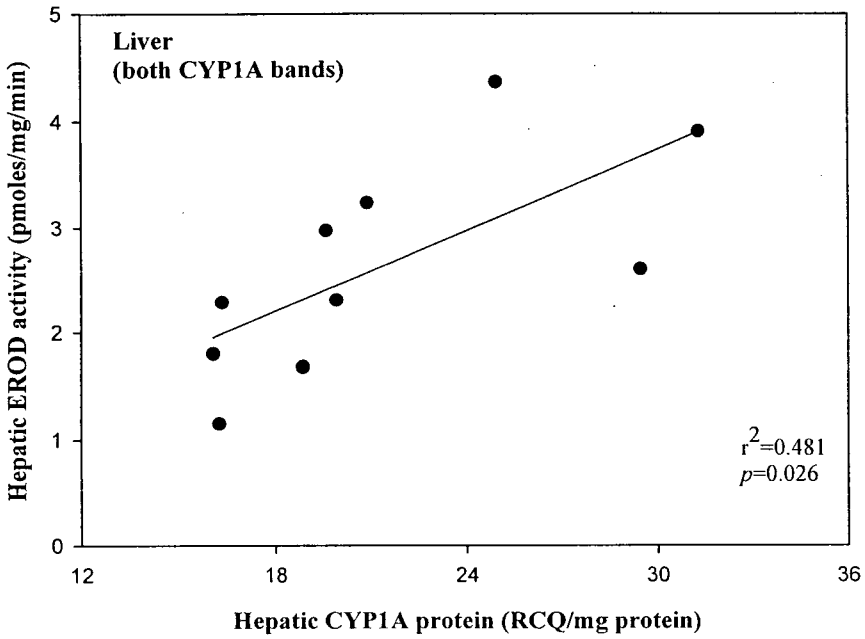


Figure 3.7: Immunoblot of captive (Fraser River) harbour seal pup liver S9 fractions probed with rabbit anti-rat polyclonal CYP1A2 serum (1:500 dilution). Lanes 1 and 9 are purified rat CYP1A2 standards, applied at 0.05 pmoles (lane 1) and 0.1 pmoles (lane 9) per lane. Lanes 2-7 are samples from seals PV0014, 16, 17, 18, 19, and 20, respectively. Lane 8 is a liver S9 fraction from a BNF-treated seal (PV0012). The S9 fractions were applied at a final concentration of 20 μg total protein per lane (lanes 2-7) and 5 μg (lane 8).

Hepatic CYP1A protein levels were positively correlated with hepatic EROD activity (r^2=0.481, p=0.026, n=10) when upper and lower putative CYP1A bands were combined (Figure 3.8A). CYP1A protein and EROD activity were also correlated when only the upper protein band was quantified (r^2= 0.538, p=0.025) (Figure 3.8B). The lower CYP1A protein band did not correlate with EROD activity (p=0.836) (data not shown).

A



B

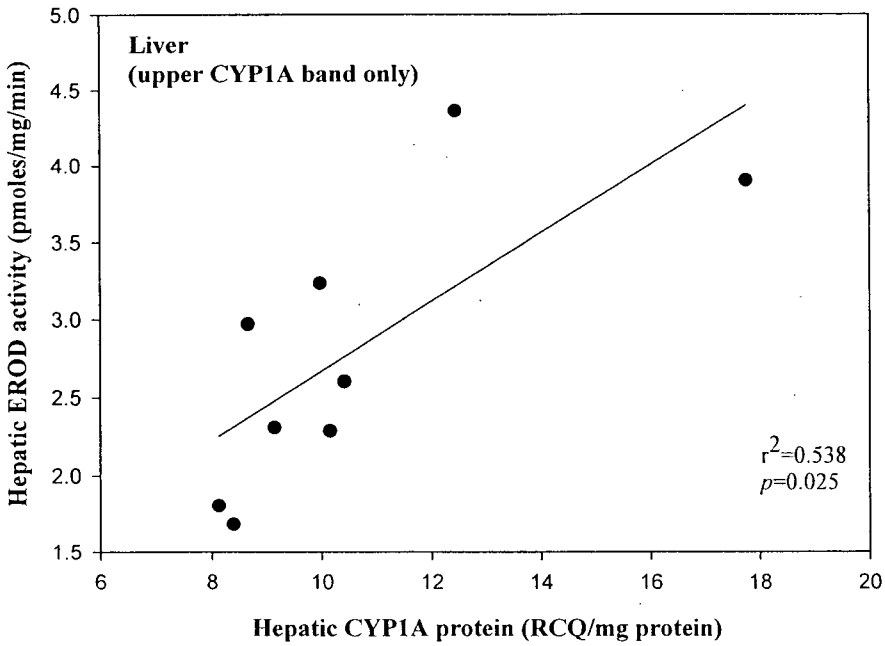


Figure 3.8: Relationship between hepatic CYP1A protein levels and EROD activity for Fraser River harbour seal pups. CYP1A protein levels shown in (A) are the combined RCQ values of upper and lower CYP1A bands. RCQ values for only the upper CYP1A band are shown in (B).

3.1.2.2 Skin

EROD activity was quantified in captive harbour seal skin S9 fractions; however, activities were below the limit of quantitation for 10 of 20 samples. Two CYP1A protein bands with the same relative mobility as CYP1A in seal liver were detected in skin samples. Average RCQs were based on duplicate or triplicate readings when possible. There were no replicates for PV006, PV0016, PV0017, or PV0018 (replicate values were either undetectable or < LOQ). CYP1A protein levels in skin correlated with EROD activity in skin when upper and lower protein bands were combined (r^2=0.494, p=0.023, n=10) (Figure 3.10). Individually, upper (p=0.399) and lower (p=0.222) protein bands did not correlate with EROD activity (data not shown). Both upper and lower bands were not detected in all samples.

Table 3.2: CYP1A expression in cutaneous S9 fractions from Fraser River harbour seal pups

Sample	Average CYP1A protein levels (RCQ/mg protein)[a]	EROD activity (pmol/mg/min)[b]
PV001	11.9	0.55
PV002	8.3	0.30
PV003	20.3	< LOQ
PV004	17.1	< LOQ
PV005	17.8	< LOQ
PV006	6.4	0.64
PV007	10.6	< LOQ
PV008	14.5	< LOQ
PV009	9.0	< LOQ
PV0010	15.8	1.00
PV0011	14.6	< LOQ
PV0012	< LOQ	< LOQ
PV0013	11.2	0.83
PV0014	16.6	0.78
PV0015	5.2	0.48
PV0016	7.4	0.45
PV0017	8.4	0.54
PV0018	16.4	< LOQ
PV0019	15.1	< LOQ
PV0020	6.7	0.49

LOQ (EROD) = 0.27 pmoles/mg/min
The EROD assay LOQ was defined in terms of pmoles resorufin formed. Because EROD activity was calculated at a different time point for Fraser River pups than the other seal groups (40 vs. 20 minutes), the LOQ, when expressed as EROD activity, is much lower for Fraser River pups. LOQ (immunoblot) = 0.3/mg total protein loaded
[a] RCQ (relative contour quantity) is the staining intensity of sample bands normalized to a 0.01 pmole/lane rat CYP1A1 standard.
[b] EROD activity was calculated at 40 minutes reaction time for all samples.

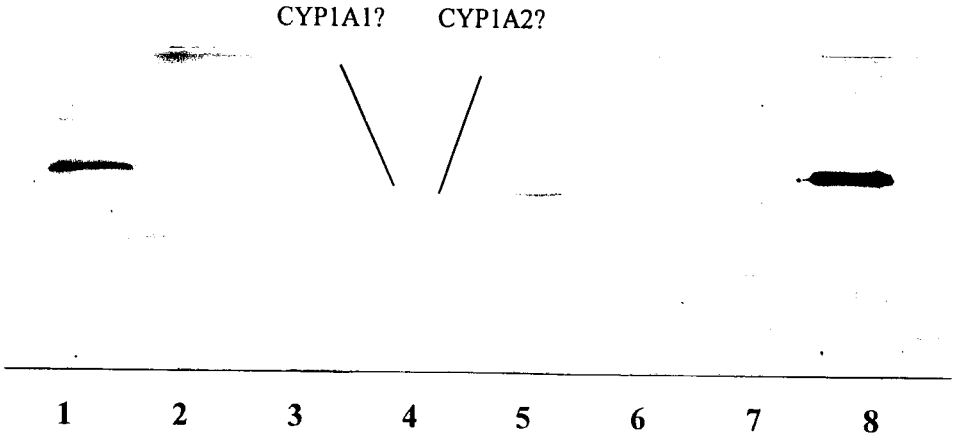


Figure 3.9: Immunoblot of captive (Fraser River) harbour seal pup skin S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lanes 1 and 8 are purified rat CYP1A1 standards, applied at 0.01 pmoles (lane 1) and 0.05 pmoles (lane 8) per lane. Lanes 3-7 are samples from seals PV0015, 16, 01, 02, and 05, respectively. Lane 2 is a skin S9 fraction from a Puget Sound seal pup (PV01-56). The S9 fractions were applied at final concentrations of 31 µg, 68 µg, 69 µg, 57 µg, 65 µg, and 48 µg (lanes 2-7), respectively.

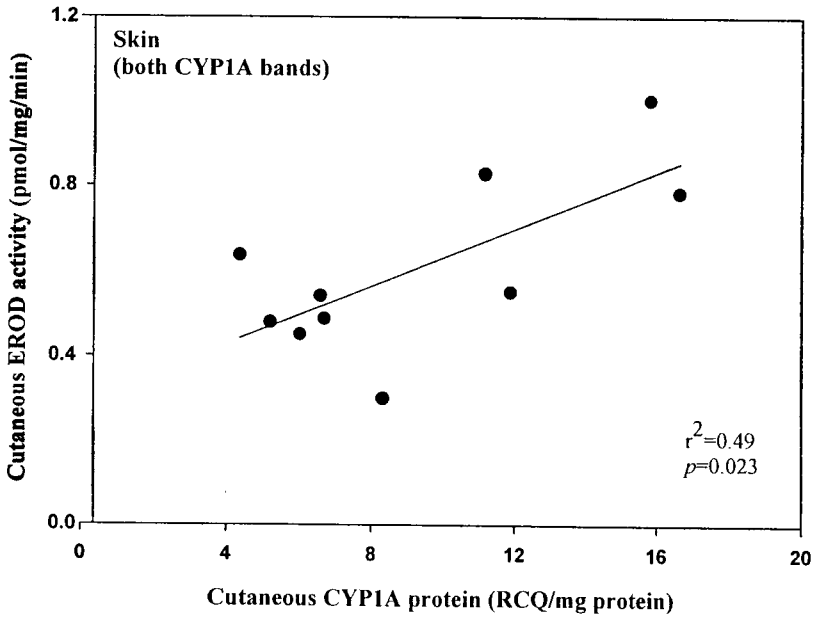


Figure 3.10: Relationship between CYP1A protein levels and EROD activity in skin of Fraser River harbour seal pups. CYP1A levels shown here are the combined RCQ values of both CYP1A bands (upper and lower).

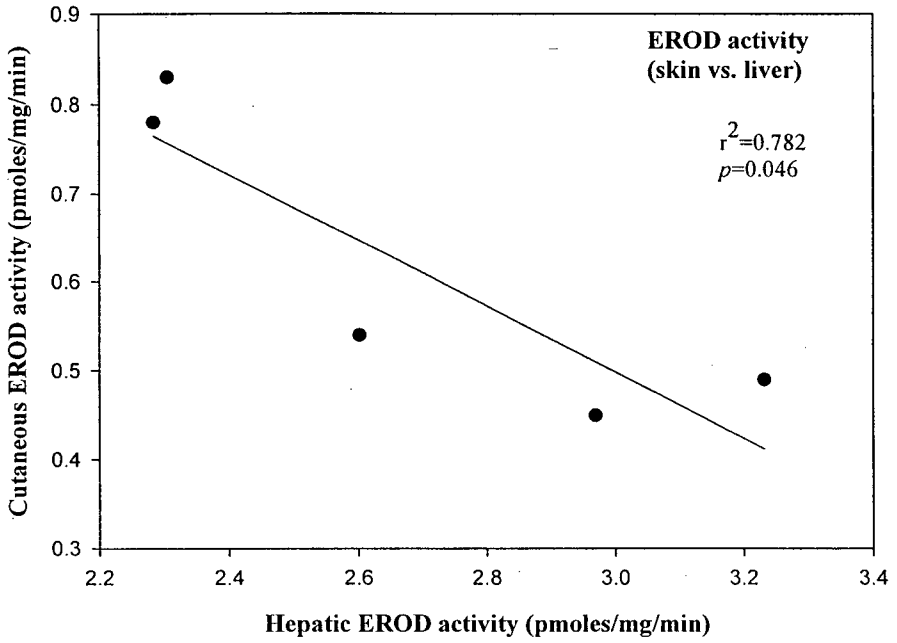
3.1.2.3 Comparison of CYP1A expression in skin and liver

EROD activity in skin was negatively correlated with EROD activity in liver (r^2=0.782, p=0.046, n=5) (Figure 3.11A). There was no significant correlation between CYP1A protein levels in the two tissues (p=0.227, n=11) (Figure 3.11B). Comparison of CYP1A protein band migration in liver and skin samples relative to each other and to the rat CYP1A standard suggested that putative CYP1A protein bands detected in skin corresponded to the same bands detected in liver (Figures 3.7, 3.9).

3.1.2.4 Effect of body weight on CYP1A expression

In order to determine whether physiological factors were affecting CYP1A expression in harbour seal tissues, I also looked at relationships between CYP1A and body weight and differences in CYP1A expression between males and females. There was a significant negative correlation between body weight at capture and hepatic EROD activity in Fraser River pups (r^2=0.572, p=0.011, n=10) (Figure 3.12A). However, there were no significant correlations between body weight and CYP1A protein levels in liver (p=0.303, n=12) (Figure 3.12B) or CYP1A expression in skin (EROD p=0.541, n=10; CYP1A p=0.975, n=19) (Figure 3.13).

A



B

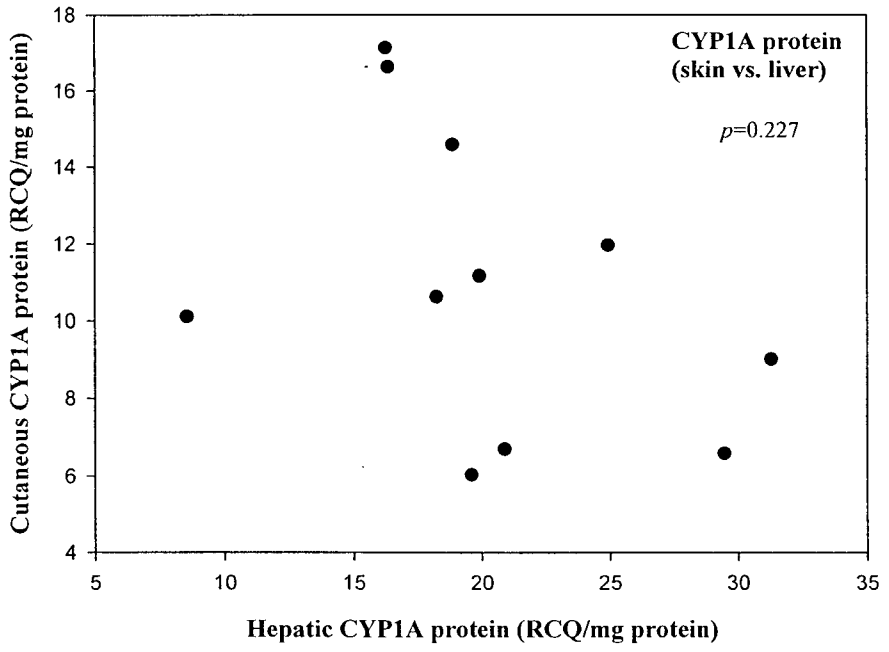
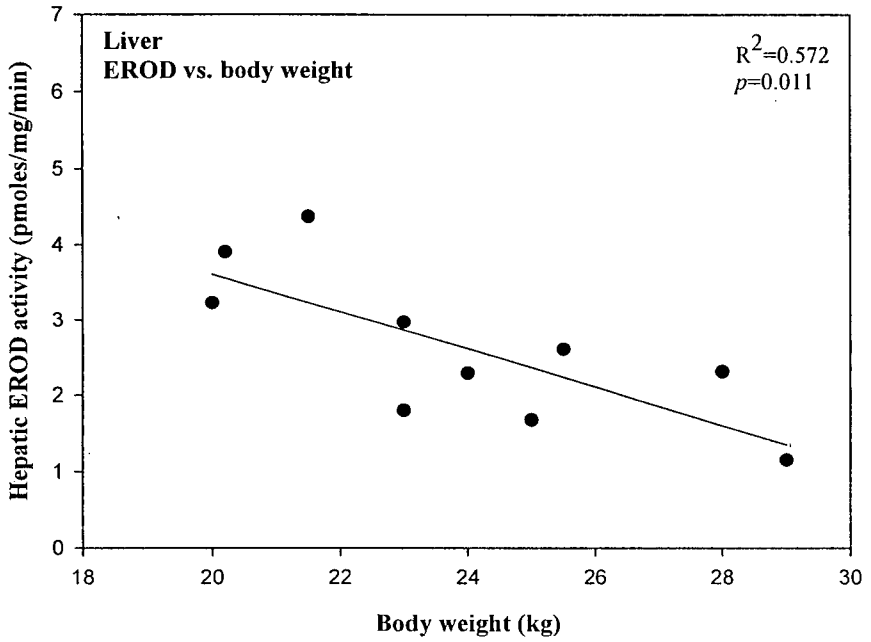


Figure 3.11: Relationship between CYP1A expression in liver and skin of Fraser River harbour seal pups. (A) EROD activity, (B) CYP1A protein levels.

A



B

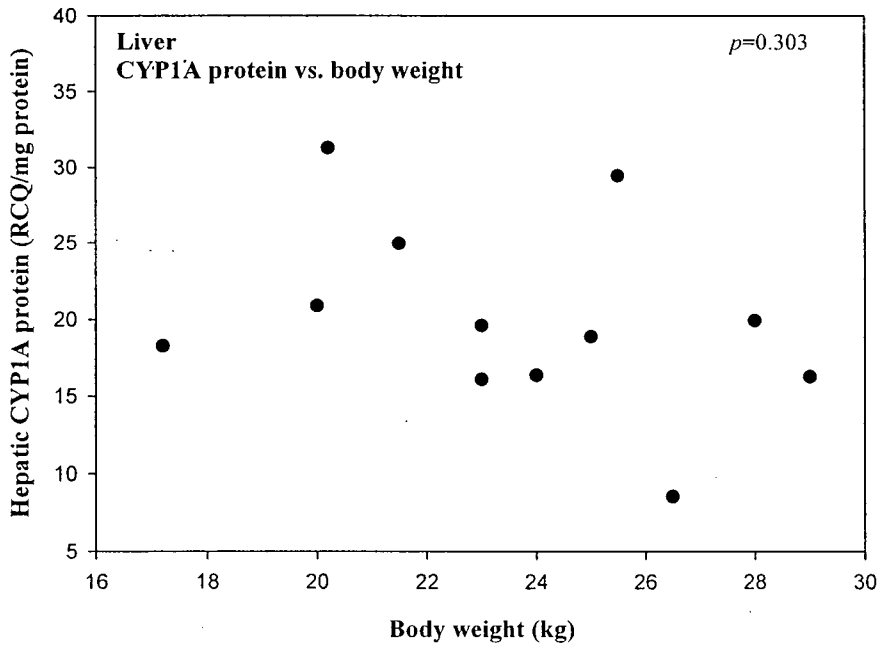
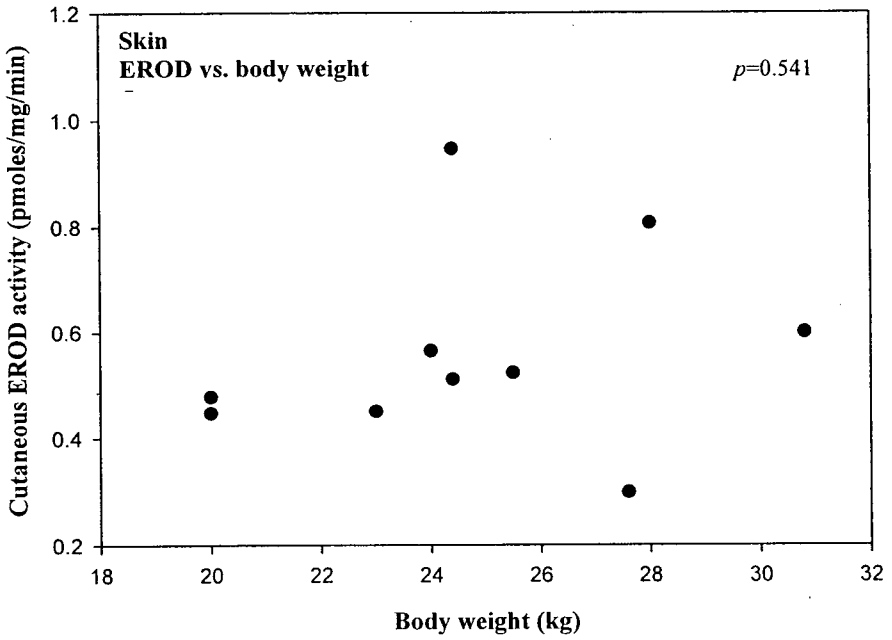


Figure 3.12: Relationship between body weight at capture and hepatic EROD activity (A) and CYP1A protein levels (B) for Fraser River harbour seal pups. CYP1A protein values shown in (B) are the combined RCQ values of upper and lower CYP1A protein bands.

A



B

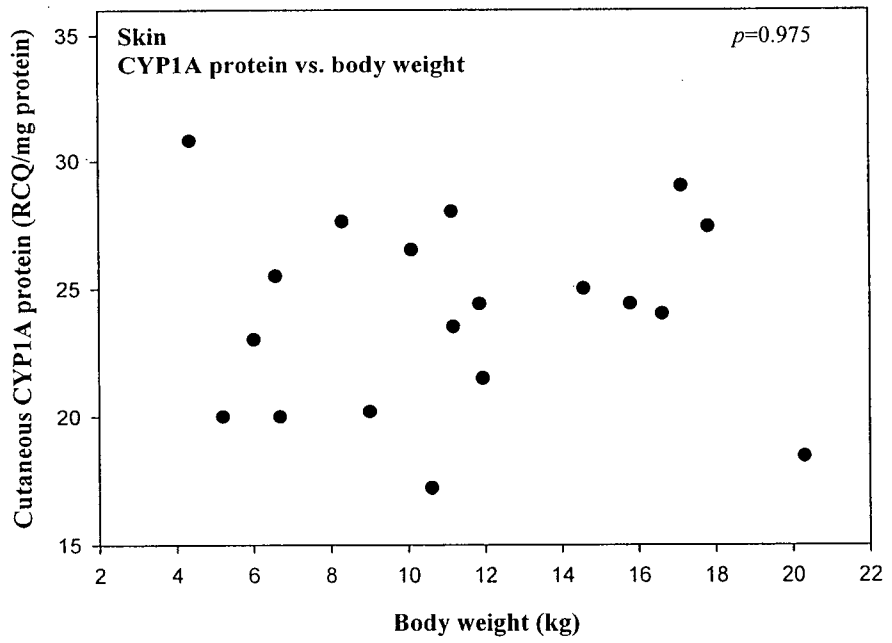


Figure 3.13: Relationship between body weight and cutaneous EROD activity (A) and CYP1A protein (B) in Fraser River (captive) harbour seal pups.

3.1.2.5 Effect of sex on CYP1A expression

There was no significant difference in hepatic EROD activity (p=0.371) or hepatic CYP1A protein (p=0.644) between males and females. There were also no sex differences in cutaneous EROD activtity (p=0.776) or CYP1A protein (p=0.854). CYP1A expression was not significantly different between males and females.

Table 3.3: Comparison of CYP1A expression between male and female harbour seal pups from the Fraser River estuary

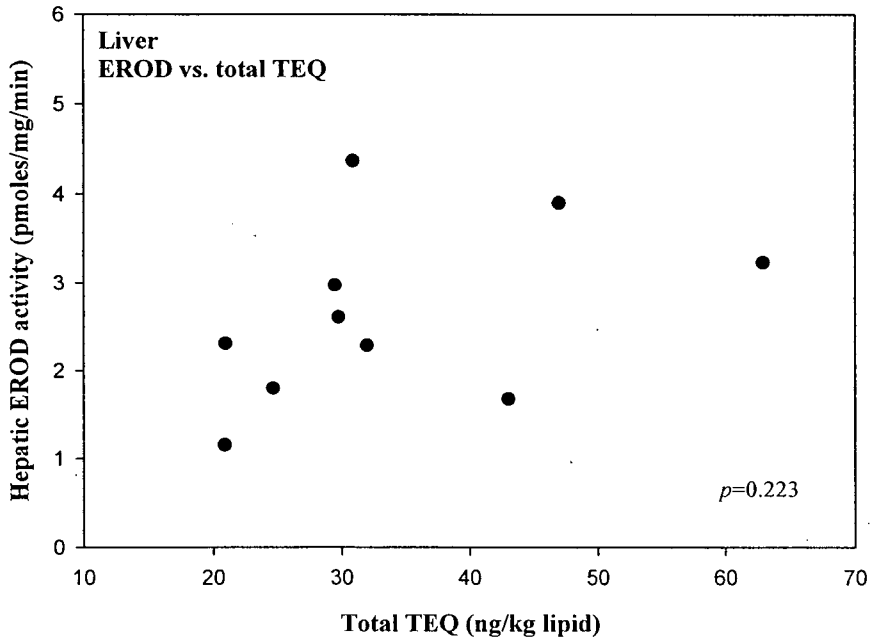
	Mean CYP1A protein levels (RCQ/mg protein)		Mean EROD activity (pmoles/mg/min)	
	Males	Females	Males	Females
Liver	19.5 ± 7.0 n=9	21.6 ± 3.1 n=3	2.43 ± 0.87 n=7	3.09 ± 1.35 n=3
Skin	12.2 ± 4.2 n=14	12.6 ± 6.0 n=5	0.56 ± 0.19 n=8	0.56 n=2

Values are presented as the mean ± SD

3.1.2.6 Effect of contaminant levels in blubber on CYP1A expression

There were no significant correlations between CYP1A expression in Fraser River harbour seal pup tissues and total TEQ for blubber (Figures 3.14A, 3.15A, 3.16). However, when mono-*ortho* PCBs were removed from the total TEQ value, there were significant positive correlations between EROD (p=0.037, n=10) and CYP1A protein (p=0.016, n=12) in liver and contaminants in blubber (Figures 3.14B, 3.15B).

A



B

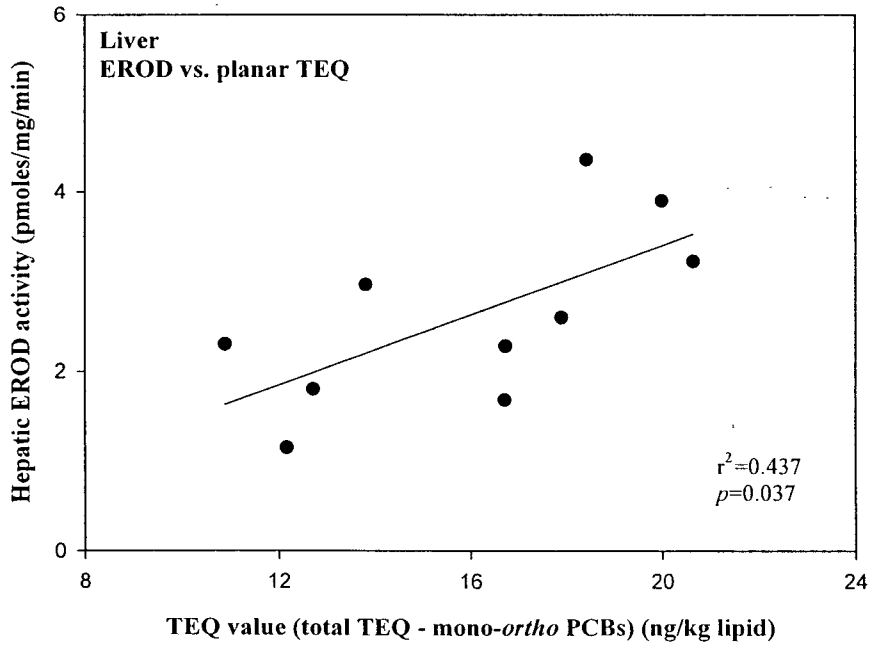


Figure 3.14: Relationship between hepatic EROD activity and contaminants in blubber. Total TEQ values are shown in (A). Total TEQ values, excluding mono-*ortho* PCBs are shown in (B).

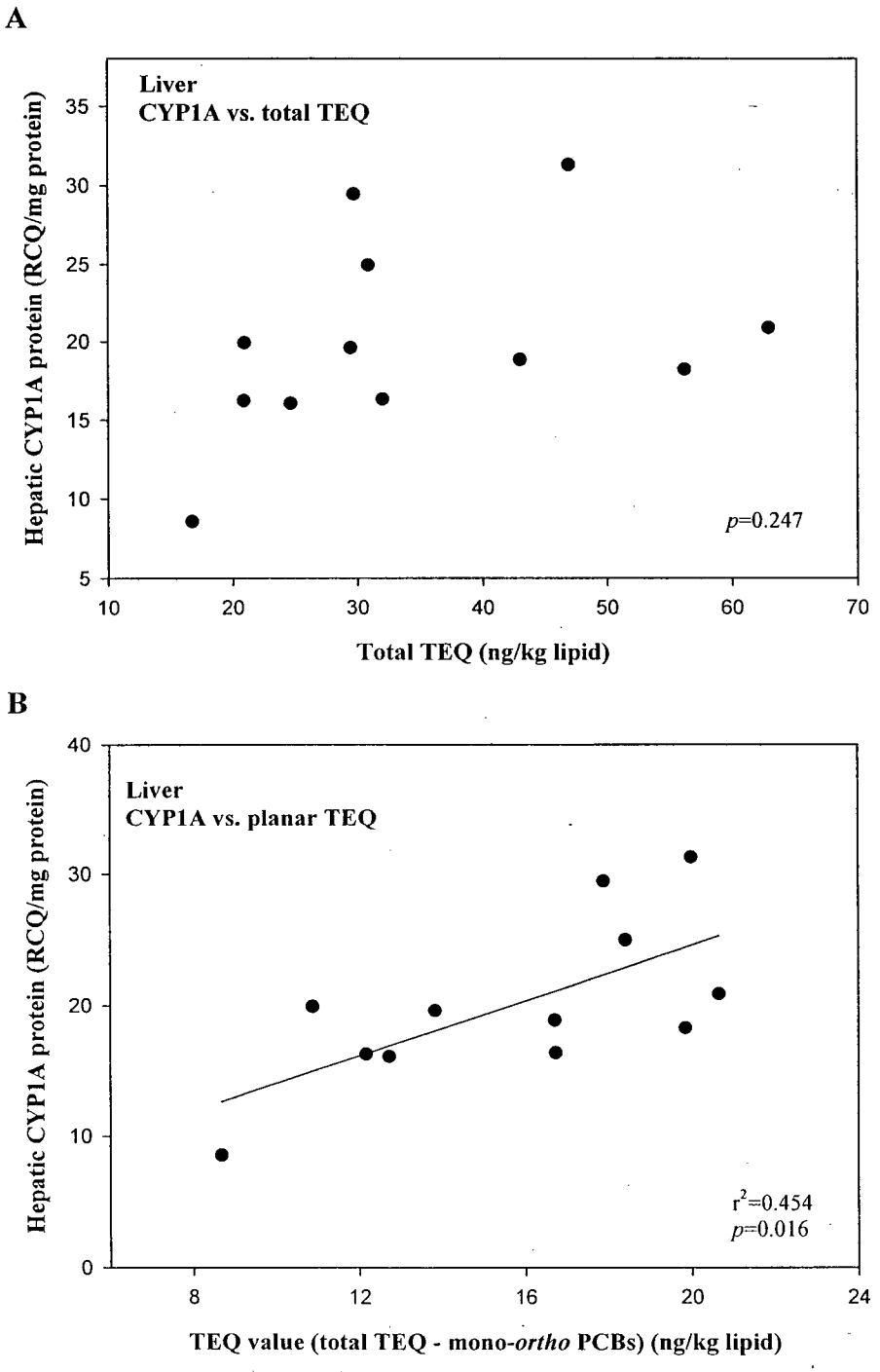


Figure 3.15: Relationship between hepatic CYP1A protein levels and contaminants in blubber. (A) shows total TEQ values. (B) shows total TEQ excluding mono-*ortho* PCBs CYP1A levels are the combined RCQ values of both CYP1A bands (upper and lower).

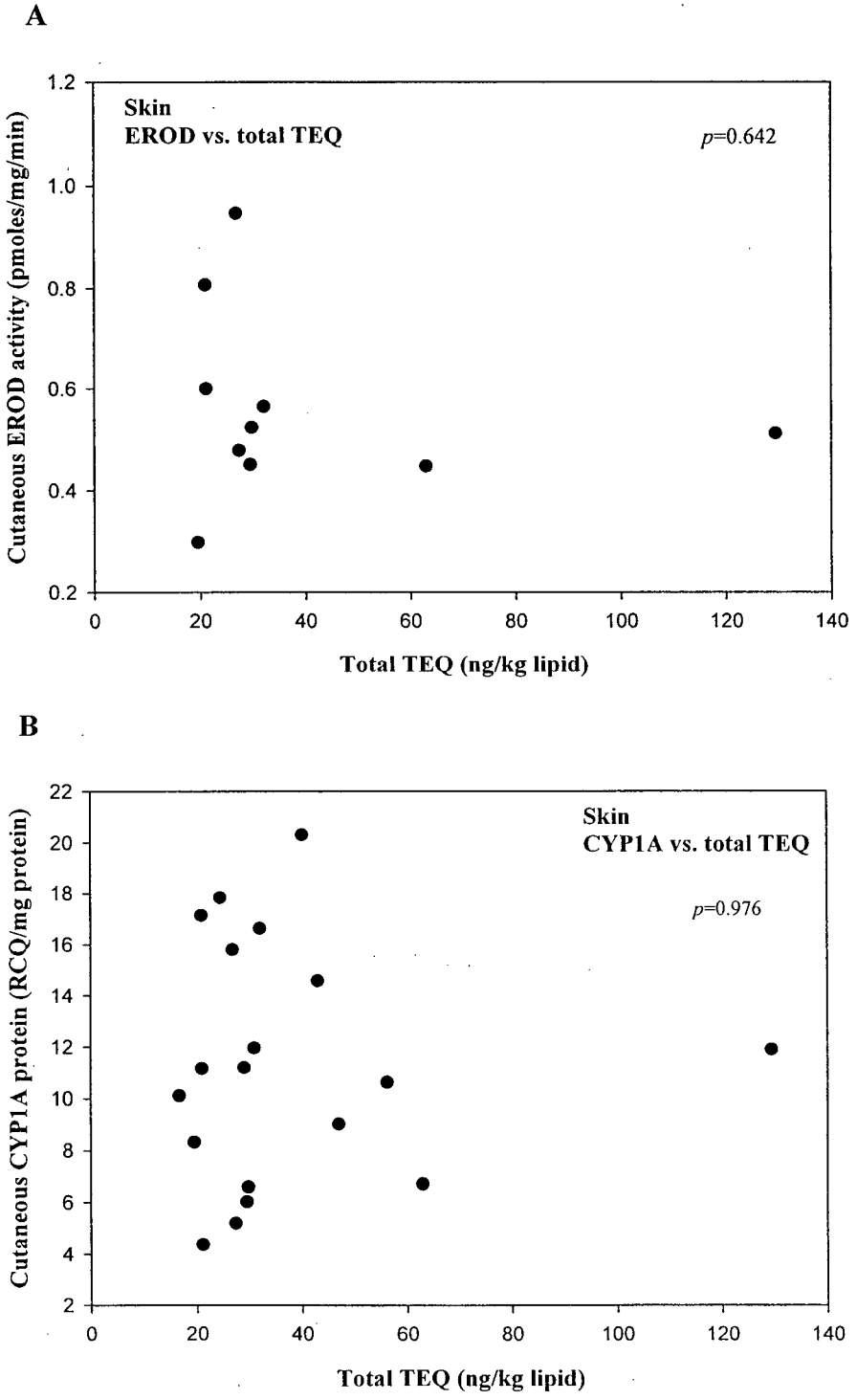


Figure 3.16: Relationship between cutaneous CYP1A expression and contaminants in blubber. CYP1A protein levels shown in (A) are the combined RCQ values of upper and lower CYP1A protein bands.

3.1.2.7 Relationship between body weight and contaminant levels in blubber

Contaminant levels in blubber (total TEQ) were negatively correlated with body weight of Fraser River pups (Figure 3.17). When a significant outlier (TEQ=129.4) ($p<0.05$) was removed from the data set, total TEQ was still negatively correlated with body weight ($p=0.0002$, $n=19$).

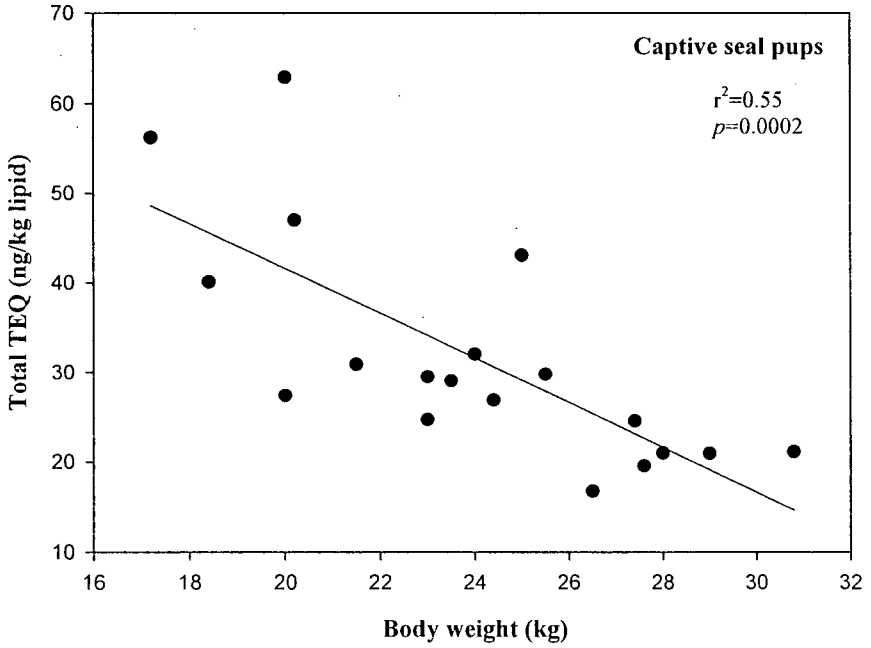


Figure 3.17: Total TEQ for blubber was negatively correlated with body weight in Fraser River harbour seal pups. A significant outlier (total TEQ =129.4) ($p<0.05$) was removed from this data set.

3.1.3 Increase in CYP1A expression over time in captive harbour seals

An increase in CYP1A expression in both liver and skin was observed in control seals ($n=3$) in the oral BNF study after three weeks in captivity. CYP1A expression in tissue biopsies that were taken during the BNF study was significantly higher than in biopsies taken three weeks earlier. Liver biopsies were obtained at both time points for

two of three control seals, and skin biopsies were obtained for all three seals at both time points.

3.1.3.1 CYP1A protein levels

CYP1A protein levels in liver were approximately seven times higher after three weeks in captivity (p=0.04), and there was a six-fold increase in CYP1A protein levels in skin from one seal (Figures 3.18, 3.19). However, due to limited sample volumes and an air bubble on one of the immunoblots, I did not obtain skin data from the second set of biopsies for the other two seals.

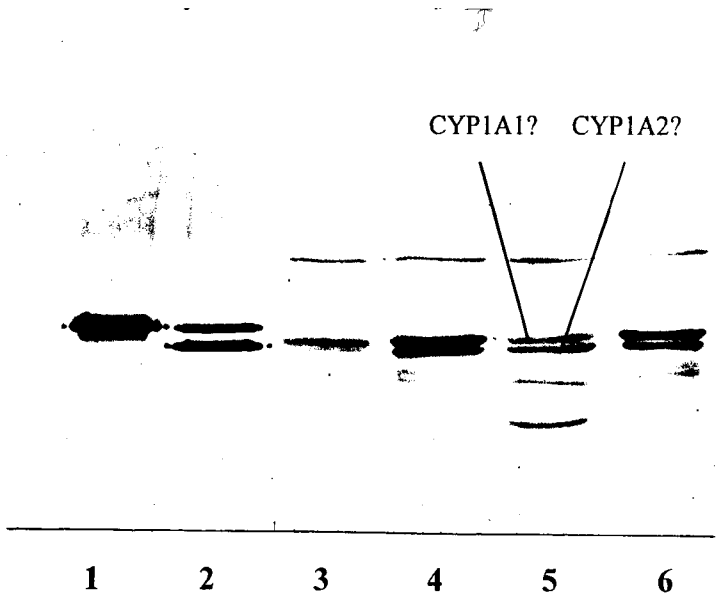


Figure 3.18: Immunoblot of harbour seal pup liver S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution) from biopsies obtained 2 days post-capture and after 3 weeks in captivity. Lane 1 is a purified CYP1A1 rat standard, applied at 0.1 pmoles per lane. Lane 2 is a 3-MC-treated rat liver microsomal sample, loaded at 0.07 µg per lane. Lanes 3 and 4 are liver S9 fractions from PV007 (biopsies taken 2 days and 3 weeks post-capture, respectively). The S9 fractions were applied at 20 µg and 10 µg total protein per lane, respectively. Lanes 5 and 6 are liver S9 fractions from PV0016 (biopsies taken 2 days and 3 weeks post-capture, respectively). The S9 fractions were loaded at 20 µg and 10 µg total protein per lane. The immunoblot was developed with substrate solution for 3.5 minutes.

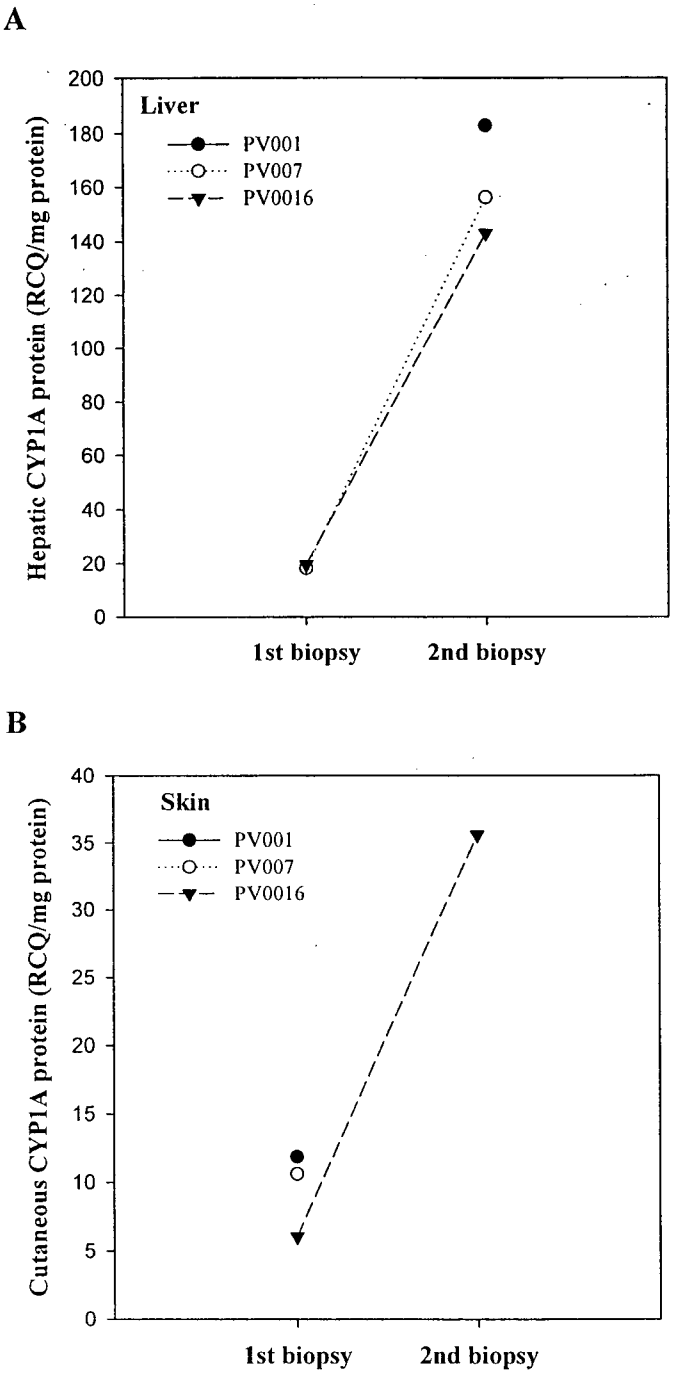


Figure 3.19: CYP1A protein levels increased in liver (A) and skin (B) of captive harbour seals during three weeks in captivity.

3.1.3.2 EROD activity

Hepatic EROD activity was approximately ten times higher in control seals after three weeks in captivity (p=0.005). In skin, there was a four to five-fold increase in EROD activity (p=0.001) (Figure 3.20).

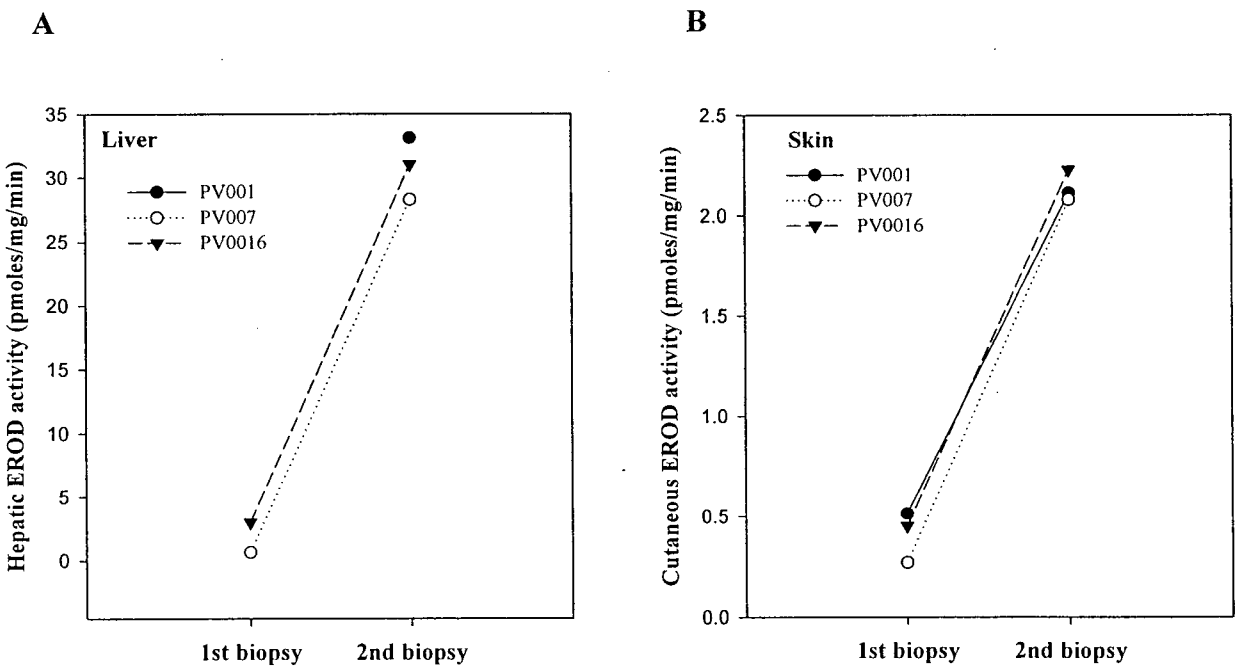


Figure 3.20: EROD activity increased in liver (A) and skin (B) of captive harbour seals during three weeks in captivity.

3.1.4 Antibody inhibition of EROD activity

To determine whether EROD activity represented a specific measure of CYP1A enzyme activity in harbour seal liver and skin, antibody inhibition experiments were conducted. In liver, anti-rat CYP1A1 IgG inhibited EROD activity by approximately 80% at the maximum antibody concentration. Inhibition occurred in pooled samples from BNF-treated and control seals (Figure 3.21 A,B). Rabbit anti-rat CYP1A2 serum inhibited EROD activity in a liver sample from a BNF-treated seal (Figure 3.21C). Rabbit anti-rat CYP1A2 serum inhibited EROD activity in a liver sample obtained 2 days post-capture from a captive seal pup (Figure 3.21D). In skin, anti-rat CYP1A1 IgG may have inhibited EROD activity slightly in BNF control seals (Figure 3.21E). Anti-CYP1A2 serum did not appear to inhibit EROD activity in skin biopsies taken immediately post-capture (Figure 3.21F).

Figure 3.21: Antibody inhibition of EROD activity in harbour seal liver and skin. EROD activity was calculated at 20 minutes reaction time for all except (F). Serum IgG concentrations were estimated, based on information in the literature.

(A) A pooled liver S9 fraction from BNF-treated seals (n=2) was incubated with anti-rat CYP1A1 IgG and loaded at 0.01 mg total protein/well. EROD activity decreased from approximately 64 pmol/mg/min (0 IgG) to 11 pmol/mg/min (0.02 mg IgG/well).

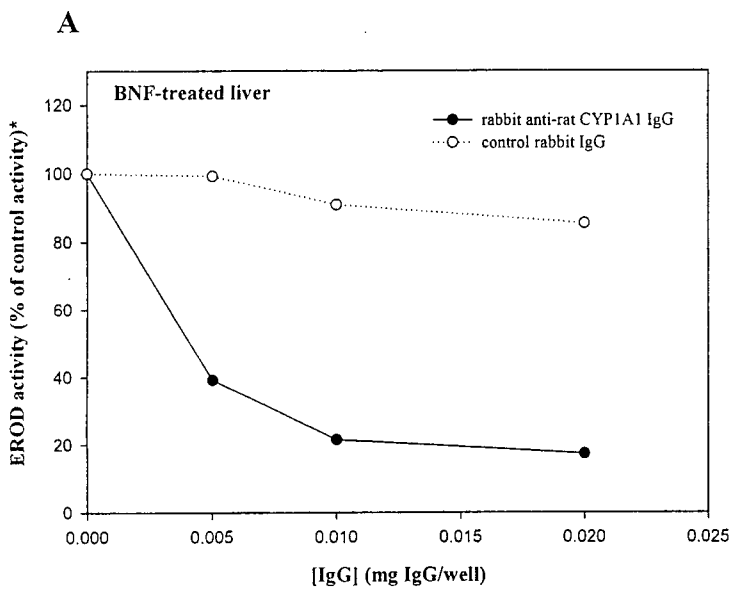
(B) A pooled S9 fraction from BNF study control seals (n=3) was incubated with anti-rat CYP1A1 IgG and loaded at 0.01 mg total protein/well. EROD activity decreased from approximately 42 pmol/mg/min (0 IgG) to 5 pmol/mg/min (0.02 mg IgG/well).

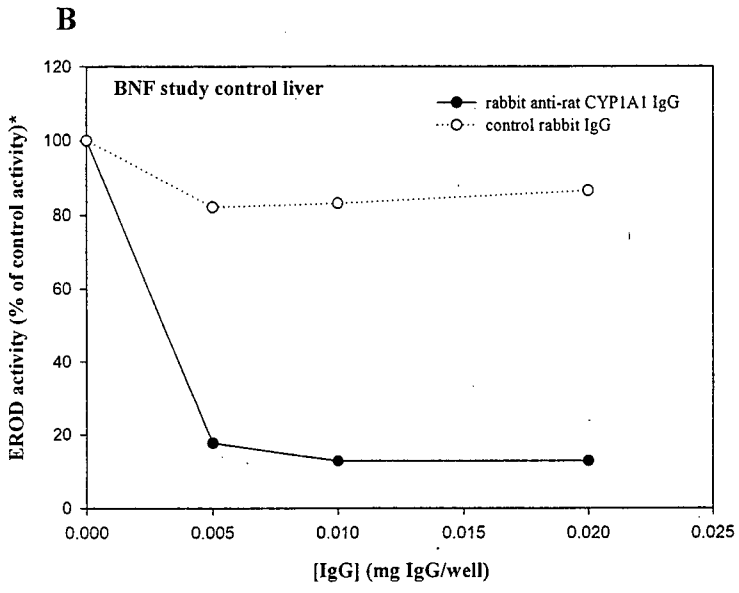
(C) A liver S9 fraction from one BNF-treated seal was incubated with anti-rat CYP1A2 serum and loaded at 0.01 mg total protein/well. EROD activity decreased from approximately 74 pmol/mg/min (0 IgG) to 28 pmol/mg/min (0.02 mg IgG/well).

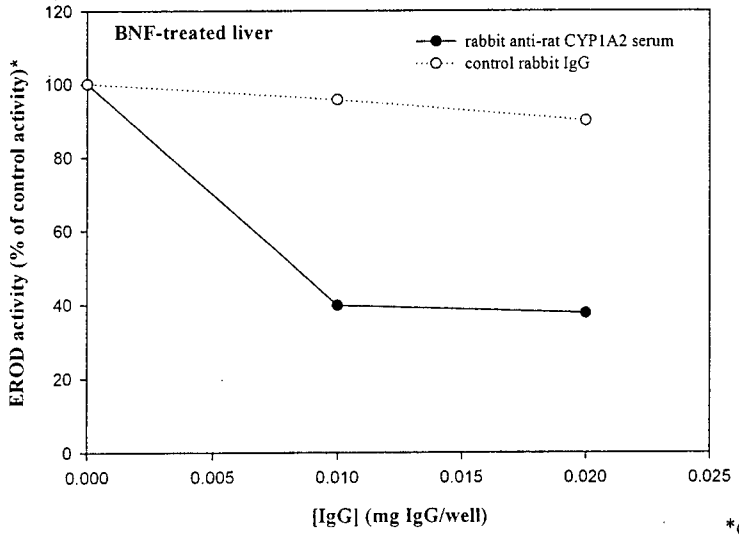
(D) A liver S9 fraction from one seal was incubated with anti-rat CYP1A2 serum and loaded at 0.1 mg total protein/well. EROD activity decreased from approximately 5 pmol/mg/min (0 IgG) to 0.9 pmol/mg/min (0.05 mg IgG/well).

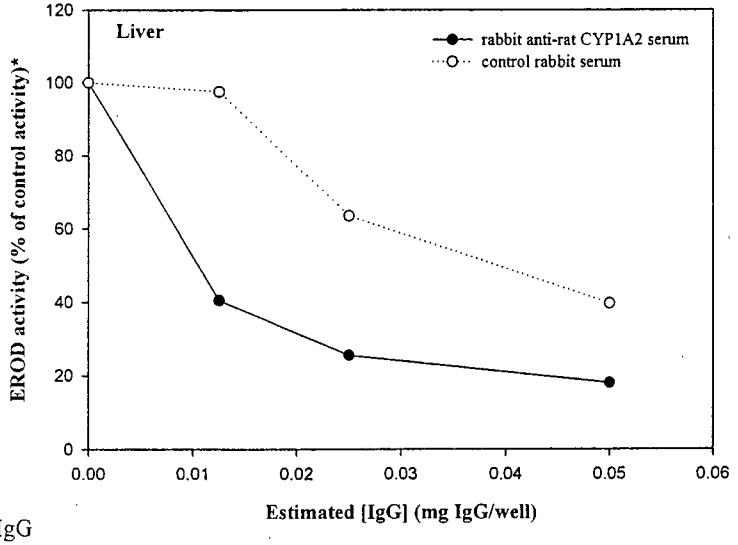
(E) A pooled skin S9 fraction from BNF study control seals (n=3) was incubated with anti-rat CYP1A1 IgG and loaded at 0.075 mg total protein/well. Only one IgG concentration was used due to limited sample volume. EROD activity decreased slightly from approximately 2 pmoles/mg/min (0 IgG) to 1.7 pmoles/mg/min (0.15 mg IgG/well). However, EROD activity of S9 fraction incubated with control serum also decreased to 1.8 pmol/mg/min (0.15 mg control IgG/well).

(F) A pooled skin S9 fraction (n=8) was incubated with anti-rat CYP1A2 serum and loaded at 0.12 mg total protein/well. At 20 minutes reaction time, EROD activity was < LOQ for all concentrations. Results shown are at a reaction time of 40 minutes.



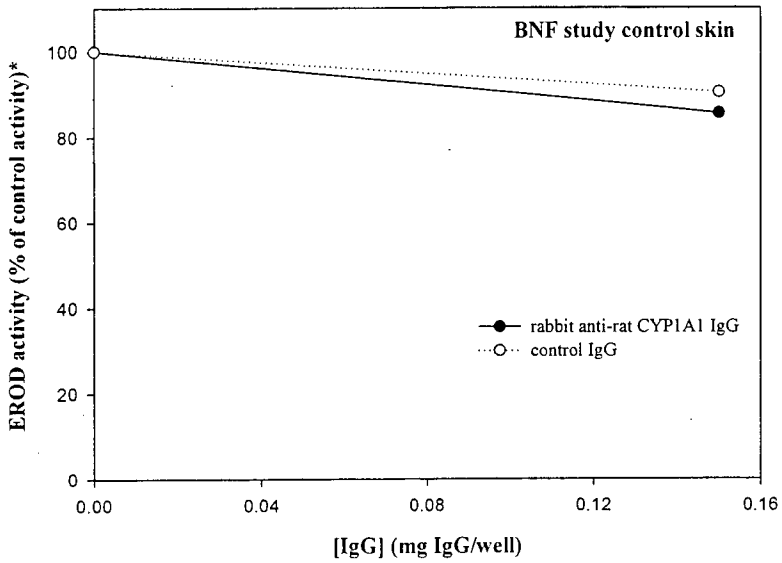






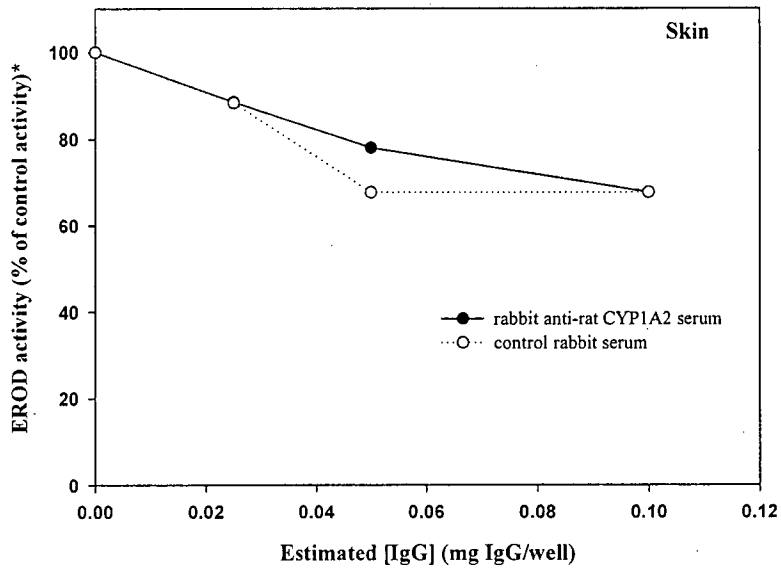
*control=0 mg IgG

E



*control = 0 mg IgG

F



3.2 Study 2: Field Study of Free-Ranging Harbour Seals

3.2.1 Hornby Island harbour seal pups

3.2.1.1 CYP1A expression in skin

Both CYP1A protein concentration and EROD activity were quantified in skin biopsies from Hornby Island seal pups. Two faint CYP1A bands having the same mobility as CYP1A protein bands in BNF-treated liver were detected on immunoblots (Figure 3.22). The lower CYP1A band was not detectable in all samples. EROD activity was below the limit of quantitation for six of ten samples. Thus, the sample size was too small to determine if EROD activity and CYP1A protein concentration were correlated in these samples (refer to Table 3.4).

Table 3.4: Cutaneous CYP1A expression in Hornby Island harbour seal pups

Sample	Average CYP1A protein levels (RCQ/mg protein)[a]	EROD activity (pmol/mg/min)[b]
PV01-01	13.4	0.64
PV01-02	12.7	< LOQ
PV01-03	14.0	0.91
PV01-04	16.0	< LOQ
PV01-05	15.1	< LOQ
PV01-06	14.0	< LOQ
PV01-07	8.6	< LOQ
PV01-08	13.1	0.66
PV01-09	9.5	0.75
PV01-10	10.1	< LOQ

LOQ (EROD) = 0.54 pmoles/mg/min
LOQ (immunoblots) = 0.3/mg total protein loaded
[a] RCQ (relative contour quantity) is the staining intensity of sample bands normalized to a 0.01 pmole/lane rat CYP1A1 standard.
[b] EROD activity was calculated at 20 mins reaction time for all samples.

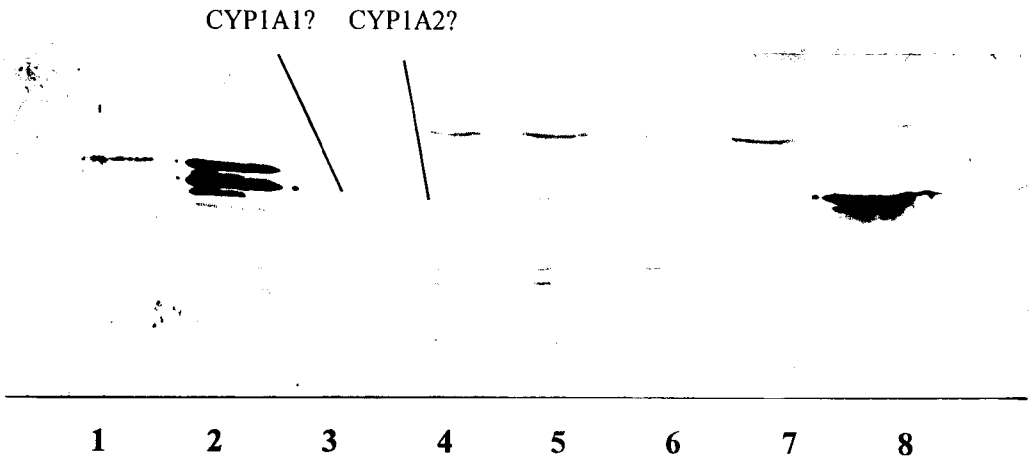


Figure 3.22: Immunoblot of Hornby Island harbour seal pup skin S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lane 1 is purified CYP1A1 rat standard, applied at 0.01 pmoles per lane. Lane 2 is a 3-MC rat liver microsomal sample, loaded at 0.07 μg per lane. Lanes 3-5 are skin S9 fractions from seals PV01-05, 06, and 07, loaded at 42 μg, 60 μg, and 60 μg, total protein per lane, respectively. Lanes 6 and 7 are skin S9 fractions from Puget Sound seal pups PV01-54 and 55, loaded at 26 μg and 45 μg, respectively. Lane 8 is a liver S9 fraction from BNF-treated seal PV0012, loaded at 2.5 μg total protein per lane. This immunoblot was developed with substrate solution for 5 minutes.

3.2.1.2 Effect of body weight on CYP1A expression in skin

Body weight was not correlated with CYP1A protein levels (p=0.834, n=10) (significant outlier removed) or EROD activity (p=0.851, n=4) for Hornby Island pups (data not shown).

3.2.1.3 Effect of sex on CYP1A expression in skin

CYP1A protein levels (p=0.207) and EROD activity (only one male above LOQ) were not significantly different between Hornby Island males (n=6) and females (n=4) (data not shown).

3.2.1.4 Effect of contaminant levels in blubber on CYP1A expression

EROD activity and CYP1A protein levels (Figure 3.23) were not significantly correlated with total TEQ for blubber. There were no correlations between CYP1A protein levels and contaminants when mono-*ortho* PCBs were removed from the total TEQ (p=0.227) (data not shown).

3.2.1.5 Relationship between body weight and contaminant levels in blubber

Contaminant levels in blubber, expressed as total TEQ, were positively correlated with body weight (r^2=0.48, p=0.026) (Figure 3.24). A single data point (39.0 kg) was largely responsible for the positive correlation, however. This data point was a significant outlier (p<0.05), and no correlation was observed when it was removed (p=0.536).

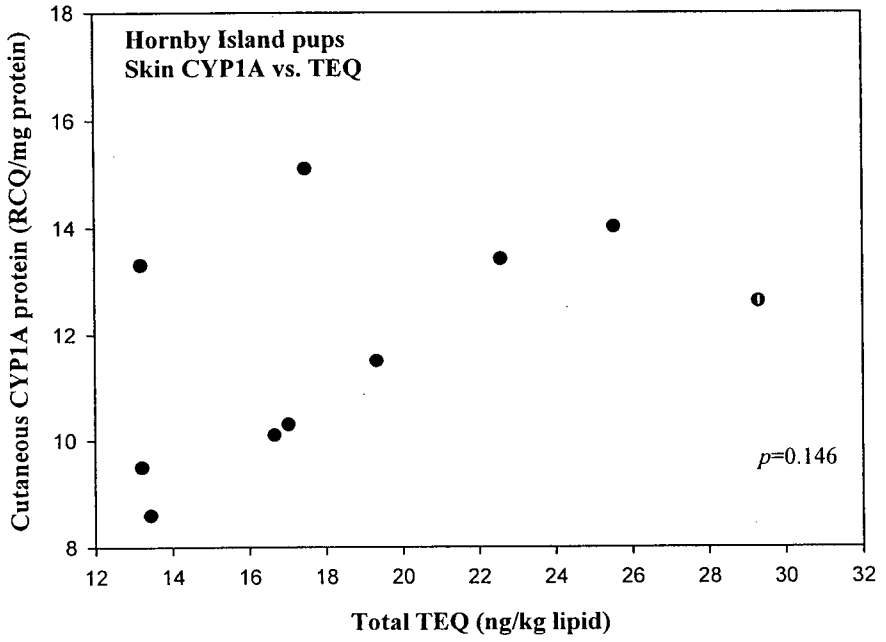


Figure 3.23: Relationship between blubber total TEQ and cutaneous CYP1A protein levels in Hornby Island harbour seal pups.

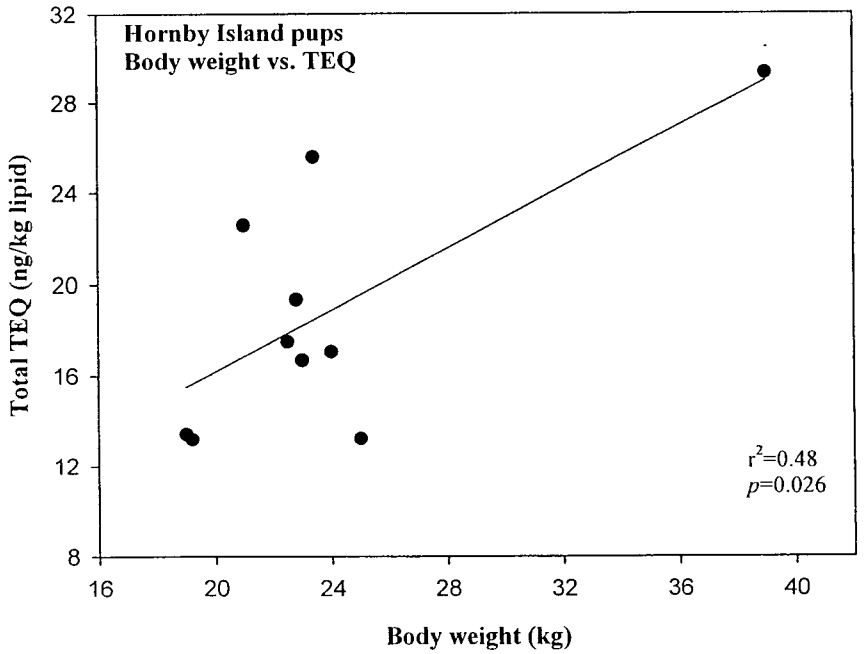


Figure 3.24: Correlation between body weight and total TEQ for blubber in Hornby Island harbour seal pups.

3.2.2 Puget Sound harbour seal pups

3.2.2.1 CYP1A expression in skin

Both CYP1A protein concentration and EROD activity were quantified in skin biopsies from Puget Sound seal pups. Two CYP1A protein bands with approximately the same mobility as the rat CYP1A1 standard were detected in skin (Figure 3.25). EROD activity was below the limit of quantitation for most samples. Thus, the sample size was too small to determine if EROD activity and CYP1A protein concentration were correlated in these samples (refer to Table 3.5).

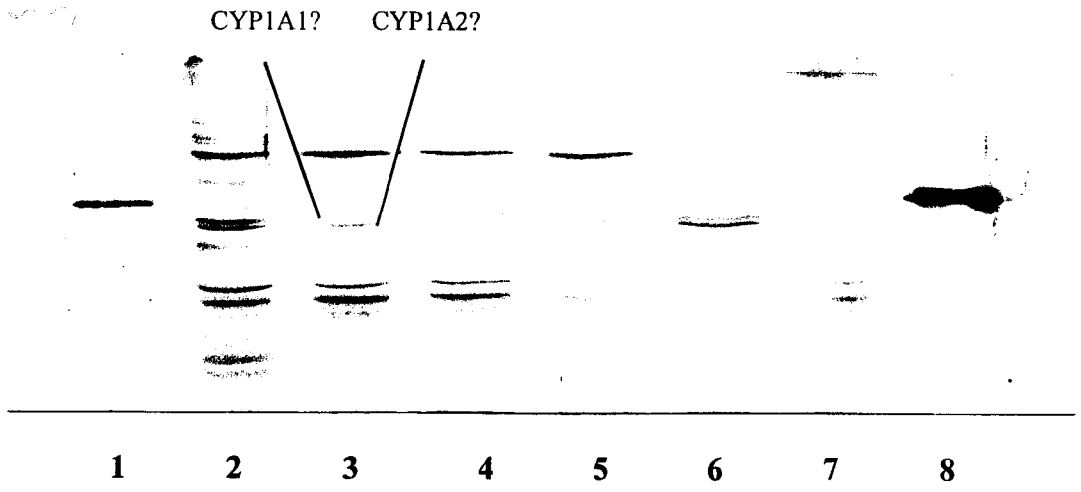


Figure 3.25: Immunoblot of Puget Sound harbour seal pup skin S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lanes 1 and 8 are purified CYP1A1 rat standards, applied at 0.01 pmoles and 0.05 pmoles per lane, respectively. Lanes 2-7 are skin S9 fractions from seals PV01-46, 47, 48, 49, 51, and 53, loaded at 31 µg, 37 µg, 60 µg, 60 µg, 37 µg, and 22 µg, respectively. The immunoblot was developed with substrate solution for 5 minutes.

Table 3.5: Cutaneous CYP1A expression in Puget Sound harbour seal pups

Sample	Average CYP1A protein levels (RCQ/mg protein)[a]	EROD activity (pmoles/mg/min)[b]
PV01-40	16.6	< LOQ
PV01-41	14.7	0.65
PV01-42	17.0	< LOQ
PV01-43	11.2	< LOQ
PV01-44	15.2	< LOD
PV01-45	19.6	< LOQ
PV01-46	21.8	< LOQ
PV01-47	23.1	< LOQ
PV01-48	5.9	0.79
PV01-49	7.8	1.10
PV01-51	22.2	< LOQ
PV01-52	11.6	< LOQ
PV01-53	< LOQ	< LOQ
PV01-54	25.0	< LOD
PV01-55	21.8	< LOD
PV01-56	21.3	< LOD

LOQ (EROD) = 0.65 pmol/mg/min
LOQ (immunoblots) = 0.3/mg total protein loaded
[a] RCQ (relative contour quantity) is the staining intensity of sample bands normalized to a 0.01 pmole/lane rat CYP1A1 standard.
[b] EROD activity was calculated at 20 mins reaction time for all samples.

3.2.2.2 Effect of body weight on CYP1A expression in skin

CYP1A protein levels were not correlated with body weight for Puget Sound pups (p=0.832, n=14) (data not shown). Correlation analysis was not performed for EROD activity, as only three values were above the LOQ.

3.2.2.3 Effect of sex on CYP1A expression in skin

CYP1A protein levels (p=0.744) and EROD activity were not significantly different between males (n=8) and females (n=7) (data not shown).

3.2.3 Adult harbour seals from British Columbia

3.2.3.1 CYP1A expression in skin

Both CYP1A protein levels and EROD activity were quantified in skin biopsies from adult seals. Two CYP1A protein bands having the same mobility as CYP1A in BNF-treated liver were detected on immunoblots (Figure 3.26). EROD activity was below the limit of quantitation for 12 of 16 samples (see Table 3.6).

3.2.3.2 Effect of body weight on CYP1A expression in skin

CYP1A protein levels (p=0.296, n=14) and EROD activity (p=0.431, n=4) were not correlated with body weight for adult harbour seals (data not shown).

3.2.3.3 Effect of sex on CYP1A expression in skin

CYP1A protein levels were not significantly different between males (n=7) and females (n=9) (p=0.439) (data not shown). EROD activity was below the LOQ for all males; therefore, a t-test was not performed.

Table 3.6: Cutaneous CYP1A expression in adult harbour seals

Sample	Average CYP1A protein levels (RCQ/mg protein)[a]	EROD activity (pmol/mg/min)[b]
PV01-17	8.2	0.69
PV01-18	17.8	< LOQ
PV01-20	< LOQ	< LOQ
PV01-22	13.7	0.84
PV01-23	< LOQ	0.81
PV01-25	9.1	< LOQ
PV01-29	6.6	< LOQ
PV01-30	10.2	< LOQ
PV01-31	13.6	< LOQ
PV01-32	11.6	< LOQ
PV01-33	18.4	< LOQ
PV01-34	48.6	< LOQ
PV01-36	24.7	< LOQ
PV01-37	12.4	< LOQ
PV01-38	12.8	< LOQ
PV01-39	5.6	1.14

LOQ (EROD) = 0.65 pmol/mg/min
LOQ (immunoblots) = 0.3/mg total protein loaded
[a] RCQ (relative contour quantity) is the staining intensity of sample bands normalized to a 0.01 pmole/lane rat CYP1A1 standard.
[b] EROD activity was calculated at a reaction time of 20 minutes for all samples.

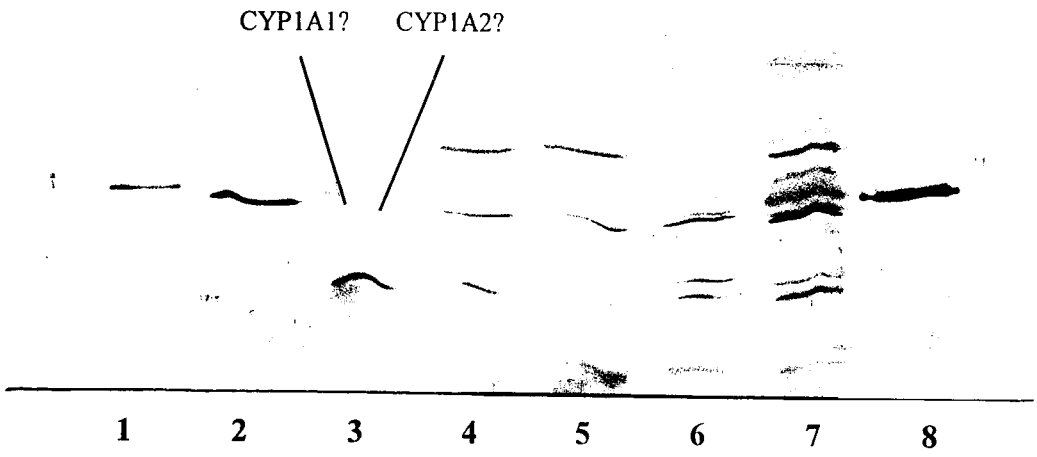


Figure 3.26: Immunoblot of adult harbour seal skin S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lanes 1 and 8 are purified CYP1A1 rat standards, applied at 0.01 pmoles and 0.05 pmoles per lane, respectively. Lane 2 is a liver S9 fraction from BNF-treated PV0012, loaded at 2.5 μg per lane. Lanes 3-5 are skin S9 fractions from seals PV01-32, 34, and 37, loaded at 60 μg, 55 μg, and 60 μg, total protein per lane, respectively. Lanes 6 and 7 are skin S9 fractions from Puget Sound seal pups (PV01-54 and 55), loaded at 26 μg and 45 μg total protein per lane, respectively. This immunoblot was developed with substrate solution for 4 minutes 45 seconds.

3.3 Harbour Seal Inter-Population Comparisons

3.3.1 Relationship between CYP1A protein levels and EROD activity

There was no correlation between CYP1A protein levels and EROD activity in skin when data from all harbour seal pup populations were combined (Fraser River, Hornby Island, Puget Sound) (p=0.122) (Figure 3.27).

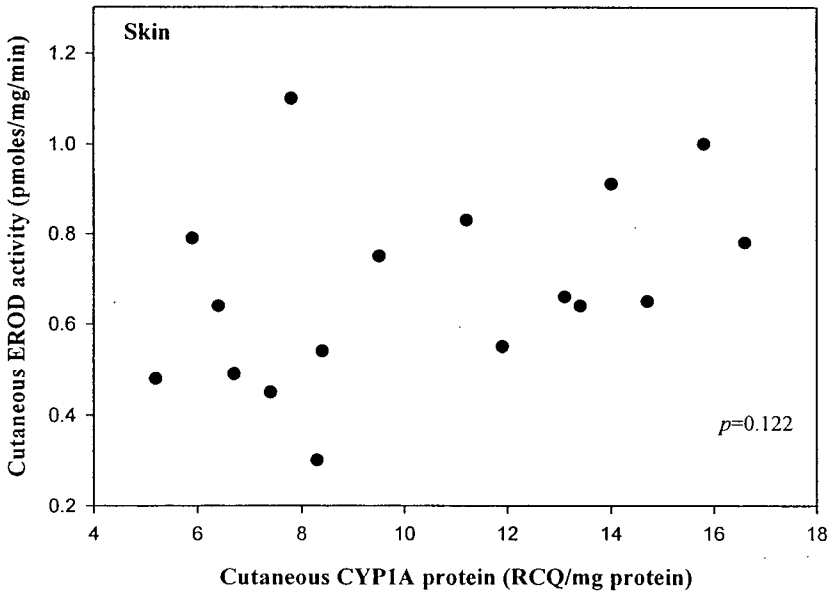


Figure 3.27: Relationship between CYP1A protein levels and EROD activity in skin of harbour seal pups from all populations (Fraser River, Hornby Island, Puget Sound)

3.3.2 Effect of body weight on CYP1A expression

Body weight was not correlated with CYP1A protein levels (p=0.821, n=43) or EROD activity (p=0.588, n=16) in skin when data from all seal pup populations were combined (data not shown).

3.3.3 Variability of cutaneous CYP1A expression between pups and adults

There was no difference in cutaneous CYP1A protein levels (p=0.529) or EROD activity (p=0.114) between harbour seal adults and pups (data not shown).

3.3.4 Effect of sex on CYP1A expression

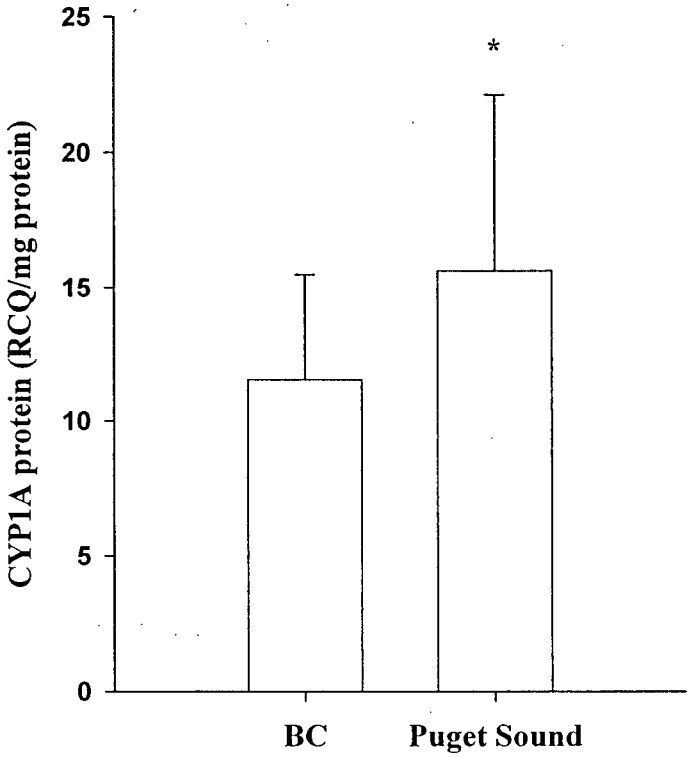
There was no difference in CYP1A expression in skin between male and female harbour seal pups when all populations were combined (EROD p=0.398; CYP1A p=0.346) (data not shown).

3.3.5 Comparison of CYP1A expression in pups from BC and Puget Sound

CYP1A protein levels were significantly higher in skin from Puget Sound harbour seal pups than in pups from British Columbia (combined data of Fraser River pups and Hornby Island pups) (p=0.003) (Figure 3.28). In each group, one sample was below the LOQ. The LOQ value was substituted for these two samples. However, if these two values were not included in the analysis, the result was still significant. When the BC populations were considered individually, CYP1A protein levels were significantly higher for Puget Sound pups than for Fraser River pups (p=0.03), but were not significantly higher than for Hornby Island pups (p=0.09). Only two Puget Sound skin samples had EROD activity above the LOQ; therefore, a statistical comparison was not performed.

3.3.6 Effect of contaminant levels in blubber on CYP1A expression

Contaminant data was only available for Fraser River and Hornby Island pups. When data from these two groups were combined, there was no correlation between contaminants in blubber and cutaneous CYP1A protein levels (p=0.643) (data not shown).



*p=0.003
Error bars=SD

Figure 3.28: Comparison of cutaneous CYP1A protein levels between harbour seal pups from British Columbia (BC) and Puget Sound, Washington

3.4 Study 3: Study of Free-Ranging Killer Whales

3.4.1 CYP1A expression in killer whale skin

EROD activity was not detected in killer whale skin, but a possible CYP1A protein band was detected on immunoblots for a few killer whale skin samples. A protein band having the same mobility as the most darkly stained protein band in beluga whale liver microsomes can be seen in lane 2 (Figure 3.29). However, the band was very faint and absent in most samples.

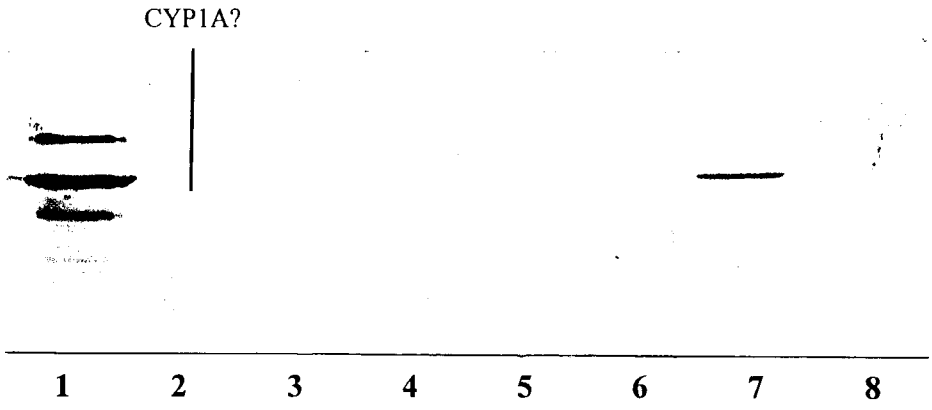


Figure 3.29: Immunoblot of killer whale skin S9 fractions probed with rabbit anti-rat CYP1A2 polyclonal serum (1:500 dilution). Lane 1 is a liver microsomal sample from beluga whale, loaded at 2 μg (estimated) total protein per lane. Lanes 2-4 are killer whale skin S9 fractions from southern resident killer whale A73 (60 μg), and transient killer whales KW00-11 (60 μg) and KW00-03 (54 μg), respectively. Lanes 5 and 6 are skin S9 fractions from Fraser River harbour seal pups PV0019 and 13, loaded at 55 μg and 60 μg, respectively. Lane 7 is a skin S9 fraction from BNF-treated seal PV0015, loaded at 10 μg total protein per lane. Lane 8 is a purified CYP1A1 rat standard, applied at 0.01 pmoles per lane. This immunoblot was developed with substrate solution for 1 minute. A short reaction time was used in order to minimize non-specific bands and to visualize the most immunoreactive protein bands.

4. DISCUSSION

The overall objective of the present study was to obtain liver and skin biopsies from marine mammals using minimally-invasive means and to validate the use of CYP1A as a biomarker of contaminant exposure in these biopsy samples. This study consisted of three groups: (1) 20 free-ranging harbour seal pups that were live-captured and then temporarily held in captivity; (2) 42 free-ranging harbour seals, including both pups and adults, sampled in British Columbia and Washington; and (3) 14 free-ranging killer whales sampled in coastal waters of British Columbia. Each of these groups offered particular advantages in the development of biomarker methods for marine mammal populations. The captive seal study allowed for biopsy of both liver and skin using general anaesthetic and provided a relatively controlled environment in which to investigate the inducibility of CYP1A in these tissues. This provided a foundation for conducting and validating field studies in which less invasive sampling was conducted (i.e. only skin-blubber biopsies). Field sampling of harbour seals provided skin biopsy samples from individuals of different age categories inhabiting both industrialized (contaminated) and more remote (relatively uncontaminated) areas. This provided an opportunity to investigate whether natural factors (eg. age) or anthropogenic factors (eg. differences in contaminant exposure) affected CYP1A expression in skin. Samples from both the captive and free-ranging harbour seal groups were used to develop and validate laboratory methods, providing an important basis for the study of killer whales, which could not be captured or sampled under controlled conditions. The availability of individual-based information for each of the killer whales sampled provided a unique

opportunity to investigate cutaneous CYP1A expression in the context of age, sex, diet, and contaminant levels.

4.1 Study 1: Captive Harbour Seal Study

4.1.1 Analysis of liver samples obtained two days post-capture

4.1.1.1 Liver biopsy approach

Our approach for measuring hepatic CYP1A in harbour seals was novel because we used small tissue biopsies from healthy animals that were live-captured and temporarily held in captivity. Whereas previously published studies of CYP1A activity in seals or other marine mammals used liver from dead animals (stranded or sacrificed), we demonstrated that an amount of tissue sufficient for CYP1A analysis could be obtained by liver biopsy under general anaesthetic. Seals appeared to recover rapidly and fully from the biopsy procedure and remained healthy while in captivity.

4.1.1.2 EROD activity

The present study is the first to report hepatic CYP1A data for marine mammals on the west coast of Canada and the first using a liver biopsy approach. EROD activity measured in S9 fractions prepared from liver biopsies obtained two days post-capture ranged from < 0.65 to 4.4 pmoles product formed/min/mg protein. Comparison of data from the present study with previously published studies shows differences in EROD activity values between seal species, as well as between age classes. Addison *et al.* (1986) reported a mean EROD activity of 6.3 ± 4.6 nmoles product formed/min/mg protein for newborn harbour seal pups of variable ages (including two fetuses) from the east coast of Canada, 1000 times higher than activities measured in the present study. EROD activities reported for other seal species (Wolkers *et al.*, 1998; Hyyti *et al.*, 2001;

Chiba *et al.*, 2002; Ruus *et al.*, 2002) were also several orders of magnitude higher than those obtained in this study. Wolkers *et al.* (2002), however, reported activities that are similar to those measured in the present study, with mean EROD activity of 9.6 ± 9.6 pmol/min/mg protein for hepatic microsomes from unweaned harp seal pups, and 11.5 ± 5.4 pmol/min/mg for harp seal mothers.

In the present study, CYP1A was measured in the S9 fraction rather than the microsomal fraction, representing one possible reason for the relatively low hepatic EROD activity observed. The S9 fraction contains many other cytosolic proteins that contribute to the total protein determination (Munkittrick *et al.*, 1993), thereby decreasing EROD activity values when expressed per mg total protein. Munkittrick *et al.* (1993) reported that EROD activity was 3.5 times higher in hepatic microsomal fractions compared to S9 fractions from white sucker (fish).

Direct comparisons of EROD activity among studies is problematic, however, if different protocols, equipment, and reagents are used. Different laboratories have reported different absolute activities when analyzing the same samples (Munkittrick *et al.*, 1993; Stagg and Addison, 1995). In one study, for example, mean EROD activities determined by eleven different laboratories for the same contaminant-exposed fish ranged from 20 to 298 pmol/min/mg protein (Munkittrick *et al.*, 1993). Different methods of measuring total protein content of samples also produced variable results. Individual samples assayed using the same method had up to 33% less or 63% greater total protein content than corresponding samples assayed with another method (Munkittrick *et al.*, 1993), and this subsequently affected EROD activities.

4.1.1.3 Immunoblot analysis

In the present study, two immunoreactive CYP1A protein bands, tentatively identified as CYP1A1 and CYP1A2, were detected in S9 fractions prepared from liver. This result was expected, as CYP1A1 and CYP1A2 gene fragments have recently been cloned in four different seal species (Teramitsu *et al.*, 2000; Tilley *et al.*, 2002). Tilley *et al.* (2002) assigned molecular weights to CYP1A1 and CYP1A2 in grey and harp seals based on deduced amino acid sequences. Molecular weights were too close together to be able to definitively distinguish between CYP1A1 and CYP1A2 (58 KDa and 57 KDa, respectively), although the greater molecular weight of CYP1A1 suggests that the upper protein band is CYP1A1 and the lower band is CYP1A2 for these two seal species. Given that the two CYP1A enzymes in grey, harp, ribbon, and largha seals were >90% identical (Teramitsu *et al.*, 2000; Tilley *et al.*, 2002), it is likely that upper and lower protein bands correspond to the same CYP1A enzymes in harbour seals. Moreover, in the present study, levels of putative CYP1A1 protein corresponding to the upper band correlated with EROD activity in liver, but the lower protein band did not correlate with EROD activity. This result suggests that the upper protein band is CYP1A1 in harbour seals, as CYP1A1 has been shown to be the primary catalyst of the EROD reaction in other species (Burke *et al.*, 1994). Additionally, in liver and skin samples from BNF-treated seals (discussed in section 4.14), the upper CYP1A band was induced to a greater extent than the lower band, providing further evidence that the upper (higher molecular weight) protein band is CYP1A1. In rats, BNF is known to induce CYP1A1 more than CYP1A2 (Thomas *et al.*, 1983).

4.1.2 Analysis of skin samples obtained two days post-capture

The present study is the first to measure CYP1A expression in skin preparations from harbour seals and is the first to report the presence of two CYP1A proteins in skin of a marine mammal. These results provide an important foundation for the future development of less-invasive approaches to biomarker research. The immunoblot analysis clearly shows that there are two CYP1A enzymes in skin. EROD activity was also detected in cutaneous S9 fractions. However, EROD activity and CYP1A protein levels were close to their respective LOQs in all skin samples, and EROD activity, in particular, was often below the LOQ. CYP1A protein values had high inter-assay variability because bands on immunoblots were often faint and were, therefore, more difficult to quantify.

4.1.3 Comparison of CYP1A expression levels between liver and skin

In the present study, EROD activity was four to five times higher in liver than skin of harbour seal pups, reflecting the general tissue distribution of CYP enzymes and the central role that the liver plays in enzymatic detoxification processes. Fossi *et al.* (1997) reported that B*a*P hydroxylase activity was five times higher in microsomal fractions prepared from liver than in homogenates prepared from skin of a sea lion, although sample size (n=1) clearly limits interpretation of this result. It is not clear if the skin homogenates analyzed by Fossi *et al.* (1997) were S9 fractions. As microsomes are more concentrated with respect to CYP1A protein than pre-microsomal homogenates, the five-fold difference in EROD activity between liver and skin reported by Fossi *et al.* (1997) was likely due in part to the analysis of different fractions. To date, the report by Fossi *et al.* (1997) is the only published comparison of hepatic and cutaneous CYP1A-

mediated enzyme activity in a marine mammal species. In our study, CYP1A protein levels were estimated to be sixteen times higher in liver than skin, but direct comparison was not possible because different internal standards were used to calculate relative staining intensities in the two tissues.

Total CYP content was not measured in liver or skin S9 fractions due to limited sample volumes, but in other species it is known that total CYP concentration is much less in skin than liver. Moreover, CYP1A makes up a small fraction of the total CYP content in uninduced animals. In liver microsomes from untreated male rats, CYP1A represents 1-3% of the total CYP content (Bandiera, 2001). In rats, the total CYP content of liver was reported to be at least 4.5 times greater than that of skin, and in mice, total CYP content was at least seventeen times greater in liver than skin (Lewis, 1996). Pham *et al.* (1989) similarly found that total CYP content was approximately seventeen times greater in microsomal fractions of liver than skin from rats. In a TCDD dosing study in mice, Diliberto *et al.* (2001) reported a 40-fold difference between EROD activity in liver and skin of control animals. Bickers *et al.* (1974) found that basal B*a*P hydroxylase activity was approximately 100 times greater in rat liver than skin, and Mukhtar and Bickers (1981) reported that skin homogenates had 7-28% of the activity of liver in B*a*P- and Arocolor 1254-treated rats, depending on the treatment group. In rats, CYP1A1 was not detected in untreated skin and was only faintly detected in skin from 3-MC-treated rats by immunoblot analysis (Pham *et al.*, 1989).

Although CYP1A expression levels in skin and liver were not positively correlated for captive harbour seal pups, the apparent molecular weights of the two immunoreactive CYP1A protein bands for skin corresponded to those for liver on

immunoblots. Additionally, CYP1A induction was observed in both skin and liver in the BNF induction study (section 4.14). If sample size was too small, or if CYP1A expression in skin was too low to detect differences among individuals, no correlation between liver and skin would be expected. However, a significant *negative* correlation between hepatic and cutaneous EROD activity was observed. A negative correlation between CYP1A in liver and skin suggests that CYP1A expression may be either tissue-dependent (eg. due to toxicokinetic or developmental differences between tissues), or that other proteins in cutaneous S9 fractions interfere with CYP1A activity in skin. It is possible that seals with higher hepatic CYP1A levels also had elevated levels of other cytosolic proteins in skin. These proteins may have 'diluted' the already low CYP1A levels in skin homogenates.

To determine if CYP1A catalyzed the EROD reaction in both liver and skin, *in vitro* antibody inhibition experiments were conducted using rabbit anti-rat CYP1A1 monoclonal IgG and rabbit anti-rat CYP1A2 polyclonal serum. Hepatic EROD activity was inhibited by up to 85%, indicating that CYP1A is responsible for catalyzing the EROD reaction in harbour seal liver samples. EROD activity was not inhibited in skin samples, however, suggesting that different enzymes may be catalyzing EROD in skin compared to liver. CYP forms other than CYP1A have been shown to contribute to EROD activity in untreated rats (Burke *et al.*, 1994). Alternatively, EROD activity in skin may have been too low (i.e. too close to the detection limit) to detect a decrease in EROD activity with the addition of antibody. Control serum IgG concentrations also may have been too high, masking actual differences between control and antibody-treated skin samples. The serum IgG concentration was not known and was, therefore, estimated

based on literature values. The serum IgG concentration may have been underestimated, resulting in greater IgG concentrations in reaction wells than expected. In fact, EROD activity in liver was significantly reduced by high concentrations of control serum (estimated concentration of 0.025 mg IgG/well) (Figure 3.21D). Antibody inhibition experiments indicated that CYP1A catalyzed the EROD reaction in liver, but results in skin were inconclusive.

Studies in rodents have provided evidence to suggest that hepatic and epidermal CYP1A1 enzyme structure and inducibility are identical. Raza *et al.* (1992) found that in rats, hepatic and epidermal CYP1A1 had common substrate specificity and similar responsiveness to inducers (both were induced by BNF treatment), common immunocross-reactivity (AHH activity was inhibited by monoclonal and polyclonal anti-CYP1A1 antibodies in both tissues, and to a similar degree), and the same HPLC elution pattern, molecular mass (SDS-PAGE), monoclonal antibody epitopes on fingerprint analysis, N-terminal amino acid sequences, and tryptic peptides (Raza *et al.*, 1992). Further evidence to support the identity of CYP1A genes in liver and skin has been provided by Shimizu *et al.* (2000). B*a*P treatment induced CYP1A1 expression in both liver and skin of mice, but did not induce CYP1A1 in either tissue of AhR knockout mice.

The results of rodent studies and the analagous response of CYP1A in harbour seal liver and skin to oral BNF treatment provide support for the idea that CYP1A enzymes in harbour seal skin are the same as those in liver. However, in the present study, relatively small sample sizes and low activities and protein levels in skin limited interpretation of the results.

4.1.4 Induction of CYP1A by in vivo BNF treatment

To my knowledge, the present study is the first to show that *in vivo* BNF treatment induces CYP1A protein levels in any tissue from a marine mammal species. This finding provides evidence that an inducible CYP1A protein is present in harbour seals and supports the development of CYP1A as a biomarker of environmental contaminant exposure in these animals. CYP1A protein levels were induced in both liver and skin, indicating that the same mechanistic response to contaminant exposure occurs in these tissues.

Unexpectedly, EROD activity was not induced in either liver or skin in BNF-treated seals. These results were confirmed for liver samples by repeating the assay, as well as by measuring EROD activity of a treated and a control sample using a different EROD assay protocol. Chemical inhibition by BNF (*in vivo*) is one possible explanation for the apparent lack of induction of EROD activity in BNF-treated harbour seals, despite increases in CYP1A protein levels. This observation is consistent with reports that EROD activity is not always induced concurrent with the induction of CYP1A protein or mRNA levels and has been attributed to competitive inhibition by the inducing agent (Gooch *et al.*, 1989; Haasch *et al.*, 1993; Goksoyr and Husoy, 1998; Petrulis and Bunce, 1999).

Haasch *et al.* (1993) found that *in vitro* BNF treatment inhibited EROD activity in liver microsomes from rainbow trout and suggested that inhibition of EROD activity after *in vivo* BNF treatment may be due to the presence of BNF in the microsomal preparations. However, the amount of BNF, if any, retained in the microsomal preparation was not known. In the present study, liver biopsies were taken 24 hours after

the last BNF treatment, and this may have been sufficient time for BNF to be eliminated from the liver. Haasch *et al.* (1993) noted that measurement of BNF tissue levels was difficult because the bioconcentration factor of BNF was not known, and radiolabeled BNF was not commercially available. Gooch *et al.* (1989) similarly suggested that a lack of EROD induction in fish given high doses of 3,3′,4,4′-tetrachlorobiphenyl (TCB) could result from inhibition by TCB retained in microsomes during subcellular fractionation. Petrulis and Bunce (1999) suggested that competitive inhibition would not be a factor when assessing environmental exposure to dioxin-like compounds, however, because these compounds would not be carried over from the intact animal to the microsomal fraction. In the present study, a subsequent inhibition of EROD activity by *in vitro* BNF treatment supported the possiblity that *in vivo* BNF treatment might result in inhibition of hepatic microsomal EROD activity.

Another suggested but less likely mechanism by which EROD activity can be inhibited is oxidative inactivation of the enzyme. Some contaminants, such as 3,3′,4,4′-tetrachlorobiphenyl (TCB) have been shown to stimulate production of reactive oxygen species (ROS) by liver microsomes, which can inactivate CYP1A by attacking its active site (Schlezinger *et al.*, 1999; Schlezinger and Stegeman, 2001).

4.1.5 Relationship between environmental contaminant levels and CYP1A expression

I hypothesized that CYP1A expression in liver and skin would be positively correlated with blubber organochlorine levels, expressed as total TEQ. Although CYP1A expression did not correlate with total TEQ (representing the sum of non-*ortho* and mono-*ortho* PCBs, PCDDs, and PCDFs), a significant relationship was apparent when mono-*ortho* PCBs were not included in this analysis (significant correlation between

contaminants and hepatic, but not cutaneous, CYP1A). Mono-*ortho* substituted PCBs tend to be mixed-type inducers (i.e. induce both CYP1A and CYP2B enzymes) and are weaker CYP1A inducers than non-*ortho* substituted PCBs, 2,3,7,8-substituted PCDDs, and 2,3,7,8-PCDFs.

Mammalian TEFs are largely based on rodent studies and may not accurately reflect the contribution of certain congeners to AhR-mediated endpoints in all species. Mono-*ortho* PCBs contributed approximately half of the total TEQ for captive harbour seal pups in the present study because their absolute concentration was the highest of the contaminant classes measured. However, it is possible that current TEFs overestimate the binding affinity of mono-*ortho* PCBs for the Ah receptor in harbour seals, and therefore a correlation between TEQ and hepatic CYP1A induction was only observed when mono-*ortho* congeners were excluded. Results of the present study suggest that mono-*ortho* PCBs are not inducing CYP1A in harbour seal liver.

High levels of mono-*ortho* PCBs in the blubber relative to PCDDs, PCDFs, and non-*ortho* PCBs may reflect the inability of harbour seals to preferentially metabolize mono-*ortho* congeners. Like rodents, marine mammals can more easily metabolize non-*ortho* (coplanar) congeners than *ortho*-substituted congeners, and the availability of adjacent unsubstituted carbon atoms at the *ortho*- and *meta*-positions, facilitates metabolism (Boon *et al.*, 1992).

Studies to date have shown that relationships between CYP1A expression and OC levels in marine mammals are variable. Although several studies reported positive correlations between hepatic CYP1A expression and contaminant levels in seals (Chiba *et al.*, 2002) or differences in hepatic CYP1A expression between seals from

contaminated and less contaminated areas (Mattson *et al.*, 1998; Nyman *et al.*, 2000) (previously discussed in section 1.2.1.2), other studies found no correlations between these variables (Chiba *et al.*, 2002; Wolkers *et al.*, 2002; Ruus *et al.*, 2002). Chiba *et al.* (2002) found positive correlations between hepatic CYP1A (EROD activity and CYP1A protein levels) and contaminant levels expressed as total TEQ in blubber of adult largha seals (9 males, 7 females), but did not find correlations in adult ribbon seals (6 males, 9 females). In another study, there was no correlation between hepatic EROD activity in harp seal mothers and pups and contaminant levels in blubber or milk (6 mother-pup pairs) (Wolkers *et al.*, 2002). Ruus *et al.* (2002) did not find a correlation between hepatic EROD activity and levels of OCs in blubber (total PCB concentrations and total TEQ) in harbour seals (5 males, 5 females; 0-11 years).

An important consideration in evaluating results of these studies is the potential contribution of confounding factors (eg. age and sex of study animals). Contaminant burdens increase in males with age, whereas reproductive females off-load a large proportion of their contaminant burdens to their offspring during lactation. Many marine mammal studies do not control for these confounding factors (samples are from both male and female adults of variable ages), and spurious correlations between contaminant levels and CYP1A expression may be reported.

In many cases, contaminant exposure may not be high enough to elicit measurable CYP1A induction responses, especially in extra-hepatic tissues, in which constitutive CYP1A levels are relatively low. Additionally, sample sizes may often be too small to detect trends. In the present study, total PCB levels in blubber of harbour seal pups were comparable to levels measured in blubber of adult largha seals, in which correlations

between contaminants and hepatic EROD activity were reported (Chiba *et al.*, 2002), but were relatively low compared to levels in harbour seals at other locations (see Table 1.1). Contaminant levels may not have been high enough in captive harbour seal pups to elicit a strong CYP1A induction response in skin.

It is also possible that a specific contaminant or group of contaminants not measured in the present study significantly contributed to the CYP1A induction pattern in harbour seal skin. Planar contaminants not included in the TEF/TEQ scheme, such as PAHs and brominated hydrocarbons, can induce CYP1A in animals (Brack *et al.*, 2000; Van den Berg *et al.*, 1998; Varanasi *et al.*, 1992). This could partially explain the lack of correlation between CYP1A expression in skin and TEQ.

4.1.6 Relationship between age and contaminant concentrations in blubber

With the exception of lactating females, we expect age and organochlorine levels to be positively correlated in adult seals (Ross and Troisi, 2001). The relationship between age and contaminant levels is strong for PCBs, but inconsistent for PCDDs and PCDFs as a result of relatively rapid metabolic elimination (Ross and Troisi, 2001). In young seals, however, the relationship between age and contaminants is less well-documented.

In the present study, body weight was negatively correlated with contaminant levels in blubber (total TEQ) for captive harbour seal pups. Addison and Stobo (1993) found that OC residue concentrations did not significantly change in grey seal pups during the nursing period. It is likely that an accompanying increase in the blubber layer effectively 'diluted' the contaminants that were also accumulating. Once pups were weaned, blubber weight decreased, and blubber residue concentrations increased

significantly (Addison and Stobo, 1993). Such a dilution effect may be partially responsible for the negative correlation observed between body weight and total TEQ in captive harbour seal pups.

4.1.7 Relationship between body weight (age) and CYP1A expression in harbour seals

Body weight can be used to approximate the age of nursing harbour seals pups (Cottrell *et al.*, 2002). Seal pups in the present study were likely at the end of their nursing period but were probably still nursing at the time of capture. It is not surprising that a correlation between body weight and CYP1A protein levels was not observed for captive harbour seal pups because ages were similar among individuals. Seals of the same approximate size were deliberately selected to try to eliminate age as a potential confounding factor (larger seal pups are typically older). However, there was a significant negative correlation between body weight and hepatic EROD activity (n=10). This result contrasts with Wolkers *et al.* (2002), in which a positive correlation between body weight and hepatic EROD activity was found for nursing harp seal pups (n=6). This correlation was attributed to larger (older) pups having a more fully developed CYP enzyme system. However, small sample sizes limit the interpretation of results in both Wolkers *et al.* (2002) and in the present study.

Although correlations between body weight or age and *contaminant* levels are frequently reported for marine mammals (Addison and Stobo, 1993; Bernt *et al.*, 1999), few studies reported correlations between body weight and *CYP1A*. During the nursing period, as body weight increases, developmental (age)-related changes in CYP1A enzymes may take place in seal pups (Addison and Brodie, 1984). However, such

developmental changes have not been well-studied in seals, and results from the present study highlight the importance of further research into some of the natural factors that affect the expression of CYP1A enzymes.

4.1.8 Relationship between sex and CYP1A expression in harbour seals

In some animal species, hepatic CYP levels and CYP enzyme activities are higher in males than in females (Goksøyr, 1995). This topic has been well-studied in fish, in which sex differences are particularly pronounced during the reproductive period. Hepatic EROD activity, for example, is lower in reproductively active females, and this suppression of CYP1A is partially attributed to increased plasma levels of the steroid hormone,17β-estradiol, in pre-spawning females (Whyte *et al.*, 2000). Sex differences in total CYP content and EROD activity have also been reported in rats (Pham *et al.*, 1989).

The evidence for sex differences in EROD activity in adult marine mammals is conflicting. Goksøyr (1995) found that male hooded seals had higher EROD activity than females, and Chiba *et al.* (2000) found that in ribbon seals, hepatic EROD activity was higher in males than females. However, no difference was detected between male and female largha seals (Chiba *et al.*, 2000), or between male and female ringed seals (Mattson *et al.*, 1998). In the present study, CYP1A expression did not differ between male and female harbour seals. However, harbour seal pups were sexually immature.

4.1.9 Increase in CYP1A expression over time in captive harbour seals

EROD activity and CYP1A protein levels increased significantly in both liver and skin of harbour seals during their three-week stay in captivity. EROD activity was approximately ten times higher in liver samples (p=0.005) and four to five times higher in skin samples (p=0.001) from control seals than in samples taken immediately post-capture (three weeks prior to the BNF study). CYP1A protein levels increased seven-fold in liver (p=0.04), and although there were insufficient data to conduct a t-test for skin, CYP1A protein increased six-fold in one seal.

4.1.9.1 CYP1A development in young seals

A plausible explanation for the increase in CYP1A expression observed in liver and skin samples from control seals is that CYP1A levels and activities changed as seal pups aged. Past studies detected EROD activity in liver samples from newborn harbour seal pups, with increases in hepatic EROD activity occurring up to 12 days post-partum in grey seal pups (Addison and Brodie, 1984b; Addison *et al.*, 1986). The developmental profile of CYP enzymes in harbour seals is not known. In general, studies in rodents and pigs have shown that development of hepatic microsomal drug-metabolizing enzymes occurs during the first four to six weeks after birth, with the phase of most rapid development occurring between birth and the third to fifth week post-partum. This developmental pattern was observed regardless of species, gestation period, or the degree of maturation at birth (Dickerson and Basu, 1975).

Previous studies of grey seals and harp seals showed that hepatic EROD activity was higher in adults than pups (Addison and Brodie, 1984b; Addison *et al.*, 1986). In the present study, EROD activity in skin samples was similar in adults and pups, and after

three weeks in captivity, EROD activity in skin samples from untreated seals was higher than that of adults. Studies in rats and rabbits suggest that young animals can actually have higher CYP expression than adults. In rats, total hepatic CYP levels were low six days after birth, increased rapidly for three to five weeks after birth (three-fold increase when expressed per gram liver weight), and then decreased to adult levels at fifty to seventy days of age (approximately 2.5 times higher than levels at six days of age) (Dickerson and Basu, 1975). A similar age-related development pattern has been shown in rabbits (Fouts and Devereux, 1972). Alterman *et al.* (1994) reported that EROD activity in liver from rats peaked in very young animals, declined until about three months of age, and thereafter did not change further. These studies raise the possibility that harbour seals experience a similar increase in CYP expression at about six weeks of age. The fact that harbour seals are known to be highly precocious, having good sight, coordination, and a highly developed immune system (Ross *et al.*, 1994) and a short period of maternal care (Cottrell *et al.*, 2002), may partially explain why CYP1A expression in pups was comparable to that in adult seals.

4.1.9.2 Factors affecting variability of CYP1A expression within and between tissues

Within tissues, one potential source of variation in CYP1A expression is protein localization. Localization of CYP1A within the liver and skin could explain both variations in expression over time when multiple biopsies are obtained from a single animal and potentially, detection of higher levels of CYP1A in an uninduced animal compared to an induced animal. In other words, one biopsy may have higher CYP1A expression than another simply because of differences in CYP1A expression within the same organ. In rat liver, CYP1A expression and the induction response vary between

centrilobular, midzonal, and periportal hepatocytes (Baron *et al.*, 1982; Wolf *et al.*, 1984). In livers of untreated rats, centrilobular hepatocytes bound 25% more rabbit anti-rat CYP1A antibody than midzonal or periportal hepatocytes (Baron *et al.*, 1982), and the degree of induction after BNF treatment also varied between regions (Baron *et al.*, 1982; Wolf *et al.*, 1984). Because our liver biopsy technique involved a 'blind' sampling of liver, it is possible that we obtained variable CYP1A yields among biopsies. However, the fact that similar CYP1A induction responses to BNF treatment and concurrent increases in CYP1A expression over time were observed in both liver and skin of captive seals suggests that these responses are real and likely do not represent an artifact of biopsy sampling.

In the present study, skin biopsies taken from harbour seals two days post-capture were from the pelvic region of the body, whereas three weeks later, biopsies were taken from the hind flipper. It is possible that differences in constitutive CYP1A expression between flipper and pelvic regions contributed to the apparent increase in cutaneous EROD activity and CYP1A protein levels over time. In rats, AHH activity was eight-fold higher in epidermis from the ear and groin than from the abdomen, back, and tail (Don *et al.*, 1987). However, there were no differences in CYP1A expression between pelvic and flipper skin samples from an adult harbour seal. Additionally, there was a parallel increase in hepatic CYP1A expression during the captive period. These results suggest that the observed increase in cutaneous CYP1A expression over time reflects a real change in CYP1A levels.

4.1.9.3 Possible effects of stress and captivity-related factors on CYP1A expression

CYP1A expression in seal pups also may have been affected by stress associated with their capture and time in captivity. Harbour seal pups were at approximate weaning age at the time of capture, meaning that they were still on a milk diet or that they had recently stopped receiving milk but were not yet eating fish. In captivity, pups were hand-fed herring, supplemented with vitamins and minerals. A change in diet from milk to whole fish may have elicited a stress or other physiological response. Contaminant levels in herring are unlikely to have had a direct effect on CYP1A, as herring has much lower levels of OCs than is predicted for fat-rich seal milk (Ross, pers. comm.).

Compounds such as flavenoids and carotenoids consumed in the diet are known to affect CYP1A expression in rats (Gradelet *et al.*, 1996; Breinholt *et al.*, 1999; Jewell and O'Brien, 1999). Carotenoids induced hepatic EROD activity forty to fifty-fold in rats in one study (Jewell and O'Brien, 1999) and 140-fold in another study (Gradelet *et al.*, 1996). Canthaxanthin (CX) and astaxanthin (AX), two of the carotenoids shown to induce CYP1A, are found in crustaceans and fish. Although dietary intake of these compounds is probably too low to cause CYP1A induction in humans (assuming a reponse in humans that is similar to rats) (Gradelet *et al.*, 1996), CYP1A levels may be affected in animals that consume large quantities of CX- and AX-containing foods.

Some CYP enzymes are suppressed and others are induced by immune and hormonal responses to stress (Morgan, 1997). Stress may be either psychological (eg. social stress caused by group housing) or physical (eg. infection or general anaesthesia) (Hinson and Raven, 1996). Seals in the captive study were subjected to many stressors, such as surgery, blood sampling, force-feeding, handling, and a change in environment.

Stress stimulates glucocorticoid secretion, and gluococorticoids are known to affect hepatic CYP activities (Harvey, 1996). In most species, cortisol is the most physiologically important glucocorticoid. In rainbow trout hepatocyte cultures, induction of EROD activity by BNF was increased two to three-fold by cortisol (Devaux *et al.*, 1992). In rats, Prough *et al.* (1989) found that *in vivo* dexamethasone (a synthetic glucocorticoid) treatment in adrenalectomized 3-MC-treated rats potentiated induction of CYP1A1 protein content and EROD activity. Down-regulation of CYP1A expression in response to stress has also been reported. In Arctic charr, Jorgensen *et al.* (2001) found that fish subjected to daily handling and confinement stress had significantly higher plasma cortisol levels than unstressed fish. However, CYP1A expression was not significantly different between stressed and unstressed fish. In the same study, in B*a*P-exposed fish, CYP1A protein levels and EROD activity were lower in stressed fish compared to unstressed fish.

A direct relationship between stress and CYP1A expression has not been observed in marine mammals, but Engelhardt (1982) found that captivity and rigorous handling caused a four-fold increase in plasma cortisol levels in juvenile ringed seals. Although harbour seals are known to be readily adaptable and acclimate quickly to captivity (Ross, pers. comm.), it is possible that stress affected gluocorticoid levels, which in turn may have increased CYP1A expression in captive harbour seals.

4.2 Study 2: Field Study of Free-Ranging Harbour Seals

4.2.1 Inter-population comparisons

4.2.1.1 Relationship between environmental contaminant levels in blubber and cutaneous CYP1A expression

Although CYP1A expression in skin did not correlate with contaminant levels (total TEQ including and excluding mono-*ortho* PCBs) in blubber of harbour seal pups from Hornby Island, higher CYP1A protein levels were found in skin of Puget Sound pups compared to British Columbia (BC) pups (Fraser River + Hornby Island), which suggests differences in contaminant exposure. Although contaminant data was not available for Puget Sound seals sampled in the present study, past studies have shown that seals from this region are several times more contaminated with organochlorines than those from BC (Ross *et al.*, submitted; Simms *et al.*, 2000b). Contaminant levels expressed as total TEQ were three times greater in blubber from recently-weaned harbour seal pups from Puget Sound compared to pups from BC (154 versus 44 ng/kg lipid) (Simms *et al.*, 2000b). The differences in CYP1A protein levels detected in the present study may reflect these known differences in contaminant exposure between regions.

4.2.1.2 Relationship between physiological factors and cutaneous CYP1A expression

As with the captive seals, pups sampled in the field were of similar body weight and age. There was no correlation between cutaneous CYP1A expression and body weight for either Hornby Island or Puget Sound pups. Sex differences were not observed for either pups or adults. CYP1A expression was not quantifiable in many skin samples, including those from adults. In fact, none of the adult male seals (n=7) and only four of nine female seals had EROD activities above the LOQ. Small sample size and low levels

of cutaneous CYP1A expression limited detection of possible relationships between physiological factors and CYP1A.

4.2.2 Significance of biomarker studies in free-ranging harbour seals

Chronic exposure to environmentally-relevant levels of organochlorines has been shown to cause immune and endocrine disruption in harbour seals (Brouwer *et al.*, 1989; De Swart *et al.*, 1994; Ross *et al.*, 1996; Simms *et al.*, 2000b). In a captive harbour seal study, seals fed fish from an area of high PCB contamination had diminished immune function (eg. reduced natural killer cell activity and T-cell function) compared to seals fed fish from a relatively uncontaminated area (De Swart *et al.*, 1994; Ross *et al.*, 1996). An adverse effect level for immunotoxicity of 17 mg/kg lipid (Σ PCB) was reported for these harbour seals. Another captive feeding study found that circulatory levels of retinol (vitamin A) were lower in seals fed a relatively more contaminated diet (Brouwer *et al.*, 1989). Additionally, Simms *et al.* (2000b) reported a correlation between circulatory retinol levels and contaminant exposure in free-ranging harbour seal pups in BC and Washington State, indicating that relatively low levels of contaminants affect retinoid homeostasis in these animals. Vitamin A is a dietary hormone that is important for growth and development and plays a role in resistance to microbial infections (Brouwer *et al.*, 1989). One mechanism of contaminant-associated disruption of vitamin A is the binding of PCB hydroxy metabolites to the plasma protein complex that transports vitamin A (and thyroxine) to target organs. As CYP1A is thought to catalyze the formation of OH-PCB metabolites from planar PCB congeners, induction of CYP1A enzymes may in fact facilitate endocrine disruption. Contaminant levels in the range of those suggested to cause immunotoxic effects in captive harbour seals have been

measured in free-ranging harbour seal pups in Washington State (Table 1.1) (Ross *et al.*, submitted), and correlations between contaminant levels and circulatory vitamin A have been observed in free-ranging harbour seal pups in BC and Washington (Simms *et al.*, 2000b). In the present study, correlations between contaminant levels and CYP1A expression were observed. Considering these combined results, we should be concerned that harbour seals in the present study are being negatively impacted by environmental contaminant exposure, and we should continue to develop and improve biomarker approaches, such as CYP1A, to assess these impacts.

4.3 Study 3: Free-Ranging Killer Whales

EROD activity was not detected in any of the S9 fractions prepared from killer whale skin. However, a faint protein band with the expected molecular weight of CYP1A was detected in some samples by immunoblot analysis. I expected to detect CYP1A in killer whale skin, as cutaneous CYP1A-mediated enzyme activity and immunohistochemical staining have been detected in skin of other whale species (fin whale and sperm whale) (Marsili et *al.*, 1998; Godard *et al.*, 2002). Additionally, killer whales sampled in the present study were highly contaminated with organochlorines relative to other marine mammals in which CYP1A has been measured, including harbour seals in this study (Tables 1.1, II.1).

Whereas BNF-treated harbour seals exhibited a strong CYP1A response to which untreated samples could be compared, there was no positive reference sample available for killer whale CYP1A. Non-specific staining on immunoblots also made it difficult to distinguish a putative CYP1A band in all killer whale samples. Structural differences

between killer whale and harbour seal skin (eg. no hair in whales) may also have affected the relative levels of CYP1A expression in these two species.

High contaminant levels in killer whales in British Columbia, and concerns about further declines of the endangered southern resident population underlie the importance of continued research into the development of minimally-invasive biomarker approaches for free-ranging cetaceans (Ross *et al.*, 2000).

4.4 Improvement of CYP1A Detection in Skin

Measurement of CYP1A in harbour seal and killer whale skin might be improved by using more sensitive techniques, such as quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Quantitative real-time RT-PCR has been suggested to be the most sensitive and reliable method for detection of low-abundance mRNA (Miller *et al.*, 1999; Bustin, 2000; Bowen *et al.*, 2000). In this process, total RNA is extracted from tissue and complementary DNA (cDNA) is synthesized via reverse transcription and amplified by PCR. Accumulation of the PCR product (DNA) is then quantified in real-time by fluorometric detection using a spectrofluorometric thermal cycler. Because CYP1A gene sequences have not yet been determined for harbour seals or killer whales, primers would have to be designed based on the sequences of other marine mammal species (eg. largha seal and minke whale) (Teramitsu *et al.*, 2000).

Immunohistochemistry is another technique frequently used to detect CYP1A expression and induction in tissues of both laboratory animals and wildlife (eg. fish, river otters, cetaceans) (Husoy *et al.*, 1994; Sarasquete *et al.*, 2000; Ben-David *et al.*, 2001; Godard *et al.*, 2002). Immunohistochemistry involves labelling and staining the protein of interest *in situ* in thin tissue sections and then assessing the degree of staining by

visualization with a microscope. One advantage of this method is that tissues do not need to be homogenized, a process that dilutes induced cells with non-induced cells (Husoy *et al.*, 1994). Disadvantages of immunohistochemistry are that a highly specific primary antibody and controls to minimize the effect of non-specific staining are needed, and this method is more qualitative than quantitative (Sarasquete *et al.*, 2000). Staining is generally scored for intensity (eg. 0=none to 4=very strong) and occurrence (eg. 0=no cells to 4=all), and these scores are multiplied to obtain a stain index on a scale of 0 to 16 (Ben-David *et al.*, 2001).

The sensitivity of immunodetection techniques, such as immunoblotting and immunohistochemistry, could also be improved by using species-specific antibodies. Although CYP1A protein sequences have now been identified for a few marine mammal species, marine mammal-specific antibodies are not yet commericially available, and immunodetection remains dependent on cross-species reactivity of antibodies prepared against CYP1A enzymes from other animal species.

Immunohistochemistry could also be used to determine CYP1A localization prior to homogenization. It may be advantageous to isolate a particular skin layer in order to obtain a more CYP1A-concentrated homogenate, or whole skin may provide a sufficiently concentrated sample as well as a larger sample volume. Mammalian skin consists of an outer epidermal layer and an inner dermal layer. In humans and rats, the epidermis is the major site of xenobiotic metabolism in skin (Chapman *et al.*, 1979; Bickers *et al.*, 1982). Mukhtar and Bickers (1981) found that in B*a*P- and Aroclor 1254-treated rats, the epidermis had the highest CYP1A enzyme activity when expressed per mg protein.

Cutaneous CYP1A activity has been shown to differ between species, and Williams (1995) suggested that these differences may be due to skin thickness and the number of hair follicles present. In rats, topical treatment with methylcholanthrene (MC) caused induction of CYP1A in hair follicles and sebaceous glands but not in the epidermis (Anderson *et al.*, 1989). Cetaceans (whales, dolphins, porpoises) have an epidermal layer that is ten to twenty times thicker than that of terrestrial mammals (Geraci *et al.*, 1986) and do not possess hair (or hair follicles). To my knowledge, information about CYP1A localization in marine mammal skin and techniques for separating skin layers has not yet been published. However, at least one laboratory has localized CYP1A in dermal endothelial cells, rather than epithelial cells, in cetaceans and in river otters (Wilson *et al.*, 2000; Ben-David *et al.*, 2001; Godard *et al.*, 2002).

4.5 Effects of Storage Conditions on CYP1A

Prolonged storage, storage temperature, and repeated freeze-thawing of tissue samples and homogenates have been shown to affect detection of CYP1A protein, and particularly enzyme activity (Pearce *et al.* 1996; Anulacion *et al.*, 1997; Yamazaki *et al.* 1997). In the present study, however, extensive precautions were taken to minimize these potential effects. Liver and skin samples were immediately frozen in liquid nitrogen following collection and were stored in liquid nitrogen (-196°C) or at -80°C until they were homogenized (from a few months to a year later). Following homogenization, S9 fractions were stored at -80°C, and CYP1A analysis was carried out a few days later (eg. killer whale skin biopsies), several months later, or more than a year later (eg. CYP1A protein analysis of captive seals). Tissue biopsies were kept frozen during sub-sampling, and homogenates were aliquotted into small volumes (eg. 60 µl) so that vials were not

repeatedly thawed and refrozen. Previously thawed samples were not used for the EROD assay but were used for the immunoblot assay if necessary. When harbour seal skin S9 fractions were thawed and refrozen several times, it did not appear that CYP1A detection by immunoblot analysis was affected.

In the present study, differences in storage time among sample groups may have introduced some variability to CYP1A expression. However, studies in the literature have shown that duration of storage may or may not affect CYP1A expression. Pearce *et al.* (1996) found that CYP content and EROD activity decreased by 20-40% after human liver samples were frozen at -80°C for up to six months. However, these same measurements were relatively unaffected by storage of hepatic microsomes at -80°C for one or two years. In hepatic microsomes from English sole (fish), AHH activity decreased by 60% after five months of storage at -80°C (Anulacion *et al.*, 1997). Yamazaki *et al.* (1997) found that there was no difference in CYP levels and enzyme activity if microsomes were prepared from fresh tissue and then stored at -80°C or made from tissues that were stored at -80°C prior to homogenization.

Anulacion *et al.* (1997) also studied the effect of storage volume on CYP1A (250 vs. 750 µl microsomal suspension/vial) and found no difference in AHH activity between volumes. In the present study, the use of smaller storage volumes (eg. 60 µl) may have affected sample stability. Additionally, S9 rather than microsomal fractions were used for CYP1A detection, and S9 fractions may be less stable than microsomal fractions during prolonged freezing due to differences in storage solutions (Tris-HCl for S9 fractions versus sucrose for microsomes).

Although every effort was made to keep samples cold during laboratory procedures (eg. samples kept on dry ice during sub-sampling and in ice baths during homogenization), unintentional warming of samples, especially during tissue homogenization, may have decreased CYP1A enzyme activity. Yamazaki *et al.* (1997) found that detection of CYP proteins by immunoblot analysis was not affected by leaving liver samples at 25°C for six hours; however, EROD activity declined to undetectable levels. The latter result highlights the sensitivity of enzyme activity to temperature and the need to keep samples cold. We expect that increasing the homogenization time will increase CYP1A protein yield up to a certain point. However, because heat is generated during homogenization, it is possible that increasing the homogenization time will also result in enzyme degradation, thereby decreasing enzyme activity.

4.6 Conclusions

1) Minimally-invasive biopsy sampling techniques were used to obtain high quality liver and skin samples from harbour seals sufficient for CYP1A analysis.

2) Both EROD activity and CYP1A protein levels were quantified in liver and skin biopsies from harbour seals.

3) Two distinct immunoreactive CYP1A protein bands, which likely correspond to CYP1A1 and CYP1A2, were detected in both liver and skin of harbour seals.

4) Oral BNF treatment induced CYP1A protein levels in both liver and skin of harbour seals, but CYP1A expression in skin was not positively correlated with that in liver.

5) CYP1A expression in harbour seal liver was positively correlated with contaminant levels in blubber, suggesting that harbour seals in British Columbia are exposed to contaminant levels that are sufficiently high to elicit induction of hepatic enzymes.

6) Cutaneous CYP1A expression did not correlate with contaminant levels, but these results were limited by small sample size.

7) Confounding factors (eg. age, sex, and condition) may have influenced CYP1A expression in liver and skin from captive harbour seals. These results further highlight the importance of characterizing and minimizing natural factors prior to assessing contaminant effects.

8) CYP1A could not be quantified in killer whale skin biopsies, possibly as a result of small sample size and/or low CYP1A expression in this tissue. Immunohistochemical localization of CYP1A and more sensitive techniques, such as RT-PCR, are needed to confirm the presence of CYP1A in killer whale skin.

4.7 Summary and Future Studies

The importance of biomarker studies in marine mammals is twofold. First, such studies can provide us with information about the health of marine mammal populations, and second, they can provide an indication of the overall health of the marine environment. The development and application of sensitive, biologically relevant, and minimally-invasive biomarkers in sentinel marine species, such as harbour seals and killer whales, will facilitate conservation-based management of these animals and improve efforts to regulate anthropogenic chemicals to minimize environmental impact.

In contrast to most other biomarker-based studies in marine mammals, we obtained tissue samples using minimally-invasive means from healthy, free-ranging animals, and we had a good understanding of the condition of each animal sampled (i.e. in the case of harbour seal pups, all animals were of approximately the same age and condition; in the case of killer whales, the age, sex, and dietary preference of each individual was known). Almost all previous biomarker studies in marine mammals have utilized tissue samples from dead animals (stranded or sacrificed), presenting both ethical concerns (killing animals for scientific purposes) and problems associated with poor sample quality (stranded animals). Sampling live, free-ranging marine mammals using minimally-invasive methods presents considerable logistical and methodological challenges. However, as conservation of these animals is the primary objective of toxicological studies, such barriers must be overcome. In the present study, CYP1A was successfully quantified in small liver and skin samples obtained by non-lethal means, providing a foundation for further development of this conservation-driven biomarker approach.

A 'weight of evidence' approach is needed to assess the effects of contaminant exposure on free-ranging marine mammals (Ross, 2000). This involves extrapolating between laboratory rodent studies involving single contaminants, captive marine mammal studies, and studies in free-ranging marine mammal populations. Laboratory-based rodent studies provide increased mechanistic understanding of biomarker responses and increased confidence of cause-effect relationships, whereas studies in free-ranging populations provide less confidence of cause and effect but increased ecological relevance (Ross, 2000). The present study showed that CYP1A is inducible in harbour seals and provided evidence that ambient contaminant levels affect CYP1A expression in free-ranging seals (ecological relevance). Combined with the observations of other ongoing marine mammal biomarker studies in our laboratory (eg. vitamin A) (Simms *et al.*, 2000b) and mechanistic laboratory-based studies in the literature, results from this study suggest that current levels of contaminant exposure are high enough to warrant continued concern and regulatory action.

Whereas many biomarker studies in marine mammals do not adequately control for natural confounding factors (eg. age and condition), the present study was designed to minimize the influence of these factors on CYP1A response. Nevertheless, natural factors, such as age and stress, may have affected CYP1A expression in harbour seal pups. This observation provides an important foundation for the design and interpretation of future biomarker studies and further highlights the importance of characterizing and minimizing the input of natural factors when assessing cause-effect relationships between contaminants and biomarker responses.

Future studies of CYP1A in marine mammals should (1) further improve the efficiency of CYP1A extraction from skin biopsies; (2) develop, validate, and apply additional quantitative methods to measure CYP1A; and (3) further explore the contribution of natural factors to CYP1A expression. This study demonstrated that CYP1A can be quantified in small liver and skin biopsies using EROD and immunoblot assays. However, as CYP1A could not be quantified in some of the skin samples, including samples from killer whales, strong conclusions about cause-effect relationships between contaminants and cutaneous CYP1A expression could not be made. More sensitive quantitative techniques will likely help to clarify contaminant responses in this tissue. Additionally, a better understanding of natural factors will help us to better assess contaminant-CYP1A relationships in future marine mammal studies.

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6. APPENDICES

6.1 Appendix I

Table I.1: Individual harbour seal data for the present study

Capture date	Site	Seal #	Sex	Weight at capture (kg)	Length (cm)	Girth (cm)	Estimated age
8/17/00	Fraser River estuary	PV001	M	24.4	95	79	3.5-5 weeks
8/17/00		PV002	M	27.6	91	82.5	3.5-5 weeks
8/17/00		PV003	F	18.4	90	70	3.5-5 weeks
8/17/00		PV004	M	29	97	84	3.5-5 weeks
8/17/00		PV005	M	27.4	101	88	3.5-5 weeks
8/17/00		PV006	F	30.8	94	89	3.5-5 weeks
8/17/00		PV007	M	17.2	84	67	3.5-5 weeks
8/17/00		PV008	M	23.5	95	75	3.5-5 weeks
8/17/00		PV009	M	20.2	89	67.5	3.5-5 weeks
8/17/00		PV0010	M	24.4	91	76	3.5-5 weeks
8/17/00		PV0011	F	25	92	83	3.5-5 weeks
8/17/00		PV0012	M	23	923	75	3.5-5 weeks
8/17/00		PV0013	M	28	100	82	3.5-5 weeks
8/17/00		PV0014	M	24	94	76	3.5-5 weeks
8/17/00		PV0015	M	20	91	69	3.5-5 weeks
8/17/00		PV0016	M	23	88	79	3.5-5 weeks
8/17/00		PV0017	M	25.5	92	80	3.5-5 weeks
8/17/00		PV0018	M	26.5	95	82	3.5-5 weeks

Capture date	Site	Seal #	Sex	Weight at capture (kg)	Length (cm)	Girth (cm)	Estimated age
8/17/00	Fraser River estuary	PV0019	F	21.5	91	75	3.5-5 weeks
8/17/00		PV0020	F	20	91	74	3.5-5 weeks
8/14/01		PV01-17	F	58.5	140	-	>4 yrs
8/14/01		PV01-18	F	78.5	152	-	>4 yrs
8/14/01		PV01-20	M	111	-	-	>5 yrs
8/15/01		PV01-22	F	48.5	-	-	4 yrs
8/15/01		PV01-23	F	62.5	150	-	>4 yrs
8/22/01	Boundary Bay	PV01-25	F	67	-	-	8-9 yrs
8/22/01		PV01-29	M	78	-	-	-
8/22/01		PV01-30	F	79	-	-	>6 yrs
8/22/01		PV01-31	M	104	-	-	12-15 yrs
8/22/01		PV01-32	M	103	-	-	10 yrs
8/22/01		PV01-33	F	61.5	-	-	-
8/22/01		PV01-34	M	89.5	-	-	-
8/22/01		PV01-36	F	63	-	-	7-11 yrs
8/22/01		PV01-37	M	50	-	-	4 yrs
8/22/01		PV01-38	M	77	-	-	6-9 yrs
8/22/01		PV01-39	F	50	-	-	-
8/8/01	Hornby Island	PV01-01	F	21	87	77	3.5-5 weeks
8/8/01		PV01-02	M	39	104	94	3.5-5 weeks
8/8/01		PV01-03	F	22.8	94	73	3.5-5 weeks
8/8/01		PV01-04	M	19.2	88	70	3.5-5 weeks
8/8/01		PV01-05	F	22.5	88.5	76.5	3.5-5 weeks

Capture date	Site	Seal #	Sex	Weight at capture (kg)	Length (cm)	Girth (cm)	Estimated age
8/8/01	Hornby Island	PV01-06	M	23.4	91	78	3.5-5 weeks
8/9/01		PV01-07	M	19	90	71	3.5-5 weeks
8/9/01		PV01-08	F	24	92	77	3.5-5 weeks
8/9/01		PV01-09	M	25	98	78.5	3.5-5 weeks
8/9/01		PV01-10	M	23	95	76	3.5-5 weeks
9/18/01	Puget Sound	PV01-40	M	-	93	76.5	3.5-5 weeks
9/18/01		PV01-41	M	-	90	71	3.5-5 weeks
9/18/01		PV01-42	M	20.3	88	67	3.5-5 weeks
9/18/01		PV01-43	F	28.8	98	83	3.5-5 weeks
9/18/01		PV01-44	F	21.8	91	78	3.5-5 weeks
9/18/01		PV01-45	F	19.8	84	67	3.5-5 weeks
9/18/01		PV01-46	F	20	90	70.6	3.5-5 weeks
9/18/01		PV01-47	M	21.2	84	72.6	3.5-5 weeks
9/18/01		PV01-48	M	25.8	87	85.7	3.5-5 weeks
9/18/01		PV01-49	F	25.6	82	78	3.5-5 weeks
9/18/01		PV01-51	F	29	94	80	3.5-5 weeks
9/18/01		PV01-52	M	24	96	80	3.5-5 weeks
9/18/01		PV01-53	F	26.2	85	77	3.5-5 weeks
9/18/01		PV01-54	F	27	95	80	3.5-5 weeks
9/18/01		PV01-55	M	28.5	100	85	3.5-5 weeks
9/18/01		PV01-56	M	29	91	87	3.5-5 weeks

6.2 Appendix II

Table II.1: Contaminant levels in blubber samples from harbour seals and killer whales in the present study

Population	Mean contaminant levels in blubber (ng/kg lipid)					
	2,3,7,8-PCDDs	2,3,7,8-PCDFs	Non-*ortho* PCBs	Mono-*ortho* PCBs	Total PCBs	Total TEQ
Fraser River harbour seal pups	58 (8)	8.5 (0.7)	-	-	2,422,489 (411,655)	36.6 (5.6)
Hornby Island harbour seal pups	225 (36)	0	23,103 (5191)	150,086 (18,815)	1,644,750 (234,617)	18.8 (1.7)
N. Resident killer whales (males)	4723 (4224)	0	18,640 (3230)	2,191,858 (537,269)	19,954,836 (3,672,056)	245.6 (48.7)
N. Resident killer whales (females)	653 (179)	0	17,011 (5755)	2,935,594 (693,227)	38,436,478 (8,568,933)	351.9 (76)
Transient killer whales (males)	237 (73)	0	5903 (1865)	6,830,536 (2,093,485)	344,015,342 (111,047,500)	779.3 (197.2)

Values are presented as mean (SE)