Physiological and Molecular Biomarkers of Environmental Contaminant-Associated Immunotoxicity in Harbour Seals (*Phoca vitulina*)

by

Elisabeth Mos
BSc/MSc, Wageningen University, 2001

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Abstract

Persistent organic pollutants (POPs) have been contaminating the marine environment since the mid 20th Century and continue to do so today. The polychlorinated biphenyls (PCBs) are of particular concern, since they are found at high concentrations in marine mammals throughout the Northern Hemisphere, and have been associated with endocrine disruption, reproductive impairment, immunotoxicity, and outbreaks of disease. In this study, samples were obtained from free-ranging harbour seal (*Phoca vitulina*) pups, which were live-captured in British Columbia, Canada, and Washington State, USA, in order to assess adverse health effects associated with POPs on the immune system of these mammals.

PCBs were the most abundant of the 31 POPs measured in seals, and represented the greatest toxicological concern on the basis of established reference values for laboratory rodents and aquatic wildlife consumers. Seal immune function was assessed using traditional measures of immunotoxicity, including hematology, innate immune function, and adaptive immune function, and related to PCB concentrations while

carefully controlling for confounding factors such as age, sex and condition. PCB concentrations negatively correlated to phagocytosis, T lymphocyte proliferative responses, (thymosin-α1-induced) lymphocyte signalling, and lymphocyte counts, and positively associated with the respiratory burst of phagocytes and aryl hydrocarbon receptor (AhR) expression of white blood cells, suggesting chemical-associated immunotoxicity. Parallel experiments, in which harbour seal white blood cells were exposed *in vitro* to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a potent immunotoxicant, further supported the hypothesis that the observed reduction in immunocompetence in free-ranging seals may be due to an AhR-mediated mechanism of immunotoxicity. Principal component analysis (PCA) of immunological endpoints combined evidence of PCB-associated effects on the immune system as a whole. However, PCA also identified a difference between the immunological profiles of urban seals and those from remote sites, consistent with elevated pathogen abundance due to biological pollution at urban sites.

In a second approach, PCB concentrations were related to concentrations of vitamin A and expression levels of its receptor, the retinoic acid receptor alpha (RARα), which are known to be sensitive to PCB exposure. More contaminated seals were characterised by lower vitamin A concentrations in circulation and in the blubber, as well as higher RARα expression in the blubber. AhR expression and concentrations of thymosin-α1 (a thymic hormone important in lymphocyte development and immune function) did not relate to contaminants when their levels were investigated in seal tissues (blubber, skin), contrasting their sensitivity in white blood cells. These results implied that PCB-associated toxicity may be reflected if a biomarker represents a primary lesion, but might not be extrapolated among tissues in all cases. Secondly, although blubber represents the primary site of PCB storage, it is not necessarily the site of the highest toxicity.

In summary, significant evidence of immunotoxicity and disruption of immune function-related biomarkers has been provided in a healthy group of free-ranging marine mammals which contributes to the weight of evidence that environmental contaminants may render marine mammal populations more vulnerable to disease through immunotoxicity. Immune function measures in free-ranging harbour seal pups exposed to contaminants *in vivo*, in combination with harbour seal white blood cells *in vitro*, furthermore suggested that immunotoxicity may take place through an AhR-mediated mechanism of action. An unexpected finding was the evidence of a second, independent, impact on the immune system of seals, consisting of biological pollution. The combination of both chemical and biological pollution, that would imply both diminished immune responses and increased pathogen loads, may represent the largest threat to the health of marine mammals in many parts of the world.

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List of Abbreviations

ANOVA	Analysis of variance
ATSDR	Agency for toxic substances and disease registry
AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
ARNT	Aryl hydrocarbon receptor nuclear translocator
BC	British Columbia
BHT	Butyl-hydroxytoluene
bp	Base pairs
BSA	Bovine serum albumin
BrdU	5'-bromo-2'-deoxyuridine
CCME	Canadian council of ministers of the environment
CD	Cluster of differentiation (cell membrane molecules)
CDV	Canine distemper virus
cDNA	Complementary DNA
ConA	Concavalin A (lectin)
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
Ct	Threshold cycle (in QPCR)
DDD	Dichloro diphenyl dichloroethane
DDE	Dichloro diphenyl ethylene
DDT	Dichloro diphenyl trichloroethane
DMQ	Dimeralized water
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive element
EDI	Estimated daily intake
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FC	Fecal coliform
GC-ECD	Gas chromatography-electron capture detection
GC-MS	Gas chromatography-mass spectrometry
GM	Geometric mean
GPC	Gel permeation chromatography
H1	Histone 1
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HPLC	High performance liquid chromatography
HRGC	High resolution gas chromatography
HRMS	High resolution mass spectrometry
Hsp90	Heat shock protein 90
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IL-2R	Interleukin-2 receptor
IU	International units
IUPAC	International union of pure and applied chemistry

LOAEL	Lowest observed adverse effect level
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
NIST	National institute of standards and testing
NK cell	Natural killer cell
NOAEL	No observed adverse effect level
NTP	Nucleic triphosphate
OC pesticide	Organochlorine pesticide
PAS	Per-ARNT-Sim (protein family)
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PBMC	Peripheral blood mononuclear cell
PC	Principal component
PCA	Principal components analysis
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofuran
PCDE	Polychlorinated diphenyl ether
PCR	Polymerase chain reaction
PDV	Phocine distemper virus
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
POP	Persistent organic pollutant
ProTα1	Prothymosin-α1
QPCR	Quantitative polymerase chain reaction
RARα	Retinoic acid receptor-α
RB	Retinoblastoma protein
RBP	Retinol-binding protein
RDL	Regional dioxin laboratory
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPL8	Ribosomal protein L8
RXR	Retinoid X receptor
SI	Stimulation index
SEM	Standard error of the mean
SMRT	Silent mediator of retinoic acid and thyroid hormone
SRBC	Sheep red blood cells
Tα1	Thymosin-α1
T_C lymphocyte	Cytotoxic T lymphocyte
T_H lymphocyte	Helper T lymphocyte
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	Toxic equivalency factor
TEQ	Toxic equivalency quotient
TF5	Thymus fraction 5 (thymus extract)
TR	Throid hormone receptor
TTR	Transthyretin
VIP	Vasoactive intestinal peptide
WA	Washington State
WBC	White blood cell
XAP2	Xenobiotic associated protein 2

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Just over four years ago, I started my PhD full of ideas and with lots of enthusiasm. And over the years, I learned many things about research of which I have loved most. I very much enjoyed the field and laboratory work, the way things came together as pieces of a puzzle when writing up the results, and presenting at conferences. It also made me realise that many people do not know that we change and destroy our world so quickly as long as they cannot see it; talking about my work to friends as well as strangers made me feel like I could make a small difference. Some days, however, it seemed like everything had already been done in research, and experiments always had to fail on days that the sun was shining but never when it rained. The last four years have made me realise a PhD is not just an education, but more a way of life, and that there is a lot you know, but that there is always more you do not. I feel both fortunate and sad that I will leave this all behind.

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We cannot discover new oceans until we have the courage to lose sight of the shore

(Muriel Chen)

CHAPTER 1

Introduction

CONTAMINATION OF THE MARINE ENVIRONMENT

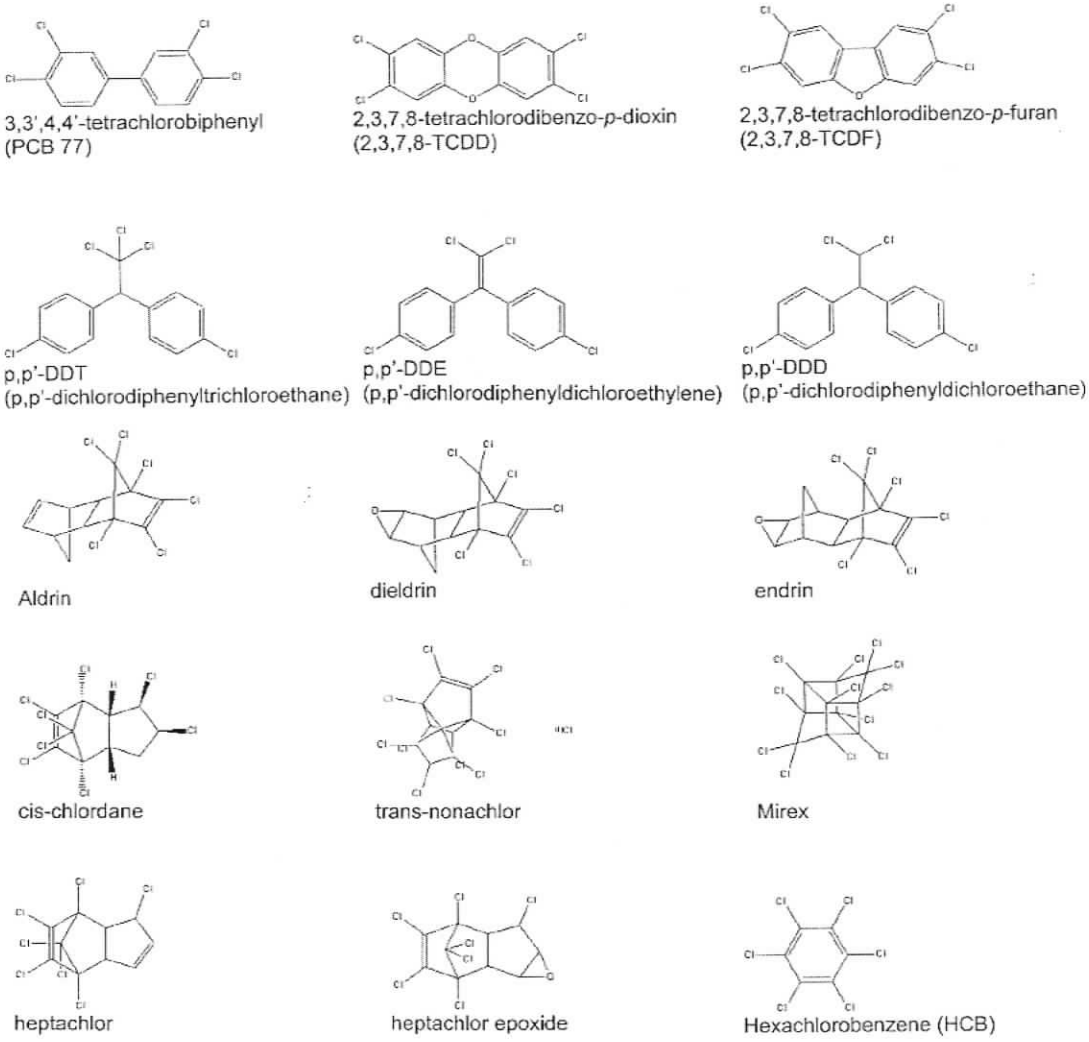
Anthropogenic contaminants in the marine environment

Marine ecosystems such as near-shore waters and estuaries belong to the most productive ecosystems in the world. However, they also typically have high human population density and an elevated level of human activities. Over the last decades, many of these ecosystems have been severely affected by humans through overfishing, physical alteration of the coastal environment, habitat destruction, as well as municipal, industrial and agricultural waste and run-off. Many of these anthropogenic activities have been shown to affect the abundance and survival of marine organisms, biodiversity, and the functioning of the marine ecosystem as a whole. However, Persistent Organic Pollutants (POPs) are especially considered a major global threat to marine life (United Nations Environment Programme, 1995).

POPs can be broadly divided into two groups. Firstly, the "legacy POPs", for which concentrations in the marine environment largely result from historical use, have been largely phased out by industrialized nations (but continue to be used in developing countries). These include industrial chemicals such as polychlorinated biphenyls (PCBs), industrial by-products sharing high structural similarity to PCBs (e.g. polychlorinated dibenzo-*p*-dioxins and –furans, PCDDs and PCDFs), and a variety of structurally unrelated organochlorine pesticides (Figure 1). In addition, there are a number of newly developed, or "emerging POPs", that were designed in part to replace certain legacy POPs. Polychlorinated diphenyl ethers (PCDEs), polybrominated diphenyl ethers (PBDEs), and polybrominated biphenyls (PBBs) are examples. Emerging POPs are commonly considered less toxic and less persistent than their legacy counterparts. However, their concentrations are increasing in the marine environment.

Figure 1. Chemical structures of selected Persistent Organic Pollutants.

Among the Persistent Organic Pollutants (POPs) are the polychlorinated biphenyls (PCBs), and the structurally related -dioxins (PCDDs) and –furans (PCDFs), of which a large number of congeners exists depending on the positioning of the chlorine atoms. A variety of other organochlorine compounds, many historically used as pesticides, are categorized as POPs due to similarity to PCBs in their persistence, bioaccumulative nature, and toxicity.



Taken from Ikonomou et al., 2001.

Although POPs show a large variety in structure and origin, they share four common properties: (1) they are highly resistant to environmental, chemical, and thermal breakdown, and therefore persistent in the environment; (2) they are semi-volatile and hydrophobic, properties that permit them to either evaporate and be transported over long ranges by atmospheric circulation, or be adsorbed onto organic particles and transported by ocean currents; (3) they are highly lipophilic, resulting in bioconcentration and bioaccumulation in organisms, especially those residing at the higher trophic levels; (4) they are toxic and can affect the health of humans and wildlife.

The organochlorine pesticide DDT (dichloro diphenyl trichloroethane) represents a well-known example of a legacy POP. DDT was developed in 1874, and its use began in 1939 as an agricultural insecticide and in the battle against insect-vectors of diseases such as malaria and typhus. Although DDT and its metabolites were detected in human fat as early as 1944, it was not until 1962, when Rachel Carson published her book *Silent Spring* (Carson, 1962), that the public became aware of the risks associated with POPs. In this book, Carson provided evidence that DDT was responsible for the egg-shell thinning that threathened the survival of many birds of prey, and warned that unbridled pesticide use would make spring mornings become silent and devoid of wildlife. Under increasing public pressure, and concern about possible human health risks, governments in most nations eventually imposed (severe) restrictions on the use of DDT in the 1970s.

The banning of DDT represented the start of many regulatory actions undertaken by governments against POPs. However, due to their physico-chemical characteristics, POPs such as DDT had already spread around the world. Today, twelve POPs (together often referred to as the "Dirty Dozen") have been designated for global action under the Stockholm Convention (2001) by the United Nations Environment Programme (Table 1). These priority pollutants have been targetted because of their impact on the environment

and human health, with the objective of reducing and eliminating emissions and discharges, to develop safe substitutes and cleaner production processes, and to promote better environmental practice in pest control. An additional 20 chemicals are currently under review for possible inclusion in the Stockholm Convention. Among the new POP candidates are the emerging PBDEs, the organochlorine pesticides endosulfan, methoxychlor and hexachlorocyclohexane (HCH), and the industrial by-product octachlorostyrene (WWF, 2005).

Table 1. POPs designated for global action, and their primary applications.

Aldrin	Insecticide against soil pests (primarly termites) on corn, cotton and potatoes
Chlordane	Insecticide used in termite control
DDT	Insecticide used in mosquito control
Dieldrin	Insecticide used on fruit, soil, and seed crops, including corn, cotton, and potatoes
Endrin	Rodenticide and insecticide used on cotton, rice, and corn
Heptachlor	Insecticide used against soil insects, especially termites, as well as against fire ants and mosquitoes
Hexachlorobenzene	Fungicide, by-product of pesticide manufacturing, and contaminant of other pesticide products
Mirex	Insecticide used against ants and termites, fire retardant
Toxaphene	Insecticide used against ticks and mites
PCBs	Used in capacitors and transformers, hydraulic and heat transfer systems, weatherproofing, carbon less copy paper, paints, adhesives, and plasticizers in synthetic resins
Dioxins	By-product of combustion (especially of plastics) and of chlorine product manufacturing and paper bleaching
Furans	By-products of especially PCB manufacturing

Taken from McGinn, 2000.

POLYCHLORINATED BIPHENYLS (PCBs)

Polychlorinated biphenyls in the marine environment

Of the many POPs, PCBs were used around the world as stable, heat-resistant oils and lubricants in electrical transformers, heavy machinery, and a variety of other applications, and approximately 1.2 million tonnes of these compounds were produced (CCREM, 1987). PCBs were banned in the 1960-1970s in industrialized nations, but continue to be used in a number of developing countries. It was estimated in 1995 that only 4% of all PCBs ever produced had been incinerated, whereas 35% had been released into the environment; the remaining 60% of PCBs is still used or is stored in dumps and landfills. From these stores, PCBs will continue to leach into freshwater or will be transported by run-off, to eventually end up in the marine environment (Orris et al., 2000).

Commerical PCB preparations (e.g. Clophen, Aroclor) consist of a mixture of up to 209 PCB congeners, differing in their degree of chlorination (between 1 to 10 chlorine atoms) and their substitution pattern. Two primary groups are recognised: PCBs that assume a globular structure and the planar PCBs that are characterized by a more flat structure, reflecting the substitution pattern of the chlorine atoms. Non-ortho substitutions on the two phenyls allow the rings to position themselves in the configuration of the lowest energy, which is a planar configuration. When the chlorines are positioned in both ortho positions, however, a planar configuration is highly unlikely due to their overlapping radii. This steric hinderance forces the phenyl rings to rotate away from each other, resulting in a globular structure. PCBs with a mono-ortho substitution pattern hold the middle between globular and planar structures (Boon et al., 1997). In the marine environment, the globular PCB congeners are present at the highest concentrations, reflecting their

higher level of persistence, whereas the planar congeners are commonly considered to be the most toxic to humans and wildlife.

When introduced into the marine environment, PCBs can absorb onto particulate matter that is either deposited into the sediments, ingested or be directly absorbed by invertebrates and fish through their skin or by the gill membranes. In gill-breathing organisms, elimination is a process of equilibrium partitioning between body lipids, blood and ambient water (Boon et al., 1992). PCBs are transferred up to the higher trophic levels, where most marine mammals and fish-eating birds reside, by the intake of prey, when the fat is burned off but the contaminants remain. Because of the lack of an exchange mechanism between blood and water, metabolism is the primary factor controlling bioaccumulation at this trophic level (Boon et al., 1992; Tanabe & Tatsukawa, 1992).

Polychlorinated biphenyls in marine mammals

Marine mammals such as striped dolphins (*Stenella frontalis*) and killer whales (*Orcinus orca*) were shown to retain about ten million times higher concentrations of PCBs than found in water (Tanabe et al., 1994). These extremely high bioconcentration factors are due to several vulnerabilities of this group of mammals. Firstly, marine mammals differ in POP exposure from most other (high trophic level) mammals by occupying marine food webs, which are typically longer than that of terrestrial ecosystems. Secondly, the long lifespan and large body size (with consequently high food intake) of marine mammals add to the elevated life-long dietary intake of PCBs. Lastly, large body size and the evolutionary loss or limited capacity of certain detoxifying enzymes (e.g. CYP2B) results in a lower rate of metabolism, and therefore slow elimination of toxic compounds (Boon et al., 1994).

Due to the hydrophobic character of PCBs, the highest concentrations of these toxic compounds are found in the lipid tissues of marine mammals, with blubber (a thick layer of subcuteanous fat) representing the major depot containing up to 90% fat. Although the blubber has likely developed as a thermoregulatory adaptation to the aquatic existence of marine mammals, knowledge has accumulated that support physiological importance of blubber as a dynamic tissue providing a source of energy and as a storage depot of hormones and nutrients (Mellish et al., 1999; Mos & Ross, 2002; Tabuchi et al., 2006). It is not clear whether PCBs are stored in an inert manner in this tissue, or are exerting toxic effects at the blubber level.

Natural influences on PCB concentrations in marine mammals

PCB concentrations vary within marine mammal species and individuals, depending on their diet, trophic levels, and age. For example, male marine mammals show an age-dependent increase in PCB concentrations in the blubber whereas females partly offload their body burden when reproducing. A small percentage (4-10% in spinner dolphins (*Stenella coeruleoalba*); (Tanabe et al., 1982) of PCBs is transferred transplacentally, while most of the reproductive transfer takes place during lactation when fat is mobilised from the mother's blubber layer in order to produce a lipid-rich milk (Addison & Brodie, 1987). Especially during the first gestation, the mother transfers considerable amounts of PCBs to her pup through this pathway (Subramanian et al., 1988). About 60% of the females body burden of contaminants are transferred from blubber into milk in harbour seals (*Phoca vitulina*), and up to 90% in striped dolphins (Boon et al., 1992).

PCB concentrations found in the blubber of nursing seal pups are similar to those received through milk, suggesting direct deposition and little or no excretion of lipophilic contaminants by the young (Addison & Stobo, 1993). Reproductive transfer of contaminants, and the consequential concentrations of PCBs in the diet of the new-born

may represent the highest exposure for a marine mammal during its life time (Shaw, 1998; Murk et al., 1998; Ross & Troisi, 2001). Taking into consideration that young mammals have a reduced enzymatic ability to metabolise xenobiotics (Boon et al., 1992) and may be more vulnerable to the toxic effects (Peterson et al., 1993; Olson & Mcgarrigle, 1992; Hoffman et al., 1996; Huuskonen et al., 1994), they may be a group at high risk.

The mechanism of PCB-associated toxicity

The complex mixtures in which PCB congeners circulate in the marine environment is reflected in the variety of toxic effects and mechanisms of toxic action that can be attributed to these compounds. Globular PCBs mediate toxicity through a number of different mechanisms of action, resulting in inhibition of certain cell functions, oxidative stress, and alteration of both dietary and endogeneous-produced hormones (e.g. vitamin A, thyroid hormones, estrogen, and prolactin) (Safe & Wormke, 2003; Dalton et al., 2002; Zile, 1992; De Krey et al., 1994; Brouwer et al., 1998). Planar and mono-ortho PCBs, as well as the dioxins and furans, on the other hand, initiate toxicity primarily through binding to a single receptor, known as the aryl hydrocarbon receptor (AhR).

The AhR is a basic helix-loop-helix (bHLH) protein, belonging to the Per-ARNT-Sim (PAS) family of transcription factors. Although AhR homologs are present in all metazoans, the distribution and function of AhR are known to be conserved in vertebrates (Hahn, 2002). The promiscuous binding site of the AhR can be activated by binding of naturally occuring dietary substances as well as xenobiotics, and likely has endogenous ligands, supporting a variety of roles for AhR (Denison & Nagy, 2003).

Firstly, AhR functions as a developmental regulator, and its presence is required for normal development of the immune and vascular systems, the liver, the ovaries and other internal organs (Denison and Nagy, 2003). AhR also influences processes of

growth and development, through the interaction with a number of other cell signaling molecules including the estrogen receptor (ERα) (Ohtake et al., 2003; Denison & Nagy, 2003). It has been suggested that these developmental roles of AhR are achieved by actions as a ligand-activated co-activator (Boutros et al., 2004; Tijet et al., 2006).

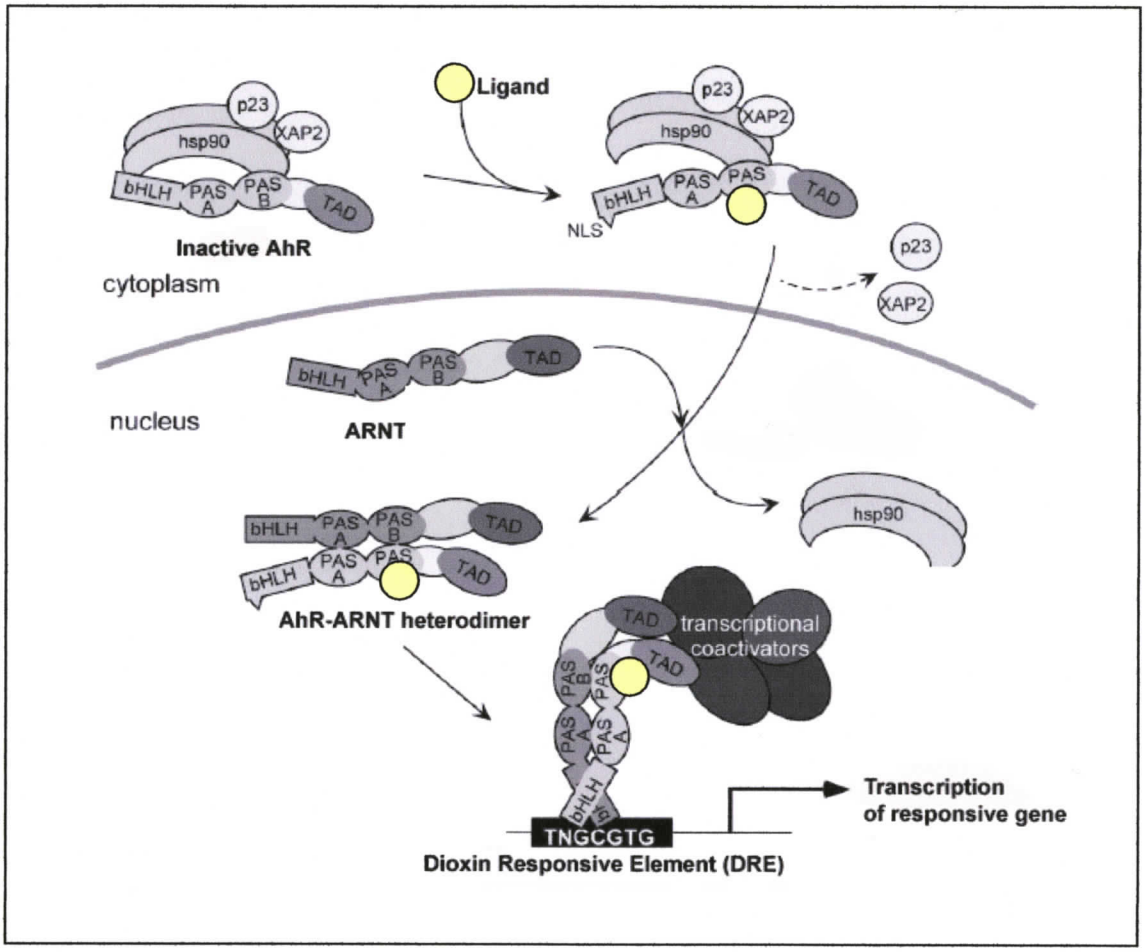
Secondly, the AhR can function as a ligand-activated transcription factor, during the adaptive response to toxic substances. This activity has been proposed to originate from the co-evolution of herbivores and plants, as a protective mechanism against plant-produced toxins. Whether it is a part of this defensive mechanism that AhR also responds to anthropogenic toxic substances, or that it is a coincidence that these chemicals bind AhR, remains unknown. However, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD represents the most potent activator, and most extensively characterised, AhR ligand to date (Denison & Nagy, 2003).

AhR-mediated signalling in response to TCDD exposure has been described in detail (reviewed in Kewley et al., 2004; Denison & Nagy, 2003; Figure 2). Due to their lipophilicity, TCDD as well as PCBs and other dioxin-like compounds easily cross cell membranes, and can bind the AhR, located in the cytoplasm of the cell. In the cytoplasm, AhR is maintained in a complex of several chaperone proteins, including heat shock protein 90 (Hsp90), XAP2, and p23, that are involved in its correct folding and stabilization (Gu et al., 2000). Upon binding of a ligand, these proteins are released, and the remaining AhR-ligand complex is translocated to the nucleus. In the nucleus, the liganded AhR forms a heterodimer with the AhR nuclear translocator (ARNT). This heterodimer can bind to specific DNA sequences, often referred to as dioxin responsive elements (DREs). AhR binding to these sequences results in enhanced transcription of the genes adjacent to DREs. Such genes include the negative regulator of AhR (AhR repressor or AhRR), and a number of enzymes that facilitate the biotransformation and elimination of toxic compounds, such as cytochrome P450s (CYP1A1, CYP1A2, and

CYP1B1), urine diphosphate glucuronosyltransferases (UDPGT), glutathione-S-transferases (GST), and flavin monooxygenases (Hankinson, 1995; Tijet et al., 2006).

Figure 2. AhR signalling in response to TCDD.

Inactive AhRs are present in the cytoplasm of most cells, in the presence of chaperone proteins that maintain its configuration. Upon binding of a ligand such as TCDD to AhR, these proteins are shed, allowing AhR to translocate to the nucleus. In the nucleus, the liganded AhR can form a heterodimer with another PAS protein called ARNT. AhR-ARNT dimers can bind to dioxin responsive elements, and initiate the transcription of responsive genes, which further induces toxic effects.



Abbreviations: AhR = aryl hydrocarbon receptor; ARNT = Aryl hydrocarbon receptor nuclear translocator; bHLH = basic helix-loop-helix domain; PAS = per-ARNT-sim domain; Hsp90 = heat-shock protein-90; NLS = nuclear localization signal; TAD = transactivation domain; XAP2 = xenobiotic associated protein-2.

Adapted from Kewley et al., 2004.

The principle of toxic equivalencies

Based on the principle of a common AhR-mediated mechanism of action among many of the PCBs, dioxins, and furans, an approach was developed that attempted to simplify the risk assessment of complex environmental mixtures of contaminants. In this approach, PCBs, PCDDs, and PCDFs are compared according to their binding affinity to the AhR, relative to that of TCDD. TCDD, which has the highest known affinity to AhR, has been given a toxic equivalency factor (TEF) of 1.0, and the potency of other compounds, as determined in laboratory experiments, decreases accordingly. TEFs are multiplied by concentrations measured in a sample to produce toxic equivalency quotients or TEQs, that combined, can estimate the overall toxicity of the mixture of total dioxin-like compounds (Table 2).

Table 2. Toxic Equivalency Factors (TEFs) for mammals and birds.

	Structure (PCB IUPAC #)	Mammalian TEFs	Avian TEFs
PCDDs	2,3,7,8-tetraCDD	1.0	1.0
	1,2,3,7,8-pentaCDD	1.0	1.0
	1,2,3,4,7,8-hexaCDD	0.1	0.05
	1,2,3,6,7,8-hexaCDD	0.1	0.01
	1,2,3,7,8,9-hexaCDD	0.1	0.1
	1,2,3,4,6,7,8-heptaCDD	0.01	<0.001
	octaCDD	0.0001	0.0001
PCDFs	2,3,7,8-tetraCDF	0.1	1.0
	2,3,4,7,8-pentaCDF	0.5	1.0
	1,2,3,7,8-pentaCDF	0.05	0.1
	1,2,3,4,7,8-hexaCDF	0.1	0.1
	2,3,4,6,7,8-hexaCDF	0.1	0.1
	1,2,3,6,7,8-hexaCDF	0.1	0.1
	1,2,3,7,8,9-hexaCDF	0.1	0.1
	1,2,3,4,6,7,8-heptaCDF	0.01	0.01
	1,2,3,4,7,8,9-heptaCDF	0.01	0.01
	octaCDF	0.0001	0.0001
Non-ortho PCBs	3,3',4,4'-tetraCB (77)	0.0001	0.05
	3,4,4',5-tetraCB (81)	0.0001	0.1
	3,3',4,4',5-pentaCB (126)	0.1	0.1
	3,3'4,4',5,5'-hexaCB (169)	0.01	0.001
Mono-ortho PCBs	2,3,3',4,4'-pentaCB (105)	0.0001	0.0001
	2,3,4,4',5-pentaCB (114)	0.0005	0.0001
	2,3',4,4',5-pentaCB (118)	0.0001	0.00001
	2',3,4,4',5-pentaCB (123)	0.0001	0.00001
	2,3,3'4,4',5-hexaCB (156)	0.0005	0.0001
	2,3,3'4,4',5'-hexaCB (157)	0.0005	0.0001
	2,3',4,4',5,5'-hexaCB (167)	0.00001	0.00001
	2,3,3',4,4',5,5'-hepatCB (189)	0.0001	0.00001

Taken from Van den Berg et al., 1998.

THE IMMUNE SYSTEM AS A TARGET OF PCB-ASSOCIATED TOXICITY

The immune system is highly sensitive to PCB-associated toxicity

The immune system is considered to be one of the most sensitive targets of PCB-associated toxicity (Birnbaum, 1995). This can, in part, be explained by AhR, the primary mediator of PCB-associated immunotoxicity, playing an important role in normal developmental processes within the immune system. In addition, AhR expression is characteristically high in immune system organs, such as the thymus and spleen, and on certain immune cell types. The following section will briefly describe the immune system of mammals, and marine mammals in particular. I will also review current knowledge of PCB-associated immunotoxicity in laboratory animals, humans, and marine mammals.

The immune system of mammals

The function of the immune system is to provide protection against pathogens (bacteria, viruses, and parasites). Due to its importance to survival, an immune system of some kind is present in all animals. Whereas this is limited to the ability to distinguish between self and non-self and cells that can remove foreign particles by phagocytosis in invertebrates, vertebrates have evolved a complex immune system where functions are divided among various cell types (Goldsby et al., 2000).

The vertebrate immune system consists of two primary components: the highly specific adaptive immune system, and a non-specific innate immune system. The immune cell types that are the principal players in the two systems develop from pluripotent stem cells. In humans, hematopoiesis begins in the embryonic yolk sac during the first weeks of development. In the third week of gestation, hematopoietic stem cells migrate into the fetal liver and then to the spleen. After the seventh month of gestation, the bone marrow becomes the primary site of immune cell development. Regulated by cytokines that stimulate the hematopoietic stem cells to proliferate and

differentiate, myeloid and lymphoid stem cells functionally separate. The myeloid lineage gives rise to monocytes, eosinophils, basophils, and the cells responsible for red blood cell and platelet production (erythroid progenitors and megakaryocytes). The lymphoid stem cells differentiate into Natural Killer (NK) cells, B lymphocytes, and pre-T lymphocytes (also called T progenitors or thymocytes). The pre-T lymphocytes migrate from the bone marrow to the thymus for further developmental processes (Goldsby et al., 2000; Roitt, 1990).

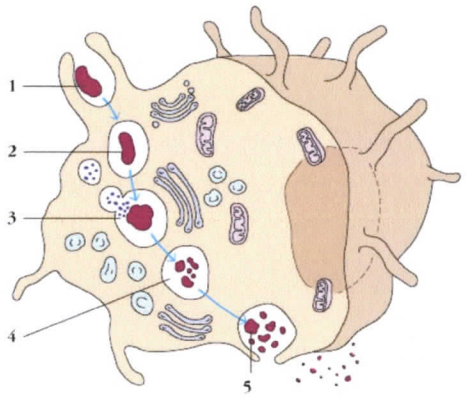
In mammals, the thymus is a primary tissue of the immune system by providing the microenvironment for maturation of thymocytes into T-lymphocytes during early life (Von Boehmer, 1988). During development, thymocytes pass through discrete phenotypic stages that can be identified by various cell surface proteins, and undergo gene rearrangements that produce the T cell receptor, while migrating through the thymus from cortex to medulla. Positive and negative selection shapes the final repertoire of T lymphocytes. All of the processes that thymocytes undergo depend upon the paracrine (hormones, chemokines and cytokines) influences of, and cell-cell interactions with, the several cell types present in the thymic microenvironment. The majority of mature T lymphocytes are released into the periphery during the prenatal period (in humans; extended into the postnatal period in mice) (Von Boehmer, 1988), and the thymus becomes less importance after birth.

Adaptive and innate immune responses

The innate immune system provides a first line of defence by means of anatomic (preventing the entry of pathogens by e.g. the skin and mucous membranes) and physiological barriers (such as increased body temperature during fever preventing the growth of some pathogens and low pH in the stomach). It also includes various cell types that are able to respond to specific pathogens in a non-specific manner. These

include cells that internalize (or phagocytose) foreign particles within an extravascular system (neutrophils, and to a lesser extent, monocytes) and in tissues (macrophages). After the engulfment process, phagocytic cells are able to destroy micro-organisms using a variety of mechanisms, including the conversion of oxygen into reactive oxygen intermediates (respiratory burst), the synthesis of nitric oxide, and the release of proteins from their granules (Figure 3). Such cell types are especially efficient against invading bacteria. Another type of cells, eosinophils, are able to attack micro-organisms that are too large in size to phagocytose, such as parasites, by releasing molecules that damage the integrity of the parasitic membrane. NK cells, a type of lymphoid cells that are also considered part of the innate immune system, respond to intracellular pathogens (such as viruses) by the release of molecules that induce apoptosis in the target cell.

Figure 3. Phagocytosis as an important mechanism of innate immunity.
Phagocytic cells play an important part in innate immunity by engulfing and destroying foreign particles in circulation (neutrophils and monocytes) and in tissues (macrophages). Uptake is mediated by the formation of pseudopodia that will surround the particle (1), and subsequently form a phagosome (2). Phagosomes fuse with intracellular lysosomes containing lysosomal enzymes and oxidizing agents (3) that will damage and digest the ingested particle (4). The remaining products are released from the cell (5).



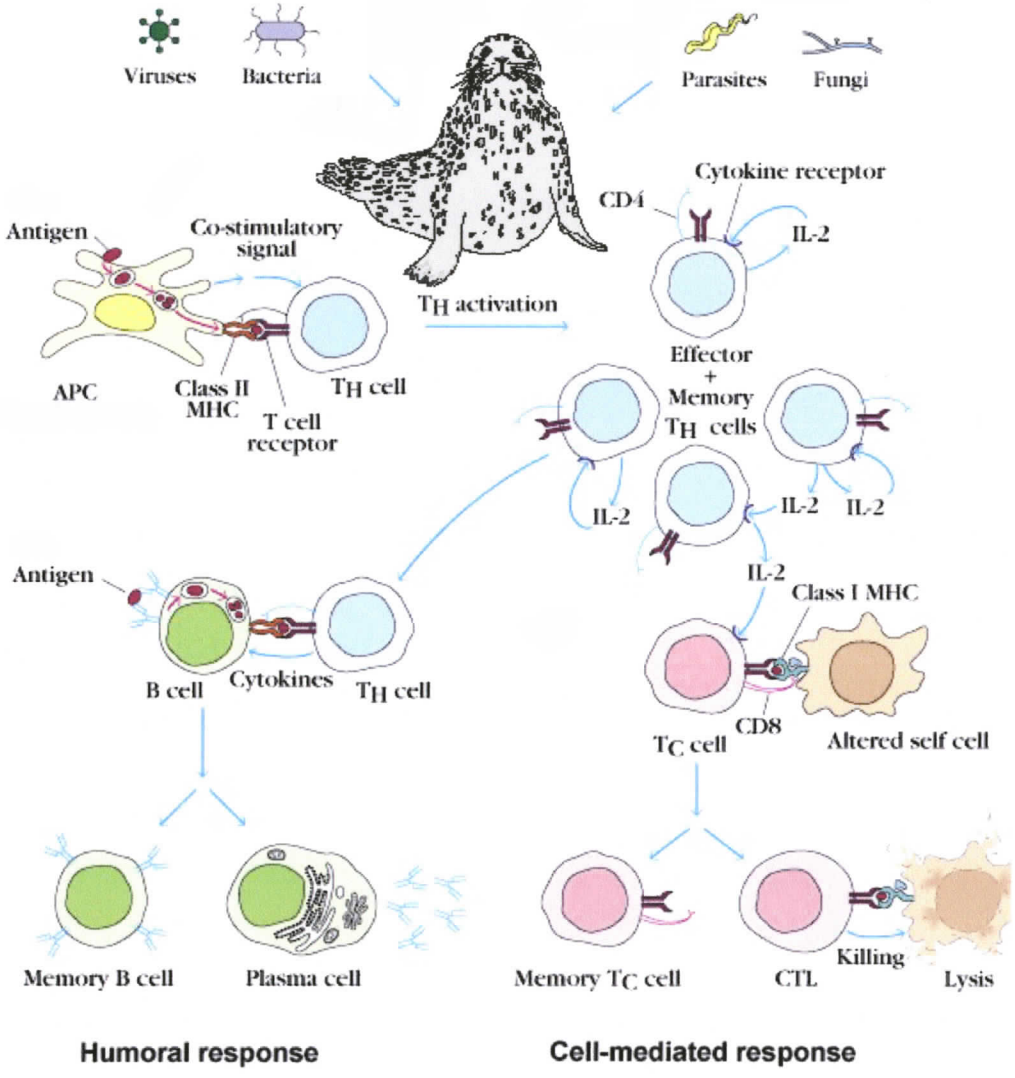
Taken from Goldsby et al., 2000.

The adaptive immune system (Figure 4) is able to respond to specific antigens or foreign molecules by displaying antigen specific-binding, cell surface receptors. In the case of B lymphocytes, this receptor consists of a membrane-bound antibody molecule (Ig receptor) and in case of T lymphocytes of a T cell receptor (TCR). When a naïve lymphocyte encounters an antigen that binds its receptor, the cell proliferates rapidly (clonal expansion). B lymphocytes directly expand in number and differentiate into antibody-secreting plasma cells after antigen binding to their receptor. T lymphocytes are employed in a more complicated manner. The TCR of T lymphocytes only binds to antigen presented in the context of major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells. This occurs in combination with a costimulatory signal from its accessory receptor (CD4 on T helper and CD8 on T cytotoxic lymphocytes). However, in some cases, clonal expansion of lymphocytes can take place independently of a specific antigen by stimulation with mitogens. This property has been extensively used in studies of adaptive immune system functioning. Concavalin A (ConA, a lectin) and lipopolysaccharide (LPS, a major component of (gram negative) bacterial cell walls) are examples of mitogens that induce a proliferative response of T and B lymphocytes, respectively.

T and B lymphocytes are especially important in the defence against viral and bacterial pathogens. T lymphocytes, in particular, are also of the greatest importance in the regulation of innate and adaptive immune responses. T lymphocytes produce and secrete cytokines, which are low molecular weight regulatory (glyco)proteins, that influence the direction of the immune response as well as its intensity and duration. Thereby, T lympochytes influence the activities of an entire network of interacting cells, including themselves (interleukin-2 (IL-2)), B lymphocytes (IL-4, -6, and –10), NK cells, macrophages, granulocytes, and hematopoietic stem cells (IL-3).

Figure 4. Overview of the adaptive immune system of vertebrates.

Pathogens invading the vertebrate body will be engulfed and digested by antigen-presenting cells (APC; macrophages, dendritic cells, B lymphocytes), followed by the presentation of antigen in the context of class II MHC to T helper (T_H) lymphocytes. T_H cells, using the T cell receptor and CD4 co-receptor, are subsequently activated and start to synthesize and secrete interleukin-2 (IL-2). IL-2 is an important lymphocyte growth factor that induces proliferation of the activated T lymphocyte clones. Activated T lymphocytes further support humoral or cell-mediated responses by contact-dependent cell stimulation as well as the secretion of specific cytokine profiles. These include IL-4, IL-5, and IL-6 in case of a humoral response, and IL-2 and IFN-γ in case of a cell-mediated response.



Adapted from Goldsby et al., 2000.

Marine mammal immunology

Of the 33 species of pinnipeds and 81 species of cetaceans, only a few have been the subject of immunological studies. These include primarily phocid seals such as the harbour, grey (*Halichoerus grypus*) and northern elephant (*Mirounga angustirostris*) seals. Based on this limited knowledge of marine mammal immunology, the animals seem to be similar to other mammals in most aspects of immune anatomy, function, and ontogeny. For example, all primary immune system organs (thymus, spleen, lymph nodes) and cell types (e.g. B and T lymphocytes, NK cells, leukocyte subpopulations, and phagocytic cells) are found in marine mammals. In addition, a number of characteristic cell surface markers present on immune cell types, as recognised in humans and mice, have been described (CD3, CD45, MHC, and Ig). Furthermore, marine mammal antibodies occur in at least three immunoglobulin classes similar to humans, namely IgG, IgM, and IgA, and are shaped alike with heavy and light chains. Consequently, the immune responses to pathogens are also similar in marine mammals to other mammals (reviewed in Ross & De Guise, 2005).

The similarities between marine mammals and other mammal species support the current understanding that many immunological processes can be extrapolated to marine mammals from humans and mice, and have allowed the modification and application of certain standard immunological testing methods in marine mammals. Blood samples of pinnipeds and cetaceans have successfully been used for cell counts, the identification of certain cell subpopulations, and in assays of cell functionality, whereas serum and milk samples have been quantified for antibody contents (Ross & De Guise, 2005).

More recent are the advances in the characterisation of the marine mammal immune system and its components at the molecular level. By use of polymerase chain reaction (PCR) assays, and cloning and sequencing techniques, efforts have been made to

understand MHC diversity in marine mammals, measure cytokine profiles, and produce recombinant marine mammal interleukins (King & Stott, 2002). This information has largely impacted the field of marine mammal medicine (e.g. rehabilitation centres). Moreover, molecular studies have demonstrated that pinnipeds and sea otters are most similar to terrestrial carnivores (members of the Canidae and Mustelidae families), while cetaceans are similar to ruminants (Bovidae and Ovidae) with respect to their immune systems (King & Stott, 2002).

Whereas many examples of similarities between the immune system of marine mammals and that of other mammals can be mentioned, little information exists on the differences. However, it is likely that small aberrations exist. For example, some marine mammals, such as many of the pinniped species, are precocious with a short nursing period and limited maternal care. It has been shown that this is reflected in the immune system of seal pups: as an adaptation to circumstances, newborn seals are more immunocompetent than for example newborn cats and dogs. For example, circulating antibody concentrations increase more rapidly (originating in the colostrum), and lymphocytes are more responsive (proliferating at a higher rate after mitogen exposure than their mothers) (Ross et al., 1993; Ross et al., 1994) in the seal pup than would be expected.

Assessing immunotoxicity associated with exposure to chemicals

The immune system is considered to be sensitive to chemicals. Prior to use, new chemical pesticides and pharmaceuticals are commonly evaluated for immunotoxicity using standard testing methods. Although small differences exist among countries and regulatory agencies, the assessment of immunotoxicity traditionally follows the tiered approach. The tiered approach to testing includes a number of endpoints, reflecting the complexity of the immune system, of both a pathological and a functional nature, and is carried out in mice (United States) or rats (Europe). In tier 1, the effects of the chemical, at a dose not producing overt toxicity, on immune system integrity are investigated. Compounds that are found to be immunotoxic in tier 1 are further evaluated to better define the toxic effects, in addition to the consequences these effects may have on the immune response to infectious agents or tumors (host resistance). The tier 2 tests depend on the nature of the findings in tier 1 assays (Anonymous, 1996; Karol, 1998; Table 3). This approach has been adapted in the testing of environmental contaminants for immunotoxic properties.

Table 3. The tiered approach to immunotoxicity testing.

Tier 1	Hematology	Total white blood cell count Differential white blood cell counts
	Thymus/spleen weights	
	Thymus/spleen cellularity	
	Lymphoid organ histology	
	IgM antibody response	Plague-forming assay (in response to SRBC)
	Lymphocyte response to mitogens	T lymphocyte proliferation in response to ConA or PHA B lymphocyte proliferation in response to LPS
	Lymphocyte response to allogeneic cells	Mixed lymphocyte response
	NK cell activity	
Tier 2	Splenic (T and B) lymphocyte count	Enumeration of cell types by surface markers
	IgG antibody response	Plague-forming assay
	Delayed-type hypersensitivity	
	Host resistance- tumor models	PYB6 sarcoma/B16F10 melanoma cells (syngeneic); requires T lymphocyte and NK cell activity
	Host resistance-bacterial models	*Lysteria monocytogenes*; requires antibodies and complement
	Host resistance-viral models	Influenza; requires antibodies and interferon
	Host resistance-parasite models	*Plasmodium yoellii*; requires T and B lymphocytes and macrophage activity

PCB-associated immunotoxicity in laboratory animals

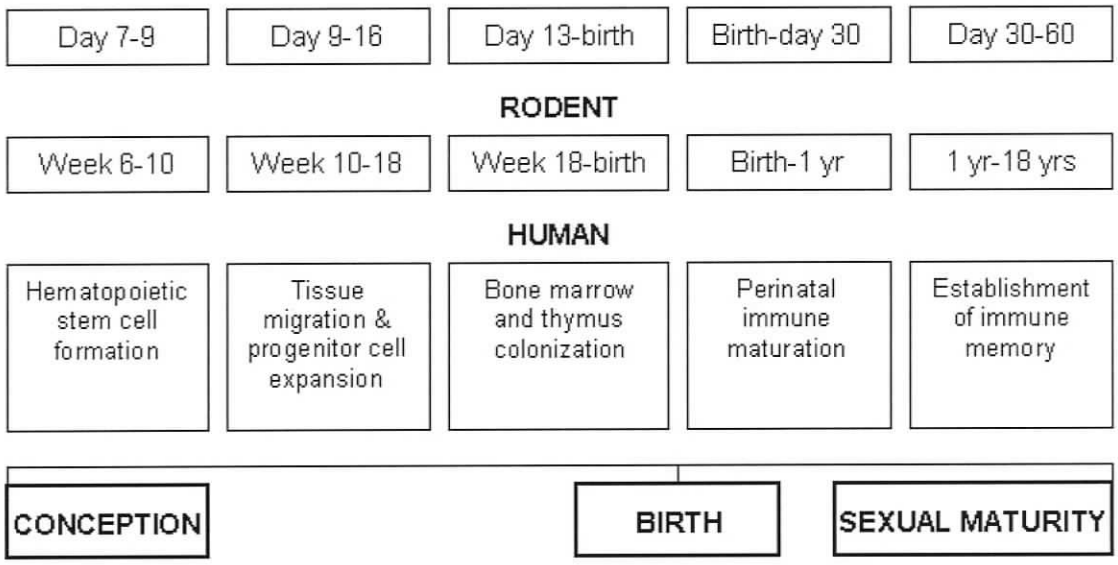
Laboratory studies have documented a number of immunotoxic effects associated with PCBs (Smialowicz et al., 2000; Agency for Toxic Substances and Disease Registry, 2000; Holsapple et al., 1991; Kerkvliet & Burleson, 1994). Experimental exposure of rats, mice, guinea pigs, rabbits, and monkeys have showed that acute, intermediate, and chronic exposure to PCB mixtures can lead to morphological and functional alterations of the immune system. Effects observed in these species typically include reduced thymus and spleen weights, cortical atrophy and reduced germinal centers in the thymus, spleen, and lymph nodes, altered T lymphocyte subsets, decreased proliferative responses of lymphocytes in response to mitogens and antigens, suppressed antibody responses, and reduced NK cell activity. Although the number of circulating lymphocytes seems to decrease consistently following PCB exposure, hematological changes in general do not appear to be a clear effect of PCBs.

PCB-associated toxicity has the most pronounced effects on immunocompetence when exposure takes place *in utero* and/or over the course of lactation, in which case exposure coincides with critical windows for the development of the immune system (Holladay & Luster, 1994; Birnbaum, 1995; Figure 5). Monkeys exposed gestationally and lactationally were immunosuppressed at PCB (Aroclor 1254) doses as low as 0.005 mg/kg/day (Tryphonas et al., 1991a). The developmental processes of the adaptive immune system, taking place in the thymus, are likely the most sensitive to toxic effects. Within the thymus, PCBs have been shown to affect the number of cells surviving the selection processes, partly by induction of apoptosis, and to change development by decreased cell-cell contact and concentrations of certain hormones and interleukins (Fine et al., 1990; McConkey et al., 1988; De Waal et al., 1992).

The immunotoxic effects resulting from PCBs exposure have been associated with an increased susceptibility to, and mortality from, bacterial and viral infection. As well,

increased invasion by parasitic organisms have been noted in host resistance experiments in laboratory rodents (House et al., 1990; Luebke et al., 1994; Thigpen et al., 1975). These findings indicate that exposure to PCBs leads to an increase in susceptibility to disease.

Figure 5. Critical windows of PCB exposure for immune system development.
If PCB exposure takes place during sensitive life stages, such as certain windows of immune system development, this can result in life-long damaging effects even at low doses of exposure. These windows coincide in even distantly related mammal species, as shown here for rodents and humans, due to the extremely high level of conservation in immune system functioning and ontogenesis.



Taken from Holsapple et al., 2004.

PCB-associated immunotoxicity in humans

Immunotoxicity has been documented in humans after accidental and environmental exposure to PCBs and structurally-related compounds. The most conclusive findings in humans came from the Yusho and Yu-Cheng populations that experienced a high level of exposure through the consumption of rice oil contaminated with polychlorinated compounds (PCBs and PCDDs) in 1968 and 1979. Immunological effects that were observed included decreased serum antibodies (IgA and IgM), decreased circulatory T lymphocytes in white blood cell counts, alterations in T lymphocyte subsets, and decreased proliferative responses of lymphocytes in response to mitogens (Chang et al., 1981; Lu & Wu, 1985). In addition, children of Yu-Cheng mothers were reported to have increased incidences of infectious diseases, including influenza, pneumonia, bronchitis, and middle ear infections (Yu et al., 1998; Rogan et al., 1988; Chao et al., 1997).

Observations in children exposed to background levels of PCBs pre- (*in utero*) and postnatally (*via* breastfeeding) further underline the sensitivity of the immune system. In Dutch pre-school children, higher prenatal exposure was associated with alterations in T lymphocyte subsets and subtle changes in the number of circulatory white blood cells at the age of 18 months (Weisglas-Kuperus et al., 1995). In a follow up study, at 3½ years of age, antibody levels against measles and mumps resulting from vaccination were significantly lower in children from mothers with higher PCB body burdens. In addition, recurrent ear infections and chicken pox were more prevalent in those children (Weisglas-Kuperus et al., 2000).

The overall consistency of PCB-associated immunotoxic effects and increased susceptibility to disease documented in humans, and the low concentration at which they take place, has raised concerns for the health of other human populations. In particular subsistence-oriented consumer groups and sports fishermen may be exposed to elevated concentrations of PCBs through environmental contamination by the

consumption of large amounts of fish and marine foods (Dewailly et al., 1994; Mos et al., 2004). For example, a study of Canadian Inuit mothers whose diets were based on marine mammal fat showed decreased serum antibody levels, changes in T lymphocyte subsets, and increased prevalence of middle ear infections, similarly to Yusho and Yu-Cheng patients (Dallaire et al., 2004; Dewailly et al., 2000). This is relevant as those consumers occupy a niche in the marine ecosystem similar to high trophic-level wildlife, and implies that PCBs can affect the immune system of species dependent on marine food webs.

Indications of PCB-associated immunotoxicity in marine mammals

POPs and PCBs have been associated with adverse health effects in marine mammals. These effects include reproductive failure in populations of highly contaminated California sea lions (*Zalophus californianus*), ringed (*Phoca hispida*), harbour and grey seals (Hutchinson & Simmonds, 1994). In addition, there are negative associations with circulatory concentrations of hormones such as vitamin A, thyroid hormone, and testosterone, and PCBs in harbour seals and Pacific Dall's porpoises (*Phocoenoides dalli*) (Subramanian et al., 1987; Brouwer et al., 1989). In the Baltic Sea, grey and harbour seals were observed in the 1990s to have skull deformations compared to specimens from before the 1960s (Bergman et al., 1992; Mortensen et al., 1992). These malformations were suggested to result from developmental toxicity of PCBs Finally, a high incidence of tumors in beluga whales (*Delphinapterus leucas*) was related to the high concentrations of PCBs and other environmental contaminants present in the Gulf of St. Lawrence in Canada (De Guise et al., 1994). With respect to immunotoxicity, evidence from marine mammals is scarce. However, the occurrence of a large number of mass mortalities among marine mammals in industrialised coastal areas

has led to concerns that PCBs also have had effects on the immune systems of marine mammals in the affected populations (Dietz et al., 1989b; Osterhaus et al., 1995).

Immunotoxicological studies of marine mammals often consist of information biased by age and condition. For example, a study into a Mediterranean Sea dolphin mortality documented large differences in PCB concentrations between victims and survivors of the event (Aguilar & Borrell, 1994). However, during disease, the condition of the animal is likely to suffer, and the consequential loss of body fat will concentrate the contaminants. An effort elsewhere documented correlative evidence of PCBs or DDT affecting immune function in a small sample size (n=5) of bottlenose dolphins (Lahvis et al., 1995). In this case, sex and age of the animals were unknown. Whereas females generally have higher immune responses than males, they also offload contaminants to their young, which may have lead to the author of the study to believe that lower contaminant concentrations are associated with higher immunocompetence.

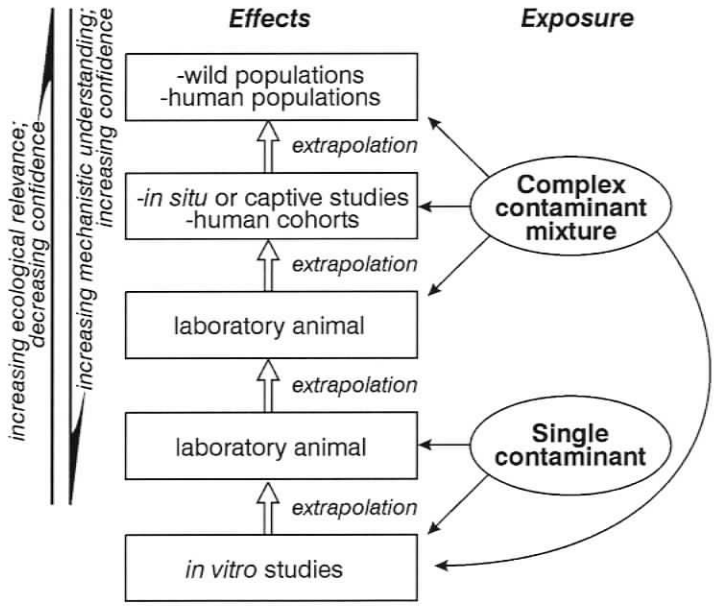
More convincing evidence was obtained through a semi-field study using harbour seals. In this 2 year study, seals were fed either herring from the contaminated Baltic Sea or herring from the cleaner Atlantic Ocean. Seals in the Baltic group developed impaired immune responses compared to those in the Atlantic group. Impairments included diminished proliferation of lymphocytes in response to mitogens and antigens (De Swart et al., 1995), reduced natural killer cell activity (Ross et al., 1996b), and diminished delayed-type hypersensitivity responses (Ross et al., 1995). When a similar diet was fed to laboratory rats, a number of the immunotoxic effects could be reproduced (Ross et al., 1997; Ross et al., 1996d), underscoring the commonality of effects between species. Chemical analysis of the two fish diets revealed that the majority of the contaminants were PCBs, and that the immune alterations could likely be attributed to those contaminants (Ross et al., 1996a).

Weight of evidence approach in immunotoxicological studies of marine mammals

Assessments of immunotoxicological health risks in marine mammals have been impeded by the fact that a tiered approach is ethically unacceptable in these animals. Free-ranging marine mammals are exposed to complex mixtures of environmental contaminants, as well as influenced by a number of natural factors that could affect immunocompetence. Under similar scenarios in regulatory assessments of human health risks, a weight of evidence approach, a conclusion based on multiple lines of evidence, is as an alternative to causal evidence.

A similar approach could be used in risk assessments concerning immunotoxicity in marine mammals (Ross, 2000). The basis for this approach is that the conserved nature of the immune system and the mechanism of toxicity through the AhR allow inter-species extrapolation of immuntoxicity among mammals. The weight of evidence suggesting that immunotoxicity affects marine mammals has so far included *in vitro* studies, experiments of laboratory rodents, semi-field studies of marine mammals, and human cohorts (Figure 6), but would benefit from additional evidence from immunotoxicological and biomarker studies of otherwise healthy free-ranging individuals.

Figure 6. Weight of evidence approach in marine mammal immunotoxicology.
The basis for a weight of evidence approach to studies of immunotoxicology in marine mammals is based on (1) the conserved nature of immune system development and function among mammals, and (2) the common mechanism of PCB-associated immunotoxicity mediated through AhR (although interspecies differences in sensitivity should be under careful consideration). *In vitro* and *in vivo* studies with laboratory animals, exposed to single contaminants and environmental mixtures, have been extensively carried out, and provided a basis of causative evidence. A single captive study of harbour seals, as well as a number of human cohorts that have been followed over periods of time and focussed on immunotoxic endpoints, have been shown to be consistent with these laboratory studies. However, studies of free-ranging marine mammals are scarce. Such studies would contribute significantly to the weight of evidence of PCB-associated immunotoxicity in environmentally exposed marine mammals.



Taken from Ross & Birnbaum, 2002.

RESEARCH QUESTIONS

Are PCBs affecting immune function in free-ranging Pacific harbour seals?

Hypothetically, exposure to PCBs, especially during developmental processes of the immune system, may affect immunocompetence throughout life. In turn, this would impair host resistance, and increase the prevalence and severity of disease. Marine mammals may be particularly vulnerable to the effects of PCBs since they carry elevated body burdens of contaminants.

In the study described in this thesis, the immune status of a large group of free-ranging harbour seals was assessed using measures of immune function, comparable in part to those commonly used in a tiered approach to assess immunotoxicity in laboratory animals. Harbour seals were selected as a study species because of the characteristics of this species consistent with factors of importance for the selection of an indicator species (reviewed in Basu et al., 2006; Table 4). This study focused on seals in coastal British Columbia (BC), Canada, and Washington State (WA), USA, in which a naturally existing gradient of contaminant concentrations was previously documented (Ross et al., 2004). Harbour seal pups were selected, to eliminate confounding effects of age, and minimize differences of sex due to low concentrations of steroid hormones in young animals.

Table 4. Characteristics of a sentinel species.

Widespread distribution
Maintained and studied in captivity
Well-known biology
Captured in sufficient numbers
Restricted home range
High trophic status
Ability to bioaccumulate contaminants
Sensitive to contaminant-related toxic effects

Taken from Basu et al., *in press.*

Can biomarkers serve as indicators of PCB-associated toxicity?

Documenting immunotoxicity in environmentally exposed, free-ranging healthy marine mammals remains extremely difficult. Many marine mammal species inhabit remote areas, are difficult to approach and capture in large numbers in order to obtain an adequate sample size, and ethical constraints hamper collecting immune tissues from free-ranging mammals. However, approaches in toxicology that rely on biomarkers, i.e. physiological, biochemical or molecular measurements that indicate toxic exposure and/or effect, could provide a means to assess the immunological health of marine mammals.

Although biomarkers have been successfully applied in wildlife toxicology to document endocrine disruption, none has so far been developed to assess immunotoxicity. Within the immune system, cell signalling molecules directly affected by PCBs, as well as a number of hormones that are disrupted by PCB exposure, could potentially indicate adverse effects on the immune system. In this study, three biologically relevant and potentially sensitive biomarkers were investigated, namely (1) AhR, (2) thymic hormones, and (3) vitamin A. As discussed earlier, AhR plays a primary role in mediating PCB-associated immunotoxicity. Thymic hormones were selected on the basis that toxic effects have been most extensively described in the thymus (characterised by high AhR expression and high sensitivity to AhR-binding compounds). Although the thymus itself is inaccessible in free-ranging marine mammals, its endocrine products could represent sensitive biomarkers of its activity. Thymic hormone concentrations in relation of contaminants, however, have been scarcely investigated in mammals, and, in addition, most of the thymic hormones have not been individually characterized in marine mammals. Lastly, circulatory concentrations of vitamin A represents one of the most frequently used biomarkers in wildlife studies of PCB-associated health effects, due to its combined physiological importance and its sensitivity to PCB-associated toxic effects

(Simms et al., 2000; Bishop et al., 1999; Elliott et al., 2001; Jenssen et al., 1995; Jenssen et al., 1995; Murk et al., 1998; Palace et al., 2001; Rolland, 2000; Spear et al., 1989; Spear et al., 1986). Although vitamin A is commonly considered to represent a biomarker of general toxicity, it functions in many immunological processes. For example, vitamin A maintains epithelial integrity to prevent pathogen entrance, and functions as a cofactor in hematopoiesis, processes of lymphocyte development, activation and proliferation, enhancement of NK cell activity and antibody secretion, and terminal differentiation and induction of apoptosis in neutrophils (Collins, 2002; Blomhoff, 1994; Allende et al., 1997; Ertesvag et al., 2002; Jiang et al., 1993; Ross & Hämmerling, 1994; Ross & Stephensen, 1996; Mehta, 2002). Therefore, this dietary hormone is relevant to the objective of the study.

Objectives

The objectives of this study were to assess the relationship between current environmental concentrations of PCBs and immunotoxicity in free-ranging harbour seals from British Columbia, Canada, and Washington State, USA, by:

- Measuring current levels of POPs in harbour seals, with special focus on PCBs
- Assessing immune function and correlating it with ambient contaminant levels in the free-ranging harbour seals of British Columbia and Washington State. I propose using traditional measures of immunotoxicity, including hematology and functional assays of innate and adaptive immune function.
- Carrying out a parallel *in vitro* study to the *in vivo* immune function measures, using harbour seal white blood cells, to validate the mechanism of action by which immunotoxic effects take place

- Developing sensitive biomarkers of immunotoxicity, consisting of physiological and molecular measurements. I propose using AhR expression, thymic hormone concentrations, vitamin A concentrations and receptor expression in harbour seals.

This information would contribute to the weight of evidence of immunotoxicity in free-ranging marine mammals and provide novel biomarkers that could be applied in future studies of other marine mammal species.

CHAPTER 2

Persistent Organic Pollutants in Pacific harbour seals (*Phoca vitulina*)

Much of the work presented in this chapter was obtained in a collaborative effort. It appears in both this thesis and the thesis of Maki Tabuchi (University of Victoria), and the authors claim equal contribution to this work.

INTRODUCTION

POPs are ambiguous environmental contaminants that, due to their persistence and lipophilicity, bioaccumulate in food chains. Marine mammals, predatory birds, and other species occupying a high trophic level are exposed to elevated levels of these compounds. Considering that many POPs are toxic, these compounds may represent a threat to the health of high trophic wildlife. A number of wildlife reports have documented the adverse health effects of POPs, of which eggshell thinning in bald eagles, reproductive failure in harbour seals and mink, and behavioural changes in gull species are examples (Lundholm, 1997; Harding et al., 1999; Reijnders, 1986; Hunt & Hunt, 1977).

Assessing the effects of POPs on the health of wildlife has remained difficult due the highly complex mixtures in which these contaminants circulate in the environment. The POP mixtures may contain up to 209 PCB congeners, 75 PCDD congeners, 135 PCDF congeners, as well as a number of organochlorine (OC) pesticides. Based on structure, OC pesticides can be divided into at least four groups: (1) dichlorodiphenylethanes, including DDT and methoxychlor; (2) cyclodienes, including chlordane, endosulfan, heptachlor, endrin, dieldrin, and aldrin; (3) chlorinated benzenes and cyclohexanes, including HCH; and (4) chlordecones, including Mirex (Klaassen, 2001).

PCBs, PCDDs, and PCDFs exert most of their toxic effects, especially in the case of the immune system, through a common mechanism of toxicity initiated through AhR binding. For many of the OC pesticides, with their considerable differences in structure, a mechanism of action remains to be elucidated. However, in laboratory studies that have tested the individual OC pesticides, it has been established that a number of them can have immunotoxic effects. These include, among others, DDT, chlordane, endosulfan, and endrin (Agency for Toxic Substances and Disease Registry (ATSDR)).

In this study, we characterised the concentrations of PCBs and OC pesticides in harbour seal pups from British Columbia, Canada, and Washington State, USA. The objective was to (1) to characterise and rank POPs in seals; (2) to compare analytical methods that quantify a subset of PCB congeners versus a full congener analysis in studies of toxicological effects.

MATERIALS & METHODS

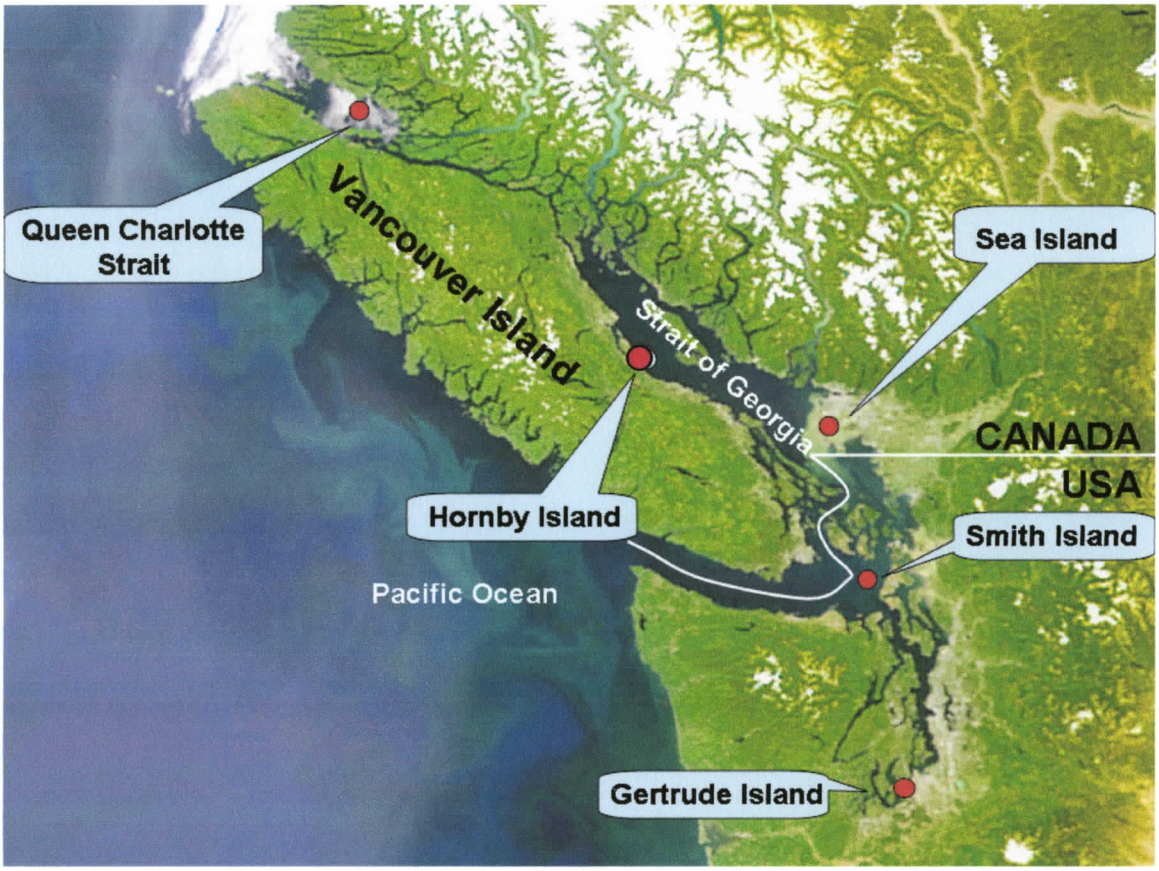
Site characterization and sampling of harbour seals

Pacific harbour seals (*Phoca vitulina richardsi*), aged 3-5 weeks, were captured at five sites in BC and WA (Figure 7), using two different techniques. Firstly, individual seal pups in rocky intertidal areas, Queen Charlotte Strait (BC; n=10; 50° 40' N, 126° 46' W) and Hornby Island (BC; n=8; 49° 30' N, 124° 35' W), were captured by hand using a selective and rapid approach by small craft and a salmon-landing net (Cottrell et al., 2002). Seals hauled-out on the mud flats of Sea Island (BC; n=8; 49° 12'N, 123° 9' W), Smith Island (WA; n=8; 48° 19' N, 122° 49' W) and Gertrude Island (WA; n=8; 47° 13' N, 122° 35' W) were captured in groups, using a large beach seine net (Jeffries et al., 1993). Seals were manually restrained while sampling. After sampling, pups were released at the capture site.

All handling and sampling procedures described in this thesis were in accordance with Canadian Council of Animal Care guidelines, and were carried out under the auspices of the Animal Care Committees and Scientific Research Permits for researchers in British Columbia (Fisheries and Oceans Canada Animal Care Committee; Fisheries and Oceans Canada Scientific Research Permit; University of Victoria Animal Care Committee protocol # 036-03), and Washington State (United States Marine Mammal Protection Act Permit No. 835).

Figure 7. Harbour seal sampling locations.

Harbour seal pups were sampled in five island locations in British Columbia, Canada, and Washington State, USA, for a study of contaminant-associated adverse health effects. Queen Charlotte Strait represented the most Northern sampling location. Hornby, Sea, and Smith Islands are located in the Strait of Georgia basin. Gertrude Island is in the Southern Puget Sound basin.



Contaminant analysis I: Trent method

Blubber (approximately 100 mg) sampled by use of 8 mm diameter biopsy punches (Acuderm, USA) was analysed for POPs using two methods, namely by the Trent and RDL methods. In the Trent method (carried out by Trent University, Ontario, Canada), a large group of seal pups (n=41 from five locations) was assessed for 34 PCB congeners and 15 OC pesticides. The PCBs analysed included the (mono- and di-ortho) congeners with International Union of Pure and Applied Chemistry (IUPAC) numbers 17, 18, 28, 31, 33, 44, 49, 52, 70, 74, 87, 95, 99, 101, 105, 110, 118, 128, 132, 138, 149, 151, 153, 156, 170, 180, 187, 191, 194, 195, 201, 205, 206, and 209. The OC pesticides were DDT and DDD/ DDE metabolites, chlordane and components, HCH isomers, endosulfan and its metabolites, endrin and metabolites, heptachlor and heptachlor epoxide B, dieldrin, aldrin, mirex, hexachlorobenzene, and metoxychlor. The industrial contaminant octachlorostyrene was also included.

Briefly, blubber samples were ground with sodium sulphate, using a mortar and pestle, and packed into a glass column, for extraction using 50:50 dichloromethane:hexane. Lipids were removed from the extract by gel permeation chromatography (GPC). The GPC procedure was conducted with BioBeads S-X3 (Biorad, Canada) equivalent to 50 gram dry weight in a 3x30 cm glass column with a glass wool plug, and eluted with hexane:dichloromethane (55:45). The eluent was, in part, used for the gravimetric analysis of lipid content of the sample, as well as collected for further sample clean-up for PCB and OC pesticide analysis. Following clean-up, the latter was fractionated by silica-gel column chromatography to yield two extracts: one containing the PCBs and some DDE, and another that contained the OC pesticides and the rest of the DDE. The concentrations of the contaminants were quantified using gas chromatography in combination with electron capture detection (GC-ECD). Quality control and quality assurance followed established laboratory procedures, and consisted

of procedural blanks and a National Institute of Standards and Testing (NIST) reference material. Further details on this method are described in (Metcalfe & Metcalfe, 1997; Metcalfe et al., 2004).

Contaminant analysis II: RDL method

In a subsample of the seals (n=24 from three out of five locations, namely Queen Charlotte Strait, Smith and Gertrude Island) congener-specific analysis of (planar, mono-ortho and di-ortho) PCBs was carried out by the Department of Fisheries and Oceans Regional Dioxin Laboratory (RDL), BC, Canada. Briefly, samples were ground with anhydrous sodium sulfate, spiked with a mixture of ^{13}C-labeled PCBs (Cambridge Isotope Laboratories, USA), and packed onto a glass extraction column. The column was eluted with 1:1 hexane:dichloromethane to extract the lipid fraction. Following sample clean-up procedures, 203 (of 209 with 6 coeluting) PCB congeners were then quantified by high-resolution gas chromatography equipped with high-resolution mass spectrometry detection (HRGC-HRMS). The HRGC consisted of a Hewlett-Packard, model 5890 Series II, with a DB^{TM}-5 (60 m x 0.25 mm, 0.1 μm) column and the HRMS was a VG-AutoSpec-S. In the analyses, a procedural blank, a certified reference material, and one random duplicate sample were included. The percent of lipid was determined using the gravimetric lipid determination by weight of extract method. Further details on sample clean-up, instrumental conditions, and quality control/assurance measures are described elsewhere (Ikonomou et al., 2001).

PCB data analysis for both analytical methods

In both PCB data sets (Trent and RDL), detection limit substitutions were made for congeners that were not detected in cases where ≥70% of the seals had detectable

values assuming the presence of the congener in concentrations below the sensitivity of the method. Congeners detected in ≤70% of the seals were replaced by zero. Contaminant concentrations were subsequently lipid-normalised before further data analysis using Equation 1:

(1) $$Lipid \cdot percentage = \frac{lipid \cdot weight \times 100\%}{sample \cdot weight}$$

The sum of PCBs was calculated from the lipid-normalised and substituted data set by concentration addition. Average concentrations and the standard error of the mean (SEM; the standard deviation of the mean divided by the square root of the sample size), were subsequently calculated.

In the comparison of the PCB analysis of the two analytical laboratories (Trent and RDL), linear regression analysis, using Spearman correlation coefficients and analysis of variance (ANOVA) for significance were used. Correlations were considered significant at $p<0.05$. For comparisons among sampling sites, the Trent data set consisting of all five locations was used. ANOVA followed by Tukey's HSD post-hoc test assessed significant differences between the locations. Outliers, identified as exceeding two times the standard deviation of the mean, were removed from the data set before further analysis.

The RDL data set of all PCB congeners allowed calculation of TEQs, using the most recent World Health Organization TEFs for mammals (Van den Berg et al., 1998), and Equation 2:

(2) $$\sum TEQ = \sum (congener \cdot i / TEF \cdot congener \cdot i) + (congener \cdot ii / TEF \cdot congener \cdot ii)$$

PCB congener patterns were also obtained using the RDL data set, by normalising individual congener concentrations to the concentration of the most persistent PCB congener (PCB153) as shown in Equation 3, after which pattern differences between the sampling locations were calculated by Equation 4:

(3) $$Normalized \cdot [PCB] = \frac{Average(congener \cdot i)}{Average(PCB153)}$$

(4) $$Normalized \cdot PCB \cdot pattern = LOG \frac{Normalized \cdot congener \cdot i \cdot of \cdot location \cdot x}{Normalized \cdot congener \cdot i \cdot of \cdot location \cdot y}$$

Organochlorine pesticide data analysis

Average concentrations and the SEM were calculated for the whole group of animals including those with undetectable concentrations as zero (i.e. no detection limit substitutions were made). The sum of related compounds, and of the parent compound and its metabolites, was calculated by concentration addition for DDT, chlordane, heptachlor, HCH, endosulfan, and endrin. In the case of DDT, a ratio between parent compounds and metabolites was calculated (Equation 5) to assess the importance of originally introduced amounts versus breakdown products. Associations between PCB and OC pesticide concentrations were assessed using Pearson correlation coefficients, since both could be considered independent variables and therefore the rules of normality did not apply. Results were considered significant at $p<0.05$.

(5) $$DDT \cdot ratio = \frac{\sum(DDT)}{\sum(DDD + DDE)}$$

Exposure estimates

Food intake can be considered the primary source of PCB exposure for marine mammals (Humphrey, 1987). In this study, the (weaned) seal pups will have derived their PCB body burden from mother-pup transfer which combines gestation and lactation. Therefore, the estimated daily intake (EDI) or exposure of seal pups from milk can be estimated using the PCB body burden, and respective body weights as a replacement of age (Cottrell et al., 2002; Equation 6). Blubber PCB concentrations

corrected for gestational transfer and dietary uptake efficiency will then estimate dietary intake according to Equation 7.

(6) $Body \cdot weight = 0.418(age) - 2.657$

(7) $\mathrm{EDImilk} = \dfrac{\left[\sum PCBs\right] - 0.04 * \left[\sum PCBs\right]}{Age * Bioavailablity}$

In this calculation, it is assumed that gestational transfer represents less than, or is equal to, 4% of the total PCB body burden (Hickie et al., 2005; Tanabe et al., 1982) and the bioavailability of PCB mixtures in milk is more than 90% (Risk Assessment Information Service (RAIS) accessed at 26/06/2006 at http://risk.lsd.ornl.gov/tox/profiles/Aroclor1254_ragsa.shtml). Exposure to PCBs from the intake of water was excluded, since this is likely to represent less than 1% of the concentrations in the food source, in accordance to the guidance provided by the Canadian Council of Ministers of the Environment (CCME, 1997). In addition, dermal and inhalation exposure can be considered insignificant compared to food intake in case of lipophilic contaminants such as PCBs that are present in foods rather than water or air (Humphrey, 1987).

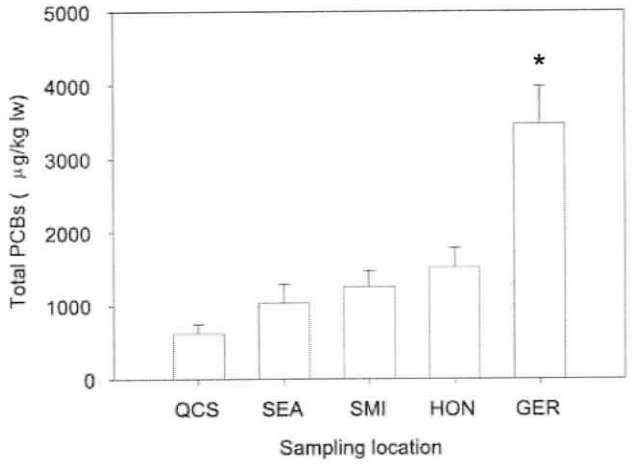
Exposure estimates of the study seal pups were compared to pups that have been weaned and successfully have started to provide themselves with marine foods by use of a harbour seal food basket study that has been described elsewhere (Cullon et al, 2005) and was adapted to our age group of seals for the purpose of this study. Values were available for seals from the Strait of Georgia (including our sampling sites of Sea Island and Hornby Island) and the Puget Sound (including our sampling location Gertrude Island). Comparisons consisted of two scenarios: (1) a comparison of averages, in which an average of the estimated EDI for food intake was divided by the average calculated EDI for milk intake in our seal pups; and (2) a worst-case-scenario, in which the lowest estimated EDI for food intake was divided by the highest calculated EDI for milk intake in our seal pups.

RESULTS & DISCUSSION

PCB concentrations in seals: Trent method

For five locations, Trent University determined PCB concentrations in seal blubber. The sum of PCBs averaged 1509 ± 202 μg/kg in seals from BC and WA (based on 34 congeners; n=41). There were large differences in PCB concentrations among the sampling locations (Figure 8). The lowest concentrations were found in seals from the most northern location, Queen Charlotte Strait (612 ± 130 μg/kg lipid weight), whereas the highest concentrations were observed at the most southern location of Gertrude Island (3614 ± 573 μg/kg lipid weight). Sea, Smith and Hornby Island represented locations of intermediate contamination. This is consistent with earlier observations of seal pups inhabiting this region (Ross et al., 2004; Cullon et al., 2005).

Figure 8. PCB concentrations in blubber of seals.
PCB concentrations (based on 34 congeners) were measured in seals from British Columbia, Canada, and Washington State, USA. The lowest concentrations of PCBs were observed in the Queen Charlotte Strait (QCS; n=10), intermediate in Sea (SEA; n=8), Smith (SMI; n=8), and Hornby (HON; n=8) Islands, and highest in Gertrude Island (GER; n=7; significantly higher than all others, p=0.000).



PCB concentrations in seals: Trent versus RDL method

At three of the five locations, Trent University and RDL carried out contaminant analyses. The RDL method quantified a larger number of PCB congeners (154 PCB peaks out of 209 theoretical congeners were present in seal samples), resulting in overall higher concentrations of total PCBs in the seals (average 2499 ± 581, ranging from 217-11138 μg/kg lipid weight). The two methods are similar when considering the seals from Queen Charlotte Strait and Smith Island (90-95% similar in concentrations), due to the many congeners that had little or no detectable concentrations in the samples examined. The PCB concentrations in Gertrude Island seals as determined by Trent, on the other hand, were significantly underestimating the actual concentrations ($p=0.001$; paired t-test), making up only 59% of their actual total load as determined by RDL (Table 5).

Table 5. Laboratory comparisons of PCB analyses.

PCBs were analysed by two laboratories, RDL and Trent, at three seal sampling locations. The analysis of PCBs in seal blubber by RDL resulted in the quantification of 154 out of the 209 possible PCB congeners. The sum of the concentrations of 154 congeners (Total PCBs ± SEM) was compared to the total PCB concentration based on 34 congeners within the RDL method and with the 34 congeners as analysed by the Trent method. Percentages indicate the portion of representation compared to the RDL method of 154 congeners.

ΣPCBs ($\mu g\ kg^{-1}$ lw)	Queen Charlotte Strait (n=10)	Smith Island (n=7)	Gertrude Island (n=7)
RDL_{154}	683 ± 144	1355 ± 177	6238 ± 1008
RDL_{34}	534 ± 118 (77%)	1071 ± 141 (79%)	4802 ± 763 (77%)
$Trent_{34}$	612 ± 130 (90%)	1256 ± 214 (95%)	3614 ± 573 (59%)

The Trent and RDL methods of PCB analysis were further compared at the level of individual seals. Total PCB concentrations as analysed by Trent correlated significantly with the total PCB concentrations as determined by RDL. In addition, this relationship persisted when the RDL data set was reduced to the 34 congeners similar to Trent (Figure 9). Using the RDL analysis, the toxic equivalency to TCDD of the PCBs (PCB-TEQ) was calculated, to assess whether the Trent analysis also accurately reflected the "dioxin-like" PCB-associated toxicity. The Trent PCB concentrations were significantly correlated to the PCB-TEQ (Figure 10), suggesting that although a PCB-TEQ cannot be calculated using the 34 (mono- and di-ortho) congeners analysed by Trent, they can be used as a measure of relative exposure and toxicity, similar to that predicted by a TEQ value.

Figure 9. Correlation analysis of two methods for PCB analysis.
Total PCB concentrations were compared among Trent and RDL using the sum of 34 identical congeners for both methods (left) and the sum of 34 versus 154 congeners for Trent and RDL, respectively (right). Trent consistently underestimated the total PCB concentration. This was due to the lower number of congeners analysed, but also appeared to be a result of lower sensitivity of the method (in a comparison of 34 congeners). However, the identical relationships between the methods in both cases support the use of either in the investigation with health effect linkages.

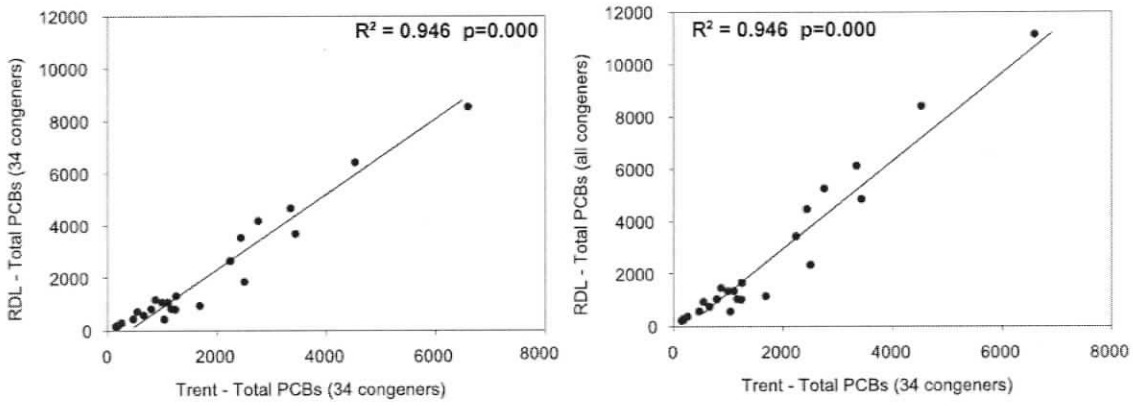
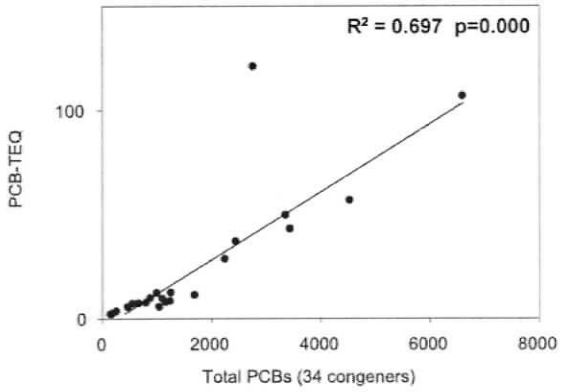


Figure 10. Correlation analysis of PCB concentrations and PCB-TEQ values.
The PCB-TEQ represents the dioxin-like toxicity of the PCB mixture in TCDD equivalents, and therefore a measure of the toxicity to immune systems as mediated through the aryl hydrocarbon receptor. PCB concentrations as quantified by the Trent method correlated significantly with the PCB-TEQ calculated from PCB concentrations as determined by the RDL method. This validates the use of the Trent method in assessing dioxin-like/AhR mediated toxicity.



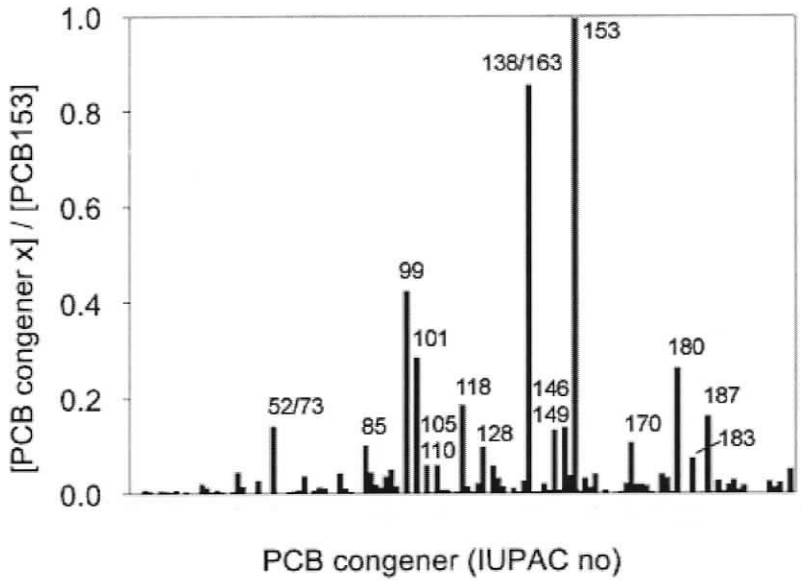
The PCB concentrations in seal pups from BC and WA (with a arithmetric mean of 2.5 mg/kg lipid weight, RDL method), can be considered moderate to low in comparison to those documented in harbour seal populations elsewhere. For example, harbour seals inhabiting industrial coastal areas in western Europe ranged from 7 to 85 mg/kg lipid weight, with the least contaminated areas being eastern Scotland and the most contaminated the Baltic Sea and German Wadden Sea. Seals from the eastern United States coast carried an average of 9 mg/kg lipid weight, whereas those inhabiting San Francisco Bay ranged from 29-50 mg/kg lipid weight (reviewed in Ross & Troisi, 2001). The PCB concentrations in BC and WA seals are also very low compared to the level of contamination found in other marine mammal species in the same region such as the killer whale (*Orcinus orca*). Depending on their dietary preference, these marine mammals were documented to carry PCB concentrations ranging from 37 to 251 mg/kg lipid weight (Ross et al., 2000a).

PCB patterns in seals

PCB patterns reflect the proximity to a source of PCBs, the harbour seal trophic position, and the susceptibility of certain congeners to metabolic breakdown (Boon et al., 1992). PCB patterns were obtained by expressing the concentrations of each congener relative to that of the most persistent, PCB 153. The patterns of the study seals (Figure 11) showed that the intermediate chlorinated PCB congeners were most abundant, whereas both the lower chlorinated and higher chlorinated congeners were present at very low concentrations. The most abundant congeners, PCB 153, 138, 99, 101, and 180, are all di-ortho substituted congeners, which were therefore quantified in both methods of PCB analyses, explaining the high level of correlation among the Trent and RDL methods.

Figure 11. PCB congener patterns in harbour seal pups.

A PCB pattern for harbour seals was obtained by using the average PCB concentration of each congener relative to that of the most persistent congener, PCB153 (here shown for Smith Island), to eliminate concentration differences. The most abundant congeners in seals were the intermediately chlorinated penta CB99 and 101, hexa CB138/163, and hepta CB180 congeners. The PCB congener pattern partly reflects the diet consumed, as well as the relative ease for metabolic and environmental breakdown over time.



In adult seals, the difference in relative abundance of PCB congeners is indicative of resistance to breakdown. Within the body, the cytochrome P450 family of enzymes is responsible for PCB metabolism. The CYP1A subfamily is being induced by, and responsible for, this process in case of the more planar PCBs, and CYP2B for globular PCBs. Harbour seals, as well as other pinniped species, have been documented to mainly rely on CYP1A for PCB metabolism, due to the relatively low activity of CYP2A in this species (Boon et al., 1992). For example, the high abundance of the PCB congeners 99, 101 and 180 can be explained by the high resistance to metabolism of these

congeners, resulting from only weak induction of CYP2B, as well as PCB 118 and 138, that are only weak inducers of CYP1A and 2B.

In seal pups, the PCB pattern reflects the contaminants of the female that were received over the course of lactation, and is therefore a combination of the pattern from the adult female as well as the mobilization processes. The PCB pattern obtained for the seal pups in this study is consistent with Ross et al., 2004 and Wolkers et al., 2004. PCB congeners 99 and 118 were present in significantly higher abundance than in adult seals, suggesting selective mobilization of these congeners. Relatively high amounts of the congeners 138, 146, 153, and 180 are consistent with their abundance in the mother (Wolkers et al., 2004). The close resemblence of PCB patterns of seal pups to milk, rather than to its mother, suggests that little metabolism is taking place in seal pups (Wolkers et al., 2004), and that blubber concentrations are reflective of their dietary exposure in concentration and pattern.

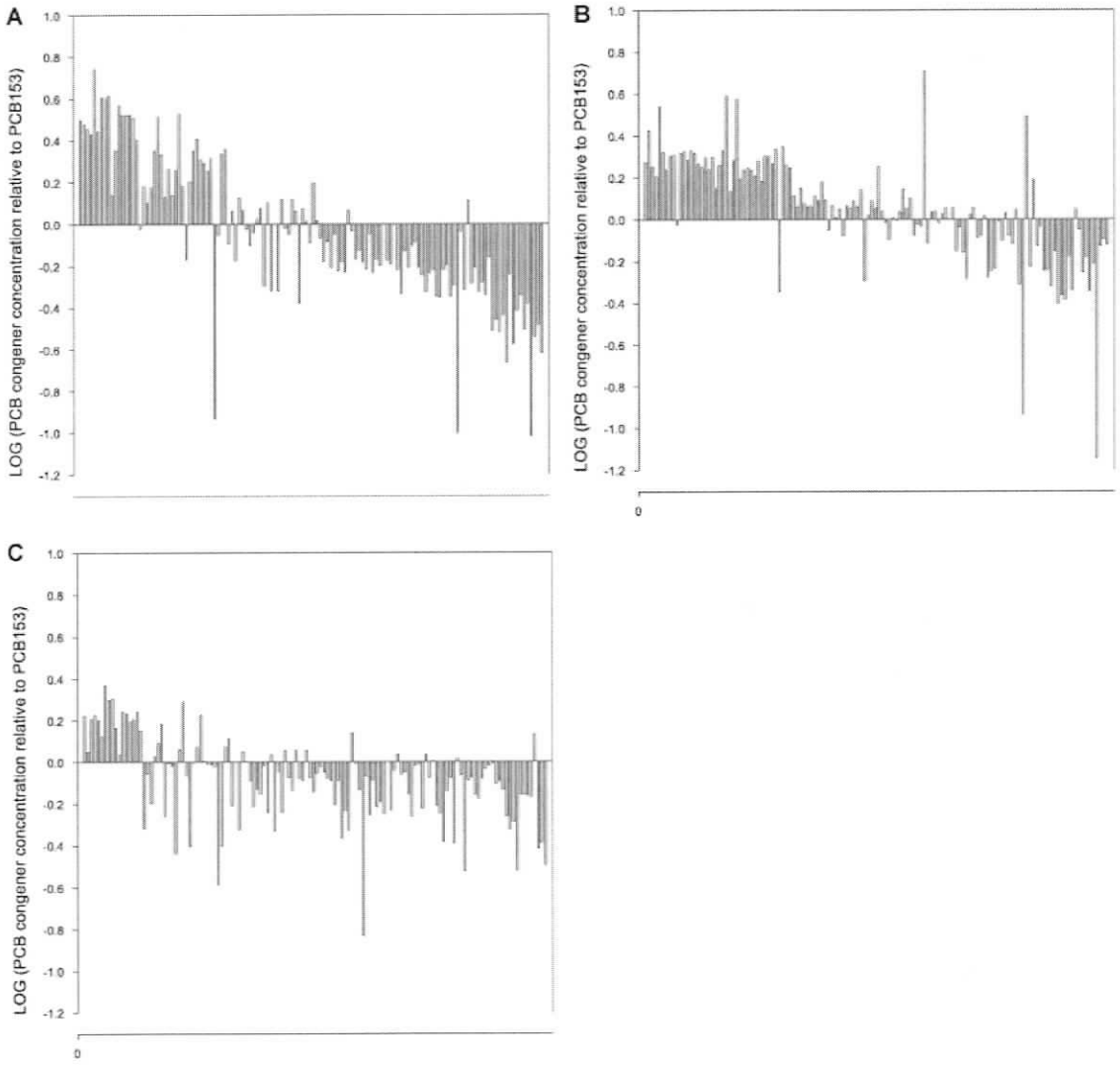
Differences in PCB patterns among seal sampling locations

Although PCB patterns in marine mammals follow a typical distribution of congeners due to the trophic level, marine food sources, and metabolic abilities of this group of mammals, pattern differences (e.g. higher presence of specific congeners in certain populations) among sampling locations are reflective of the source and long-range transport of PCBs. In this study, Queen Charlotte Strait seal pups were characterised by a relatively high abundance of the lower chlorinated congeners and a relatively low abundance of higher chlorinated PCBs, compared to Gertrude Island and Smith Island (Figure 12). Little difference in pattern existed between Smith and Gertrude Islands. This geographical change from North to South is probably due to the ability of lower chlorinated PCBs (with lower molecular weights) to be more easily transported by atmospheric circulation, whereas the higher chlorinated PCBs remain closer to the

source (Ross et al., 2004). Therefore, Gertrude and Smith Island seals are subjected to a PCB mixture reflective of the contamination of the industrial Puget Sound basin, whereas long-range transport is probably the largest input of contaminants in remote regions such as the Queen Charlotte Strait. Current investigations in the area will provide conclusive evidence on these PCB transport processes by study of environmental concentrations.

Figure 12. PCB congener patterns in three populations of harbour seals.

A pattern of individual PCB congeners (by IUPAC number) of the Queen Charlotte Strait (QCS) sampling location was compared to that of the Gertrude (A) and Smith Islands (B), and the pattern of Smith Island was compared to that of Gertrude Island (C). At QCS, domination by lower chlorinated (lighter) PCB congeners was observed relative to Smith and Gertrude Islands. The latter had more abundant higher (heavier) chlorinated PCBs (with up to ten-fold differences in relative concentrations compared to QCS). In a comparison of Smith and Gertrude Islands less difference in pattern was observed.



POP concentrations in seals

Among the POPs characterised in this study, PCBs were the contaminants present in the highest concentrations. PCBs represented 50% of the total POP concentration in seal blubber (based on the RDL method). The structurally related PCDDs and PCDFs were at least 1000-fold lower than PCB in concentrations, and a minority of the total TEQ in most seals (<40% on average in the group of seals, 57% in Queen Charlotte Strait and 10% in Gertrude Island seals). Due to analytical difficulties and the resulting discrepancies with previous studies, this data will not be discussed further. Within the group of OC pesticides, all compounds could be detected in seal blubber. Aldrin represented the lowest abundance (detected in 7 out of 42 seals) and the lowest average concentration, whereas DDT and its metabolites represented the most abundant (41 out of 42 seals) and highest in concentration. Chlordane, heptachlor, and HCH were also present in high concentrations in the blubber of seals. Concentrations of all other OC pesticides were generally low (Table 6).

Total DDT (as the sum of the original compounds, 4,4-DDT and 2,4-DDT, and its metabolites, DDE and DDD) ranged from 187 to 4467 μg/kg on a lipid basis, with an average of 1031 ± 138 μg/kg (n=41; compared to an average of 1509 ± 202 μg/kg for PCBs). DDT was detected in all but one seal, the latter originating from Hornby Island. Similarly to PCBs, the highest concentrations of DDT were found in Gertrude Island seals, and the lowest in the Queen Charlotte Strait. DDT concentrations were higher than PCB concentrations (based on the Trent method) in all Hornby seals. Seals from Smith and Gertrude Islands, inhabiting areas in vicinity of the highly industrialized Puget Sound, however, had (with one Smith exception) higher PCB than DDT concentrations, reflecting their proximity to a PCB source rather than (historically) lower use of DDT.

Table 6. Organochlorine pesticide concentrations in harbour seals.

Concentrations of the organochlorine pesticides (mean ± standard error of the mean; n=41) compared to the concentration of PCBs (n=24) in harbour seal blubber. All concentrations in μg/kg lipid weight.

	Concentration ± SEM
Total PCBs (RDL_{154})	**2499.20 ± 580.57**
4,4-DDE	979.17 ± 133.35
2,4-DDE	13.06 ± 2.19
2,4-DDD	7.80 ± 0.92
4,4-DDD	13.23 ± 0.94
2,4-DDT	0.35 ± 0.11
4,4-DDT	43.22 ± 4.35
Total DDT[1]	**1031.06 ± 137.91**
G Chlordane	2.37 ± 0.95
A Chlordane	20.24 ± 4.39
Trans nonochlor	69.25 ± 6.15
Cis nonochlor	6.77 ± 0.56
Total Chlordane[2]	**96.22 ± 8.88**
Heptachlor	0.34 ± 0.19
Heptachlor epoxide B	71.06 ± 7.67
Total Heptachlor[3]	**71.39 ± 7.68**
α HCH	33.39 ± 1.72
β HCH	13.43 ± 3.51
γ HCH	7.59 ± 0.63
δ HCH	1.66 ± 0.36
Total HCH[4]	**54.70 ± 4.01**
Endosulfan	0.90 ± 0.20
Endosulfan II	1.91 ± 0.32
Endosulfan sulfate	1.48 ± 0.32
Total Endosulfan	**4.18 ± 0..62**
Endrin	1.55 ± 0.52
Endrin aldehyde	1.90 ± 0.35
Endrin ketone	0.25 ± 0.15
Total Endrin	**3.70 ± 0.74**
Dieldrin	9.44 ± 0.63
Hexachlorobenzene	5.72 ± 0.46
Mirex	3.62 ± 0.49
Methoxychlor	1.68 ± 1.21
Octachlorostyrene	1.28 ± 0.23
Aldrin	0.02 ± 0.01

[1]Technical grade DDT consists of a mixture of 65-80% 4,4-DDT, 15-20% 2,4-DDT, and less than 4% 4,4-DDD. Metabolites of DDT are DDE and DDD. [2]Technical grade Chlordane consists of 10 major components [3]Heptachlor, an insecticide as well as a chlordane breakdown product, is rapidly converted into heptachlor epoxide (20% within hours in the environment and within the body). [4]Technical grade HCH consists mainly of the α-isomer (60-70%) and around 10% of the γ-, β-, and δ- isomers.

DDT concentrations represent a legacy from historical use, as indicated by the 1: 22 ratio of DDT (the parent compound) to its metabolites. Furthermore, the most common form of DDT in concentration was 4,4-DDE (or p,p-DDE), a metabolic product more persistent than DDT itself.

Associations between PCBs and organochlorine pesticides

Among the POPs, many compounds were correlated in concentration to PCBs in seals (Table 7). These compounds included the immunotoxic DDT and chlordane, as well as heptachlor, which were also the three most abundant POPs in seals. Correlations between POPs may reflect similarity in physical and chemical properties. For example, PCBs and DDT (especially in the form of DDE) share the properties of high persistence and lipophilicity, whereas HCB and HCH are highly volatile subjecting them to long range transport processes (also known as the grasshopper effect). In the cases of PCBs and octachlor (an accidental by-product of high temperature industrial processes), a geographically similar location of the source, namely the abundant industry in the Puget Sound basin, can explain their correlated concentrations in seals.

Immunotoxicity of PCBs and organochlorine pesticides

The toxicity of PCBs and the model compound TCDD were compared to other POPs using established threshold levels for immunotoxicity in rodents (Table 8). According to these values, the most immunotoxic compounds of the POPs studied are TCDD, PCB mixtures and dieldrin/aldrin. The more abundant organochlorines DDT, chlordane, and endosulfan can be considered only moderately immunotoxic, with threshold concentrations approximately 15-40 times lower than PCBs causing effects in rodents. Due to the low concentrations of dieldrin/aldrin in seal blubber, and the low immunotoxic

potential of the three abundant organochlorine pesticides, PCBs and structurally related compounds are likely to represent the largest immunotoxicological risk to seals in both concentration (abundance) and toxicity.

Table 7. Correlation analysis of PCBs and organochlorine pesticides.

Significant correlations (shaded grey) exist between many OC pesticides and PCBs, making PCBs a suitable alternative to complex environmental mixtures of chemical contaminants to compare toxic effects among individuals exposed. Contaminants are ranked by concentration, with the most abundant ones in the top left corner and the contaminants lowest in concentrations in the bottom right. Values represent the regression coefficients (R) and p values. OCS=Octachlorostyrene.

	ΣPCB	ΣDDT	ΣChlordane	ΣHepta chlor	ΣHCH	ΣEndo sulfan	ΣEndrin	Dieldrin	HCB	Mirex	Methoxy chlor	OCS	Aldrin
ΣPCB		**0.365** **0.017**	**0.681** **0.000**	**0.342** **0.026**	0.029 0.856	0.074 0.640	-0.030 0.850	0.283 0.070	0.185 0.241	**0.703** **0.000**	0.058 0.716	**0.825** **0.000**	-0.142 0.369
ΣDDT	**0.365** **0.017**		**0.375** **0.014**	**0.462** **0.002**	0.120 0.448	0.160 0.311	-0.168 0.287	0.039 0.808	0.264 0.092	**0.618** **0.000**	-0.105 0.508	0.070 0.661	-0.019 0.907
ΣChlordane	**0.681** **0.000**	**0.375** **0.014**		**0.462** **0.002**	0.287 0.066	0.222 0.157	0.089 0.577	**0.367** **0.017**	0.241 0.124	**0.448** **0.003**	-0.066 0.678	**0.535** **0.000**	-0.141 0.373
ΣHeptachlor	**0.342** **0.026**	**0.462** **0.002**	**0.462** **0.002**		**0.389** **0.011**	0.146 0.357	-0.102 0.518	0.132 0.403	**0.448** **0.003**	**0.486** **0.001**	-0.151 0.340	0.069 0.662	-0.030 0.850
ΣHCH	0.029 0.856	0.120 0.448	0.287 0.066	**0.389** **0.011**		**0.370** **0.016**	**0.445** **0.003**	**0.412** **0.007**	**0.307** **0.048**	-0.093 0.559	-0.083 0.601	-0.067 0.676	-0.074 0.643
ΣEndosulfan	0.074 0.640	0.160 0.311	0.222 0.157	0.146 0.357	**0.370** **0.016**		**0.555** **0.000**	**0.813** **0.000**	0.029 0.853	0.107 0.498	-0.097 0.542	-0.262 0.093	-0.034 0.833
ΣEndrin	-0.030 0.850	-0.168 0.287	0.089 0.577	-0.102 0.518	**0.445** **0.003**	**0.555** **0.000**		**0.559** **0.000**	-0.006 0.971	-0.164 0.298	0.237 0.131	-0.130 0.412	-0.043 0.788
Dieldrin	0.283 0.070	0.039 0.808	**0.367** **0.017**	0.132 0.403	**0.412** **0.007**	**0.813** **0.000**	**0.559** **0.000**		0.146 0.357	0.177 0.262	0.217 0.168	0.081 0.608	-0.100 0.529
HCB	0.185 0.241	0.264 0.092	0.241 0.124	**0.448** **0.003**	**0.307** **0.048**	0.029 0.853	-0.006 0.971	0.146 0.357		0.136 0.389	-0.124 0.435	0.199 0.206	0.255 0.104
Mirex	**0.703** **0.000**	**0.618** **0.000**	**0.448** **0.003**	**0.486** **0.001**	-0.093 0.559	0.107 0.498	-0.164 0.298	0.177 0.262	0.136 0.389		0.021 0.897	**0.383** **0.012**	0.032 0.840
Methoxychlor	0.058 0.716	-0.105 0.508	-0.066 0.678	-0.151 0.340	-0.083 0.601	-0.097 0.542	0.237 0.131	0.217 0.168	-0.124 0.435	0.021 0.897		0.172 0.275	-0.077 0.629
OCS	**0.825** **0.000**	0.070 0.661	**0.535** **0.000**	0.069 0.662	-0.067 0.676	-0.262 0.093	-0.130 0.412	0.081 0.608	0.199 0.206	**0.383** **0.012**	0.172 0.275		-0.188 0.232
Aldrin	-0.142 0.369	-0.019 0.907	-0.141 0.373	-0.030 0.850	-0.074 0.643	-0.034 0.833	-0.043 0.788	-0.100 0.529	0.255 0.104	0.032 0.840	-0.077 0.629	-0.188 0.232	

Table 8. Established effect levels for immunotoxicity in the mouse by oral exposure.

Toxicity reference values for the organochlorine pesticides identified in seals were compared to PCBs and dioxin to establish an understanding of their immunotoxic potential. (A) Acute exposure (B) Intermediate exposure (C) Chronic exposure. (Source: Agency for Toxic Substances & Disease Registry).

A

Compound	Reference value*		Year of evaluation
2,3,7,8-TCDD	NOAEL	0.005 µg/kg/day	1998
PCB mixture		N/A	2000
DDT		N/A	2002
Chlordane	NOAEL	8 mg/kg/day	1994
HCH		N/A	2003
Endosulfan		N/A	2000
Endrin		N/A	1996
Heptachlor / Heptachlor epoxide		(-)	2005
Dieldrin / Aldrin	NOAEL	12 mg/kg/day	2002
Hexachlorobenzene		N/A	2002
Mirex		N/A	1995
Methoxychlor		(-)	2002
Octochlorostyrene		N/A	N/A

B

Compound	Reference value*		Year of evaluation
2,3,7,8-TCDD	NOAEL	0.15 - 0.5 µg/kg/day	1998
PCB mixture	NOAEL	0.5 mg/kg/day	2000
DDT	NOAEL	10.5 mg/kg/day	2002
Chlordane	NOAEL	8 mg/kg/day	1994
HCH	NOAEL	20 mg/kg/day	2003
Endosulfan	NOAEL	2.1 mg/kg/day	2000
Endrin		N/A	1996
Heptachlor / Heptachlor epoxide	NOAEL	19 mg/kg/day	2005
Dieldrin / Aldrin	LOAEL	0.13 mg/kg/day	2002
Hexachlorobenzene	NOAEL	22 mg/kg/day	2002
Mirex		N/A	1995
Methoxychlor		(-)	2002
Octochlorostyrene		N/A	N/A

C

Compound	Reference value*		Year of evaluation
2,3,7,8-TCDD	NOAEL	0.03 – 0.3 µg/kg/day	1998
PCB mixture		N/A	2000
DDT	NOAEL	10.5 mg/kg/day	2002
Chlordane		N/A	1994
HCH		N/A	2003
Endosulfan		N/A	2000
Endrin		N/A	1996
Heptachlor / Heptachlor epoxide		N/A	2005
Dieldrin / Aldrin	NOAEL	0.003 mg/kg/day	2002
Hexachlorobenzene		N/A	2002
Mirex		N/A	1995
Methoxychlor	NOAEL	599 mg/kg/day	2002
Octochlorostyrene		N/A	N/A

* NOAEL = No observed adverse effect level; LOAEL = Lowest observed adverse effect level; N/A = Not available; (-) = Not considered immunotoxic after review of all available data by ATSDR

Hazard assessment of PCB exposure in seals

As part of ecological risk assessments, the hazard that adverse health effects take place is commonly addressed by comparing EDIs to regulatory guidelines. EDIs of PCBs for adult seals in the study region were recently reported by Cullon *et al.* (2005). Based on information of seal feeding ecology, Cullon *et al.* compiled a food basket of preferred dietary items of harbour seals that could be assessed for contaminant concentrations. The food basket was adapted for the purpose of this study to accurately reflect the higher energy demands and associated higher food intakes of young animals. According to this information, a 24-kg seal pup would consume 2.0 kg of marine foods, which equalled 1.4 μg PCBs/kg/day in the Strait of Georgia (including our sampling sites Hornby and Sea Islands; Table 9). Its counterpart in the more contaminated Puget Sound (including Gertrude Island) would feed on 3-fold higher concentrations (4.5 μg PCBs/kg/day; Table 9) (D. Cullon, personal communication).

When the rodent-based toxicity threshold value for intermediate exposure to PCBs (Table 8) is adapted for interspecies differences in sensitivity (by a safety factor of 10; CCME, 1997), a guideline of 50 μg PCBs/kg/day would be considered acceptable. This value is approximately ten-fold higher than the intake of seal pups in the area with the highest PCB concentrations (Gertrude Island).

The high sensitivity of certain aquatic wildlife consumers and their chronic exposure has resulted in the adaptation of a guideline within Canada which is not based on laboratory studies (CCME, 1999). This toxicity threshold value, referred to as the tissue residue guideline for the protection of wildlife consumers of aquatic biota against adverse health effects of PCBs, is based on a hypothetical animal with the highest possible exposure and metabolism. When comparing seal pup EDIs from the Cullon *et al.* study (0.6 and 1.3 ng TEQ kg^{-1} diet) to this highly conservative threshold (0.79 ng TEQ kg^{-1}), seal pup exposures imply a risk of adverse health effects in seals.

Lactating seal pups represent a group of risk for PCB-associated health effects

In a recent study where PCB concentrations in grey seal mother and pup pairs were followed over the course of lactation, it was shown that the pup had up to 8 times higher circulatory PCB concentrations than the mother (Debier et al., 2003). This supports the notion that lactating seal pups feed higher in the food chain than juveniles and adults. Furthermore, this suggests that the actual exposure of lactating pups is underestimated when using EDIs based on the consumption of marine foods.

No data was available to measure the PCB intake from milk for seals in our study, however, under the assumption that all PCBs in seal pup blubber are derived from the mother (until weaning), EDIs for milk intake could be calculated. Based on the per body weight values (Table 9), the exposure of seal pups consuming marine foods and milk is at least 10-fold different. This difference may be as high as 100-fold different among seal pups from different locations, e.g. a milk consuming pup from Gertrude Island versus a food consuming pup in the Strait of Georgia.

Due to the effects of developmental toxicity common to POPs, and PCBs in particular, young marine mammals may represent a group of high risk. This is especially of concern when assessing toxic effects on the immune system, relying on the neonatal period to obtain immunocompetence for life long protection against pathogens.

Table 9. Exposure estimates for harbour seal pups.

Harbour seal pup exposures were calculated using blubber PCB concentrations and compared to estimates from a harbour seal food basket (Cullon *et al.*, 2005). For the EDI_{milk}, blubber PCB concentrations were corrected for placental transfer, and the remainder was assumed to be derived from milk. For the $EDI_{marine\ foods}$, values were based on a seal pup with a body weight of 24 kg that would consume 2.0 kg of foods per day, based on energetic tables. PCB intakes through inhalation or water intake were not considered to be significant (less than 1% of food intakes), and, based on the latter, dermal absorption was not applicable. Values are averages plus/minus SEM, with ranges given in brackets. Ranges represent PCB intakes based on the RDL analysis of 24 animals in case of the EDI_{milk} and the difference between the values for a Strait of Georgia and Puget Sound food basket in case of the $EDI_{marine\ foods}$.

		EDI_{milk}	$EDI_{marine\ foods}$
PCB intake	$\mu g\ day^{-1}$	588 ± 138 (53 – 2677)	71 (34 – 108)
	$\mu g\ kg^{-1}\ day^{-1}$	32.4 ± 7.8 (2.0 – 145)	3.0 (1.4 – 4.5)
TEQ intake	$ng\ day^{-1}$		1.2 – 2.6
	$ng\ kg^{-1}\ day^{-1}$		0.05 – 0.11

Conclusions

Harbour seals inhabiting the coastal areas of British Columbia and Washington State were subjected to a relatively low level of contamination compared to elsewhere. PCBs were the primary POP detected in blubber biopsies of the seals. An evaluation of toxicity reference values in combination with concentrations of POPs showed that PCBs are likely the primary immunotoxicant in seals. Although the PCB concentrations can be considered low for a marine mammal species, they may lead to adverse health effects in free-ranging harbour seals of this coastal region according to a hazard assessment, in particular in lactating seal pups.

PCB concentrations correlated to the concentrations of many other POPs, including all OC pesticides with immunotoxic properties. This supports the use of PCBs as an alternative to complex mixtures in the assessment of immunotoxicity associated with the real world exposures to environmental contaminants. Within the group of PCB congeners, the two methods of PCB analyses were highly similar. These results validate the interchangable use of PCB analytical methods depending on the objective of the study. A partial, cost-effective, PCB congener analysis should be used when a larger sample size is needed and overall exposure is significant, for instance in toxicological studies, but provides limited capacity to interpret source-transport-fate processes, as well as mechanisms of action by which adverse health effects take place.

CHAPTER 3

Chemical and biological pollution contribute to the immunological profiles of free-ranging harbour seals

Portions of this chapter first appeared in the following publication:

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INTRODUCTION

In recent decades, disease-related mass mortalities have affected several marine mammal populations around the world, leading to significant conservation concerns. Morbilliviruses, in particular, have been notable for their role in the deaths of thousands of harbour seals (*Phoca vitulina*) in northwestern Europe in 1988 and 2002, thousands of Baikal (*Pusa sibirica*) and Caspian (*Pusa caspica*) seals in 1987-88 and 2000, and several thousand striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea in 1990-92 (Osterhaus et al., 1995; Kennedy et al., 2000; Harding et al., 2002). Periodic reports of outbreaks of leptospirosis among California sea lions (*Zalophus californianus*) (Gulland et al., 1996), further underscore the extent to which pathogens play a role in morbidity and mortality of free-ranging marine mammals.

Evidence suggests that environmental contamination by PCBs and structurally related compounds (e.g. PCDDs and PCDFs) may have contributed to some of these events through immunotoxicity (Ross, 2002). Many marine mammals are at particular risk of immunotoxicity due to their high trophic level, long life span and limited metabolic capacity to eliminate PCBs, resulting in the bioaccumulation of these compounds. In addition, reproductive transfer of the lipophilic PCBs, through fat-rich milk produced from the mother's blubber (Addison & Brodie, 1987), exposes young marine mammals at a sensitive developmental stage of the immune system.

The immunotoxic effects of PCBs have been documented in a number of laboratory studies, and are most pronounced when exposure takes place early in life (pre- and/or early postnatal period) (Birnbaum, 1995). Effects on the adaptive immune system include reduced thymus and spleen weights, altered T lymphocyte subsets, decreased in T lymphocyte number and functionality, decreased antibody responses of B lymphocytes, and suppression of immunization-induced proliferation of lymphocytes

(Blaylock et al., 1992; Tucker et al., 1986; Sharma et al., 1978; Nohara et al., 2005). PCBs can affect innate immunity by decreasing phagocytosis by leukocytes and macrophages, and decreasing the activity of NK cells (Funseth & Ilbäck, 1992; Ganey et al., 1993; Tryphonas et al., 1991a). Reduced immune responses due to PCB-associated immunotoxicity have been associated with an increase in susceptibility to disease in laboratory animals (Ross et al., 1996d), in wildlife species including birds and fish (Vethaak et al., 1996; Sagerup et al., 2000), and in humans (Dallaire et al., 2004).

Despite the logistical and ethical challenges associated with immunotoxicological studies in marine mammals, a weight of evidence supports the idea of chemical contaminant-associated immunotoxicity in pinnipeds and cetaceans. This weight of evidence is based on observations in PCB exposed laboratory rodents, and limited with respect to marine mammals to a captive study of harbour seals (De Swart et al., 1996), and the elevated levels of PCBs in harbour seals, harbour porpoises (*Phocoena phocoena*), and striped dolphins that died during virus-associated mass mortalities in Northwestern Europe and the Mediterranean Sea, respectively, compared to healthy individuals (Aguilar & Borrell, 1994; Jepson et al., 1999).

PCBs are the primary environmental immunotoxicant in concentration and toxicity to the free-ranging harbour seals of BC and WA (Chapter 2). In a recent effort to provide preliminary evidence of immunotoxicity; a correlative relationship was observed between PCB concentrations and the adaptive immune response of BC seals (Levin et al., 2005). The objective of the present study was to assess immune status in a larger group of free-ranging harbour seals, from a broader geographical region, covering a wider range of contaminant concentrations, and with an expanded array of immunological tests.

MATERIALS & METHODS

Seal sampling

Harbour seal pups captured at four sites in BC and WA (as described in Chapter 2), namely Hornby Island, Sea Island, Smith Island, and Gertrude Island, were included in the study. Seals were manually restrained while collecting blood from the extradural vein, using a 3.2-cm 18-gauge needle, into EDTA- (for hematology) and heparin-containing tubes (for isolation of peripheral blood mononuclear cells (PBMCs) and leukocytes) (both Becton-Dickinson, USA), and kept at 4°C until processing (within 24 hrs). Blubber (for contaminant analysis) was sampled using 8 mm biopsy punches (Acuderm, USA). In addition, body weight, sex, length, girth, and nursing status were recorded to estimate age, condition, and basic overall health. The time spanning capture and sampling was documented to avoid handling-related stress influences.

Mitogen- and thymosin α_1-induced cell proliferation

PBMCs were assessed for functionality using their proliferative responses by methods adapted from De Swart *et al* (De Swart et al., 1993). Heparinized whole blood was diluted one-on-one with RPMI-1640 (Gibco, Canada) containing 10 IU/mL of heparin (Gibco, Canada). Blood was subsequently gently layered onto Lymphoprep^{TM} (Biolynx, Canada) to isolate the PBMCs by density gradient centrifugation (30 minutes at 600 g, RT). PBMCs were washed three times, counted, and the concentration adjusted into RMPI-1640 Glutamax supplemented with 10% fetal bovine serum (Cansera, Canada), penicillin-streptomycin (100 IU and 100 μg mL^{-1}, respectively; Gibco, Canada), and 2 x 10^{-5} M 2-mercapto- ethanol (Sigma-Aldrich, Canada). Cells were cultured in flat-bottom 96 well plates (Falcon, USA), at 10^5 cells per well, in a constant environment of 37°C 5% CO_2 in air, with or without stimulant. Two mitogens, Concavalin A and Lipopolysaccharide (ConA and LPS), were used at concentrations found to be optimal

by previous studies (Mori et al., 2005) of 1 and 5 μg mL^{-1}, respectively (both from Sigma-Aldrich, Canada). Thymosin α_1 (bovine, Sigma-Aldrich, Canada), a highly conserved thymic hormone, was used at an optimal concentration of 6.25 ng mL^{-1} (based on average circulatory values reported for other species and preliminary experiments). After a 68-hour incubation, cell proliferation was determined using a BrdU-incorporation enzyme-linked immunosorbent assay (ELISA; Roche, Germany), as per manufacturer's instructions. This assay detects the incorporation of 5'-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, by an anti-BrdU antibody and a colorimetric enzymatic reaction (690 nm; Multiskan EX 1.0 ELISA plate reader). The relative response of stimulated cells versus non-stimulated cells is reported as a stimulation index (SI).

Phagocytosis and respiratory burst

Phagocytosis and respiratory burst were analysed as described in De Guise *et al* (De Guise et al., 1995). Briefly, heparinized whole blood was centrifuged for 10 minutes at 250 g, and the cell pellet collected. Red blood cell lysis, by shock with NH_4Cl, provided a leukocyte sample. Leukocytes were resuspended in Hanks Balanced Salt Solution (Gibco, Canada), washed three times, counted, and adjusted to a concentration of 2.10^6 cells. For phagocytosis, leukocytes were incubated with fluorescent latex beads (Molecular Probes, USA) at 100 beads per leukocyte. Free beads were removed by layering the cell suspension on a 3% albumin gradient and centrifugation for 8 minutes at 150 at 4 °C. The fluorescence of approximately 10,000 leukocytes was quantified using flow cytometry, and reported as the percentage of cells that had taken up at least one bead. Respiratory burst was quantified by incubating leukocytes (for 15 minutes at 37 °C in the dark) with 5 μM 2,7-dichlorofluorescin diacetate (Invitrogen, USA), a probe becoming fluorescent upon production of H_2O_2. Subsequently, the oxygen dependent

pathway of phagocytes was activated with 10^{-9} phorbol myristate acetate (PMA) (Sigma-Aldrich, USA). Labelled cells were kept in the dark until flow cytometric analysis of approximately 10,000 leukocytes, and quantified using the increase in activity in PMA stimulated cells compared to unstimulated cells, reported as SI.

Hematology and fecal coliform analyses

The Central Lab for Veterinarians, Langley, BC, carried out hematology by use of a blood smear from EDTA-containing samples on a glass slide, followed by Giemsa staining. White blood cell (WBC) counts were reported as total WBC count, and the numbers and proportions of the different circulating WBC subpopulations (neutrophils, monocytes, lymphocytes, eosinophils and basophils). Fecal coliform (FC) concentrations in water were provided by Environment Canada (Hornby Island), the Greater Vancouver Regional District (Sea Island), and the Washington State Department of Health (Smith and Gertrude Island), and reported as the number of FC bacteria per 100 mL.

Data analysis

Data were presented as the mean plus/minus the SEM. Comparisons of immune responses between sexes were done by Student's t-test (two-tailed), assuming equal variances. Linear regression analyses (R^2) were applied to associations between immune status, confounding factors and contaminant concentrations (using contaminant data from the Trent data set, consisting of 34 (mono and di-ortho) PCBs, to achieve the largest sample size of seals (as described in Chapter 2). For lymphocyte function, the SI was multiplied by 10, and log transformed for regression against contaminant concentrations. A cumulative measure of adaptive immune response (total SI) for each animal was calculated, using the sum of mean-adjusted SI of ConA, LPS and thymosin

α_1. Associations that failed the criterion for normality (i.e. lymphocyte count) were assessed by Spearman's rho correlation. Results were considered significant if $p \leq 0.05$.

Because the basis of measurement differed for the immune parameters, each parameter was divided by the maximum value observed for the parameter prior to principal components analysis (PCA). Hematological values were scaled to the maximum WBC count because individual counts were fractions of the total WBC count, rather than independent measures. Arcsine transformation (calculation of the square root followed by the arcsine) of this proportional data set returned it to a normal distribution, and was then autoscaled before PCA. Missing data (WBC values for one Smith seal and adaptive responses for one Hornby seal) were replaced by the average value of seals from the same location. Varimax rotation was used to position the axes to maximum possible sum of the variances of the loadings, while maintaining orthogonal eigenvectors. Sample scores for significant principal components (PC), and the geometric mean (GM) regression of PC1 and PC2 (as the exploratory value of a combination of PC1 and PC2) (Yunker et al., 2005), were correlated to contaminant concentrations and confounding factors to assess which were attributing to differences in immune function among seals.

RESULTS & DISCUSSION

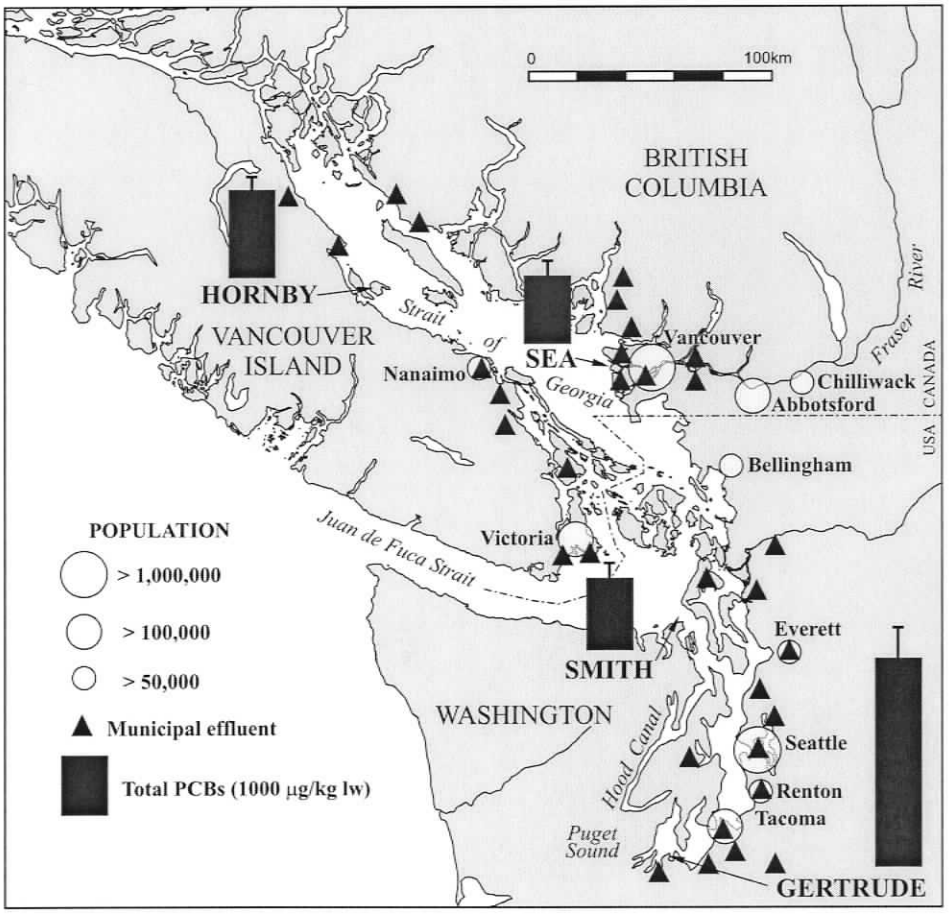
An immunotoxicological study of free-ranging harbour seals

A number of virus-associated mass mortalities have taken place among marine mammal populations inhabiting industrial coastal areas during recent decades, suggesting that chemical contamination may have played a role in the occurrence and severity of these events. The relatively high concentrations of PCBs in marine mammals, and their established immunotoxicity in a variety of species (Vos et al., 1973; Grasman et al., 1996; Dallaire et al., 2004) highlight the risk that this chemical class, in particular, may pose a risk to the health of marine mammals in many parts of the world (Ross et al., 2000b).

Immunotoxicity associated with chemical exposure can affect the quality and the quantity of circulating white blood cells, thereby diminishing defences against invading pathogens. In this study, we characterised immune function in a total of 31 harbour seal pups, which were similar in body weight (20.1 $\pm$ 0.5 kg) and therefore inferred to be of the same age (Cottrell et al., 2002), with an equivalent sample size between sexes (females n=17, males n=14). This limited the influence of potentially confounding factors. In addition, we did not detect a correlation between any of the measures of immune function and body weight ($p>0.05$; results not shown), or contaminant concentrations and body weight ($R^2=0.011$, $p=0.578$). There were no significant differences in immune function and contaminant concentrations between sexes (Student's t-test, $p>0.05$; results not shown). Contaminant concentrations varied widely among seals (396 to 6587 $\mu g\ kg^{-1}$ lipid weight; n=31) based on the sum of 34 PCB congeners, with the most contaminated animals originating from Gertrude Island in the Southern Puget Sound (Washington State, USA; Figure 13). Our study design therefore provided a setting

within which potentially confounding factors were minimized, while the range of contaminant concentrations was maximized.

Figure 13. Sampling location characteristics.
Harbour seal pups, sampled at four island locations in the transboundary region between British Columbia, Canada, and Washington State, USA, represented a range of blubber PCB concentrations (396 to 6587 μg kg^{-1} lipid weight; averages per location as shown). The sites spanned remote (Hornby and Smith Islands; n=8 and 8) and near-urban (Sea and Gertrude Islands; n=7 and 8) environments, with the latter close to urban centers, municipal effluent, and agricultural activities.



PCB-associated toxic effects on the adaptive immune system of seals

PCB concentrations were negatively correlated with measures of the adaptive immune response of seals. The proliferative responses of T lymphocytes to ConA, but not of B lymphocytes to LPS, were significantly decreased in more contaminated animals (Figure 14A-B). This is consistent with an immunotoxic mechanism of action attributed to the AhR in laboratory animals (Ross et al., 1997), and *in vitro* exposed cells of marine mammals (De Guise et al., 1998). Although the sensitivity of T lymphocytes to immunotoxic compounds is well established, few studies have evaluated lymphocytes recovered from free-ranging marine mammals. In a small group of cetaceans (n=5), decreased T lymphocyte proliferation was attributed to either PCBs or DDT (Lahvis et al., 1995). Levin *et al* (2005), on the other hand, reported recently that PCBs may also increase the proliferative response of (sub-optimally stimulated) lymphocytes in harbour seals (n=18). Therefore, immunotoxic compounds may both suppress and stimulate, but stimulation may reflect a response of only the most immunocompetent cells. Alternatively, it has been suggested that some toxic compounds may stimulate at lower doses while inhibiting at higher doses, also referred to as hormesis (Stebbing, 1982).

Lymphocyte signalling, an endpoint not previously assessed in free-ranging marine mammals, was also significantly reduced in more contaminated seal pups (Figure 14C), as indicated by the decrease in *in vitro* responsiveness to thymosin α_1. Thymosin α_1 induces interleukin-2 (IL-2) secretion, an important lymphocyte growth factor, and stimulates synthesis of the IL-2 (high affinity) receptor, both of which are important in lymphocyte proliferation following pathogen recognition. Although PCBs could be mediating a disruption of IL-2 signalling via the thymosin α_1 receptor, our results more likely reflect a direct effect. PCBs are known to disrupt cytokines (Jensen et al., 2003; Nohara et al., 2002) and cytokine regulation (Boverhof et al., 2004) through AhR-

dependent mechanisms of action, and harbour seal PBMCs exposed *in vitro* to PCBs were shown to decrease in functionality at least in part due to a disruption of cell signalling and cytokine (IL-1 and IL-2) production (Neale et al., 2005).

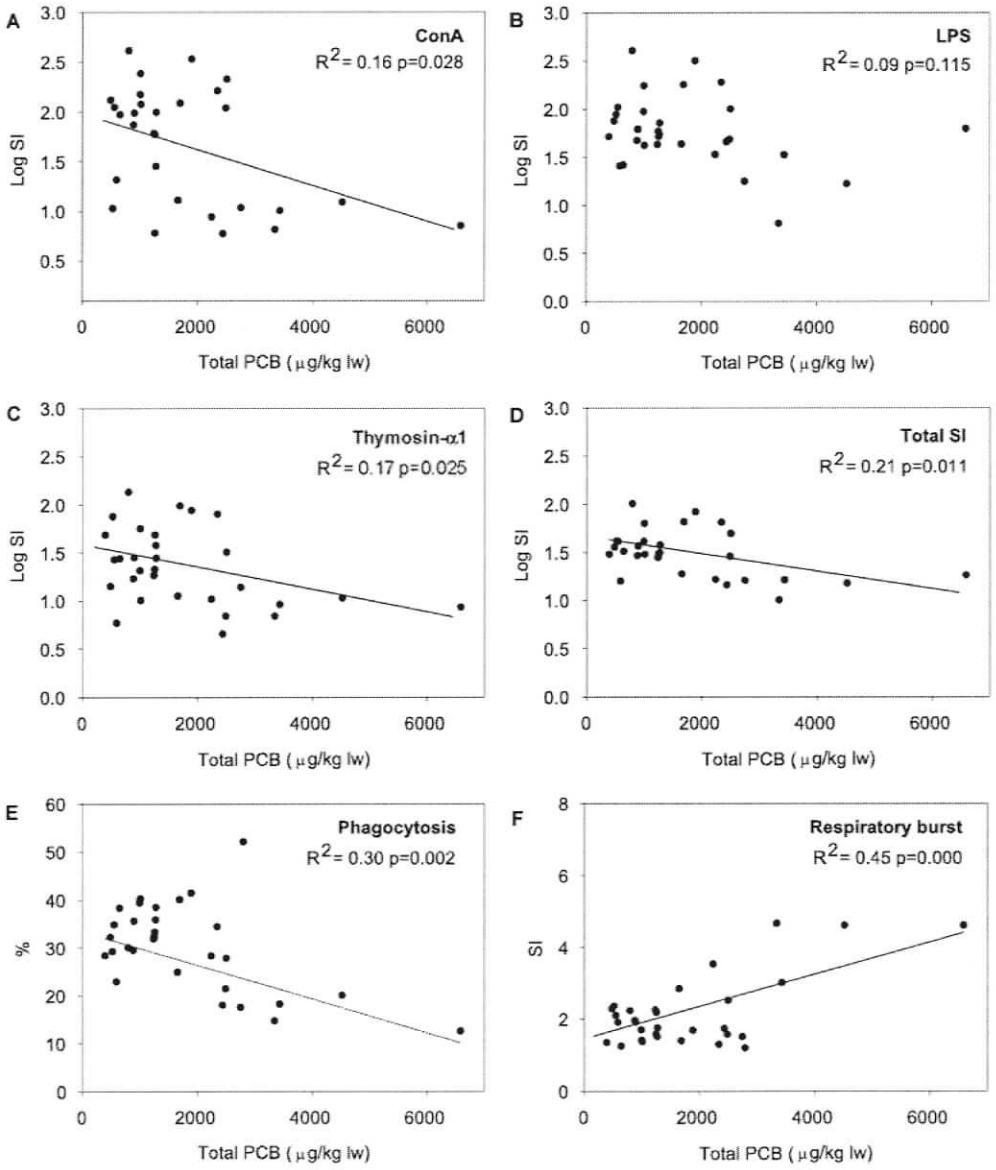
Reduced overall lymphocyte proliferation, as captured by a total stimulation index (Figure 14D), indicates that seals exposed to contaminants had lower overall (mean-adjusted) lymphocyte functionality. In addition to this reduced functionality, the more contaminated seals had decreased circulating concentrations (Spearman's rho, R= -0.53; p=0.002) and decreased percentages of lymphocytes (Pearson, R= -0.26; p=0.004) in the white blood cell counts. Since PCBs appear to be affecting both the quality and quantity of lymphocytes, the adaptive immune system of the more contaminated seals, as a whole, may be less able to respond to infectious agents.

PCB-associated toxic effects on the innate immune system of seals

Reduced phagocytic activity of neutrophils and monocytes in more contaminated animals (Figure 14E) suggested another immunotoxic effect of contaminants. However, increased respiratory burst (Figure 14F), an intracellular process in which reactive oxygen intermediates are produced in order to kill and digest ingested pathogens, by the same cell types, could indicate an immunotoxic enhancement. These observations are supported by decreases in phagocytosis in cells of marine mammals (Levin et al., 2004; Hammond et al., 2005), and increased respiratory burst in human cells, in response to *in vitro* exposure to PCBs (Voie et al., 2000). Altered function of phagocytic cells appear to be largely explained by AhR-independent mechanisms of action, and may result from oxidative stress, disturbance of particle uptake, or interference with anti-oxidant enzymes (Dalton et al., 2002; Narayanan et al., 1998). A PCB-associated disruption of innate immunity is of concern, since these cell types represent a rapid, non-memory based, immunological defense against many types of invading pathogens.

Figure 14. Associations between immune function and PCBs in seals.

Regression analysis indicated that lymphocyte function in harbour seals was negatively associated with PCB concentrations in response to a T lymphocyte mitogen (ConA) and the IL-2-stimulating hormone thymosin α_1, but not in response to a B cell mitogen (LPS; A-C). The total Stimulation Index (SI), representing the mean-adjusted cumulative lymphocyte response (ConA, LPS, and thymosin α_1) of each seal, indicates that the more contaminated seals had reduced lymphocyte performance (D). Increasing PCB concentrations were also significantly associated with reduced particle uptake (E) and elevated respiratory burst (F) by phagocytic cells (i.e. neutrophils and monocytes).



Immunological profiles of seals as determined by principal components analysis

Given the complexity of the interacting components of the immune system, a PCA model was used to characterise overall immune status in our harbour seals. The PCA separated seals into three overlapping clusters, with the remote Hornby and Smith Island (H/S) seals projecting in the upper right quadrant, the near-urban Sea Island (V) seals forming an intermediate group to the left of axis centre, and the near-urban Gertrude Island (G) seals located in the lower left quadrant (as circled in Figure 15A). The seal samples with missing values projected near the centre of the cluster for each location and, accordingly, the substitution of average values had not effected the separation by location. PC3 was not used for interpretation due to a low eigenvalue and the strong influence of a few outlier samples (not shown).

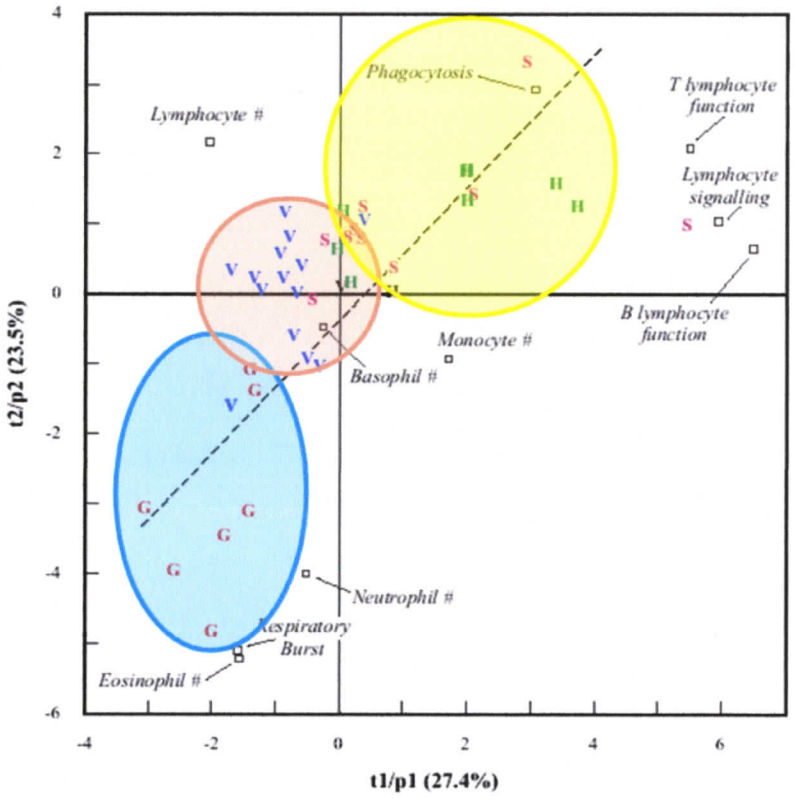
The corresponding variable projections indicated a pattern of low lymphocyte and phagocyte functionality, and high respiratory burst activity for the G compared to the H/S animals. The low importance of lymphocyte/monocyte numbers in PC1 and PC2 (projecting in the upper left and close to axis centre, respectively, indicating little influence on the model), and relatively higher neutrophil counts in G seals, suggest that low functionality rather than decreases in cell numbers characterised the changes in immune status of G animals. Many of the variables projected to both PC1 and PC2 (Table 10), suggesting two large influences on immunological profiles.

PC1, PC2, and the GM regression of PC1 with PC2, correlated significantly with PCB concentrations (Table 11). The variable projections associated with the spacing of individual seals on the biplot were furthermore consistent with the univariate correlations of immune function measures and PCB concentrations. The relationship between immunological profile and PCB concentrations was strongest with PC2. PC2 therefore appears to reflect the contribution of immunotoxic contaminants to the differences in immune function among seals.

Figure 15. PCA model of immune function variables in seals.

Varimax rotated projections of a Principal Components Analysis (PCA) model of 10 immune function measures in harbour seals revealed that two key factors influenced the immunological profiles of harbour seals, namely chemical (A) and biological (B) contaminants.

A. PCA separated seals into three overlapping groups, with H/S seals in the upper right quadrant, G seals in the lower left, and V seals in the intermediate area. The significant relationship between, in particular, the vertical scores (PC2) of seals with PCB concentrations (Table 11) confirmed univariate results which indicated that seals from more contaminated sites in this coastal region may suffer from immunotoxicity. The immunotoxic effects in these seals include lower lymphocyte function and phagocytosis and higher respiratory bursts in more contaminated individuals.



Footnote: The dashed line represents the geometric mean regression relationship between the samples in PC1 and PC2.

B. The horizontal separation in PC1 is consistent with that of urban (G and V; in blue) and non-urban (H/S; in yellow) seals. The variable projections of high eosinophil and neutrophil counts as well as high respiratory burst in urban seals that are coupled to these groups suggests that proximity to sources of biological pollution also contributes to the immunological profiles of seals (Table 12).

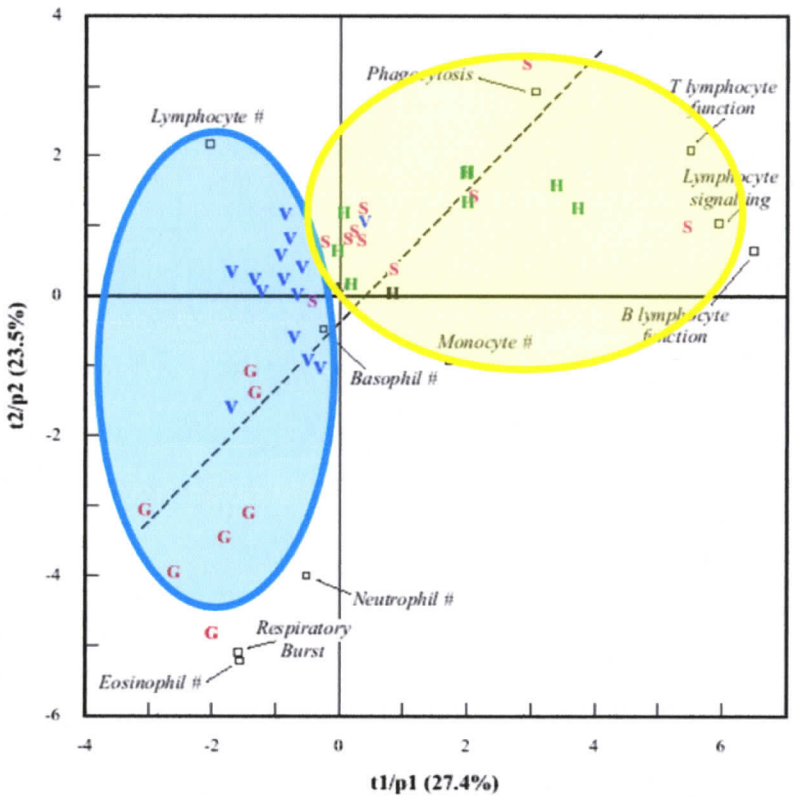


Table 10. PCA component matrix.

Component matrix of the 10 immune function variables in the PCA model show contributions of lymphocyte signaling and proliferation, phagocytosis, respiratory burst, neutrophil count, lymphocyte count, and eosinophil count to PC1 and PC2, whereas the other hematological parameters contributed to PC3.

PCA variables	PC1	PC2	PC3
Lymphocyte signaling	0.521	0.113	-0.004
T lymphocyte proliferation	0.483	0.224	0.002
B lymphocyte proliferation	0.570	0.072	0.017
Phagocytosis	0.269	0.312	-0.076
Respiratory burst	-0.141	-0.537	-0.068
Neutrophil count	-0.048	-0.421	0.372
Lymphocyte count	-0.181	0.233	0.460
Monocyte count	0.149	-0.097	0.553
Eosinophil count	-0.139	-0.550	0.016
Basophil count	-0.023	-0.049	0.577

Table 11. Regression analysis (R^2) for PCA of immune function variables.

Principal component (PC) 1 and 2, the sum of PCBs, body weight, and the time between capture and sampling of the seals.

	PC1 Eigenvalue 2.7	PC2 Eigenvalue 2.4	GM regression PC1 with PC2
ΣPCB	**-0.15** **p=0.032**	**-0.50** **p=0.000**	**0.15**
Body weight	0.01 p=0.572	0.01 p=0.604	
Sampling time	0.02 p=0.451	0.05 p=0.169	

PC1 provided a clear separation between seals sampled at near-urban locations (G/V) versus the more remote (H/S) sites, independent of PCB concentrations (Figure 15B). Within the near-urban sampling locations, PCB concentrations are much higher in seals from G than in V, suggesting that their (horizontal) clustering is not associated with chemical pollution. G and V sites share features, however, as both are influenced by freshwater run-off, and are in close proximity to major human population centers (greater Seattle and Vancouver, respectively), agriculture, and sewage treatment plants (for the latter, plants are within 5-10 km, compared to more than 30 km for remote H/S sites; Figure 13).

The variable projections of high neutrophil and eosinophil counts, that could not be explained by univariate associations with PCBs (both Spearman's rho, R=0.222; p=0.237 and R=0.281; p=0.132, respectively) but characterise PC1, suggest that seals sampled at near-urban sites experience a higher exposure to bacteria and parasites, respectively. In addition, increased respiratory burst could indicate an upregulation of mechanisms involved in bacterial destruction. This immunological profile could be reflective of a higher susceptibility to disease due to PCB exposure. However, the separation between near-urban and remote locations independent of contaminant concentrations strongly suggests that biological pollution is more likely to be the underlying factor.

Indications of biological pollution in seal habitat

In order to characterise biological pollution in the vicinity of our four sampling locations by means of organic waste input, we obtained FC counts from the responsible public health agencies. FC bacteria, living in the digestive tract of warm-blooded animals and excreted in their feces, are routinely used as an indicator of biological (fecal) pollution of

drinking and recreational waters. FC counts were high and fluctuating at both of the near-urban (G/V) seal sampling sites, whereas they were low to undetectable at both remote (H/S) sites (Table 12), consistent with a role of biological pollution in explaining the differences in immunological profiles between groups of harbour seals. The source of FC at the seal sampling locations is not clear, but could be due to either human sources (sewage discharge, septic fields, stormwater drainage), agricultural activities (runoff from livestock and soil fertilization), or wildlife. However, all sites have high abundance of seals (Table 12), and wildlife generally contributes only chronically low levels of FC (H. Osachoff, personal communication). Therefore, the source of FC at the G and V sites is likely related to the upstream contributions of humans, pets and livestock into these two coastal areas.

Evidence for a role of biological pollution also arises from the recent isolation of a number of bacterial pathogens from rehabitation seals from BC and WA (1999-2003) that are typically associated with sewage and agricultural runoff. These include *Escherichia coli, Enterococcus* spp, *Salmonella* spp, *Clostridium difficile* and *Cl perfringens*, *Klebsiella* spp, *Coxiella burnetti*, *Listeria monocytogenes,* and *Pseudomonas* sp (S. Raverty, unpublished observations). Furthermore, the presence of the protozoa *Toxoplasma gondii* (associated with cat feces in freshwater runoff), *Giardia* (from canids and other terrestrial wildlife), and *Sarcocystis neurona* (diagnosed in BC horses, but also affecting cats and other terrestrial vertebrates i.e. opossums) in harbour seals within our study area provides direct evidence of spill-over of terrestrial pathogens into coastal marine mammals (Lambourn et al., 2001; J. Gados, personal communication; S. Raverty, unpublished observations).

Table 12. Fecal coliform measurements nearby seal sampling sites.

Feceal coliform (FC) measured in water indicate a higher level of biological pollution at the urban sites of Gertrude Island (G) and Sea Island (V), compared to the remote Hornby (H) and Smith Islands (S). Similar seal abundance at the four locations suggest that FC was likely not of seal origin, but rather a result of nearby human activities. FC data from Environment Canada (H), Washington State Department of Health (S, G), and Greater Vancouver Regional District (V).

Location	Haul-out size (# of seals)[1]	Human activities in close proximity[2]		Mean FC/100mL (Range)[3]
		Population density (people/km^2)	Agricultural activities (yes/no)	
Hornby Island	≥1500	25	No	3.3 (<2-5)
Smith Island	~500	None	No	5.6 (<2-13)
Gertrude Island	≥500	97	Yes	15.6 (<2-110)
Sea Island	≥1000	715	Yes	32.5 (23-55)

[1] Data for H from (Olesiuk, 1999), S/G/V from (Jeffries et al., 2000). [2] Data for H/V from Statistics Canada (year 2001). Population density for V includes the cities of Vancouver and Richmond. Data for G from (Grant & Ross, 2002) based on the coastal Puget Sound area (year 2000). [3] H: mean value based on nine sampling stations sampled once each during February 2004 (low tide; no data available for the summer of 2003-2004). S: mean value based on three sampling stations at Point Partridge, sampled once each during September 2003 (low tide). G: mean value based on nine sampling stations in Wycoff Shoals, sampled once each during September 2003 (low tide). V: 30-day mean based on five samplings at Iona Beach, September 2003, range based on 30-day mean FC values from May-September 2003 (tide unknown).

Conclusions

Our study documents a negative relationship between immune function and PCB concentrations in free-ranging harbour seals in BC and WA. These observations are supported by a weight of evidence from mechanistic studies with laboratory animals and captive seals, and suggest impaired immune responses in contaminated seals. In addition, although our study was not explicitly designed to assess the impact of biological pollution, we detected a distinct immunological signature that was consistent with elevated pathogen exposure in seals inhabiting near-urban, coliform contaminated areas. With an impaired immune response, seals from more contaminated coastal regions (e.g. Puget Sound) may be particularly vulnerable to infection by new pathogens introduced into their habitat by anthropogenic activities.

CHAPTER 4

Evidence of Aryl hydrocarbon Receptor (AhR)-mediated mechanisms of immunotoxicity in harbour seals

INTRODUCTION

Polychlorinated biphenyls are environmental contaminants with immunotoxic properties that have become widespread due to their persistence and bioaccumulative nature, and have affected the health of humans and wildlife (Ross et al., 2000b). Marine mammals, in particular, remain exposed to and affected by elevated levels of these compounds through the consumption of contaminated prey over a long life span. It is of increasing concern that the conservation of marine mammals may be impeded by contaminant-related immunotoxicity, resulting in frequent mass mortalities during recent decades among these wildlife species.

PCB-associated immunotoxicity, on the adaptive immune system in particular, is mediated primarily through the AhR. This is supported by correlation between *in vivo* immunotoxicity and the binding affinity of TCDD and individual PCB congeners (Kerkvliet et al., 1985; Kerkvliet et al., 1990; Silkworth et al., 1984; Silkworth & Grabstein, 1982) as well as large differences in sensitivities to immunotoxicity in inbred strains of mice based on AhR genotypes (Vecchi et al., 1983; Silkworth et al., 1986). In mice, resistant DBA/2 and sensitive C57BL/6 strains require a ten-fold difference in TCDD dose to elicit the same toxic effects (Poland & Knutson, 1982). This difference is attributed to a single amino acid substitution in the ligand binding domain (Poland et al., 1991). The generation of mouse strains lacking functional AhRs ($AhR^{-/-}$) has shown that exposure to TCDD did not cause thymic atrophy or changes in thymocyte development, and neither the cellular nor the humoral response was suppressed by exposure of these animals to TCDD (Fernandez-Salguero et al., 1995; Vorderstrasse et al., 2001; Hundeiker et al., 1999).

Toxic responses to dioxin exposure are variable among species due to the polymorphic nature of the AhR (Peterson et al., 1993). The harbour seal AhR was

recently cloned and sequenced (Kim & Hahn, 2001). Using this sequence, subsequent ligand binding affinity experiments of *in vitro* expressed harbour seal AhRs were able to show that harbour seals are sensitive to dioxin toxicity, similar to the dioxin-responsive C57BL/6 mouse strain (Kim & Hahn, 2001). In comparison, humans were more similar to the unresponsive DBA/2 strain (Kim & Hahn, 2001). High AhR binding affinity implies that harbour seals may represent a susceptible species with respect to AhR-mediated immunotoxic effects.

Among harbour seal pups from BC and WA, differences in immune function were observed, consistent with exposure to immunotoxic PCBs (Chapter 3). Harbour seals may be susceptible to PCB-associated toxicity based on their AhR structure, and, furthermore, AhR may represent the primary mediator of immunotoxicity. The role of AhR in immunotoxic effects in harbour seals was therefore investigated. Two strategies were employed, consisting of an *in vitro* dosing approach to provide evidence that a potent AhR activator, namely TCDD, can induce a decrease in harbour seal lymphocyte function and alter AhR expression. As well, direct measurements of AhR expression in seals resulting from *in vivo* exposure provide evidence of a link between TCDD (and therefore dioxin-like PCB) exposure and a decrease in immunocompetence in free-ranging seals.

MATERIALS & METHODS

Seal sampling and sample preparation

Blood and blubber samples were obtained from seals of all five locations, Queen Charlotte Strait, Hornby Island, Sea Island, Smith Island, and Gertrude Island, during the field season of 2003, as described in Chapter 2. For comparative purposes, additional

blood samples of Gertrude Island seal pups were obtained during the field season of 2004, following identical methods and procedures.

PBMCs were isolated from heparinized whole blood as described in Chapter 3. At all locations, a sample equal to 10^6 cells in RPMI-1640 was separated for RNA isolation. Cells were centrifuged to a pellet in a 15 mL cell culture tube, and transferred to a RNase free microcentrifuge tube. A second centrifugation step was used to remove all remaining culture medium, and the cell pellet redissolved in 1.5 mL RNA stabilising solution (RNALater™; Ambion, USA) for storage at –20°C until further analysis. In case of the Hornby seal samples, an additional sample was used for a cell culture experiment involving *in vitro* exposure to TCDD for assessment of proliferative responses. In case of the Sea and Gertrude Island seal samples, an additional sample was used for a cell culture experiment involving *in vitro* exposure to TCDD, followed by RNA isolation for assessment of changes in AhR expression.

From all locations, two blubber biopsies of each seal were used. Firstly, for contaminant analysis, a subsample of a 8 mm blubber biopsy was frozen in liquid nitrogen (Chapter 2). Secondly, a complete 6 mm biopsy was stored in 1.0 mL RNALater™, kept cool until returning to the laboratory, and then moved to –20°C until further processing for RNA isolation.

PBMC culture and *in vitro* exposure to TCDD

For details on cell isolation and cell culture conditions see Chapter 3. PBMCs were cultured in presence of a proliferation-inducing compound (1 μg/mL ConA or 25 ng/mL thymosin-α1; both from Sigma-Aldrich, Canada) and a single dose of TCDD (0, 50, 100, and 200 pg/mL; Ultra Scientific RPC-088) for 68 hours. In an assessment of the toxic effects on cell proliferation, PBMCs were subjected to a BrdU incorporation ELISA

(Chapter 3) after the incubation period. For determination of effects on AhR expression, three triplicate wells of cultured PBMCs (600.000 before culture) were pooled and centrifuged to pellet. The cell culture medium was removed, the cells resuspended in 1.5 mL RNALater™, and stored at –20°C until RNA isolation.

RNA isolation

PBMC samples in RNALater™ were centrifuged for 1 hour at 12.000 *g* / 4°C to pellet the cells. All RNALater™ was removed, and the cell pellet resuspended in 1 mL TRIzol™ reagent (Invitrogen, Canada), a solution of phenol and guanidine isothiocyanate for the isolation of RNA. This reagent maintains the integrity of RNA while disrupting cells and cell components. A centrifugation step of 10 minutes separated a clear TRIzol™ phase from cell debris, and was transferred to a clean 1.5 mL microcentrifuge tube. Chloroform (200 μl) was added to separate the solution into an organic and an aqueous RNA-containing phase. In case of the (smaller and cleaner) PBMC samples from cell cultures, chloroform was directly added to the TRIzol. Tubes were inverted 20 times, incubated for two minutes at room temperature, and centrifuged for 15 minutes to obtain phase separation. The aqueous layer was transferred into a new tube that contained 1 μl of 20 μg/μl glycogen to facilitate the visualising of the RNA pellet in later stages. Samples were kept on ice while 500 μl isopropanol (Sigma-Aldrich, Canada) was added for RNA precipitation, and samples were inverted twice before storage overnight at -80°C. The next day, samples were thawed on ice, and upon thawing, centrifuged for 1 hour at 4°C to pellet the RNA sample. The supernatant was discarded, and 1 mL 75% ethanol in water added to wash the pellet. After a short centrifugation step (10 minutes / 7500 x *g*) the wash was discarded, and the RNA pellet air dried for 10 minutes before redissolving the sample into 20 µl RNase-free water (10 µl in case of cultured PBMCs). RNA samples

were then incubated at 55°C for ten minutes to ensure all RNA was in solution, and frozen at -80°C.

To extract total RNA from blubber, samples could easily be removed from the RNALater™ solution using autoclaved tweezers. Blubber biopsies were separated from the skin, and subsequently divided into two equal (4 mm) sections, of which solely the lower portion (closest to the muscle) was used to avoid possible influences of stratification, as validated and described by Tabuchi et al. (2006). This study showed no significant differences in steady state expression levels of the normalizer gene ribosomal protein L8 (RPL8) in the lower portion of the blubber among seal sampling locations.

The lower portion of blubber was homogenized using a Retsch MM301 mixer mill as described elsewhere (Tabuchi et al., 2006; Veldhoen & Helbing, 2001). Briefly, each blubber tissue sample was homogenized in a 1.5 mL microcentrifuge tube with the addition of 400 μl of TRIzol™ and a 3 mm diameter tungsten-carbide bead for a period of 6 minutes. An additional 3 to 6 min of mixing was performed if unhomogenised tissue remained following the initial homogenisation period. Samples were then centrifuged to obtain a clear TRIzol™ phase, and all subsequent steps were similar to those as described for PBMCs.

Total cDNA was generated from total RNA, using Superscript II RNase reverse transcriptase, as described by the manufacturer (Invitrogen, Canada). Briefly, one microgram of total RNA, as determined by spectrophotometry, from each sample was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Canada) in presence of 10 mM dNTPs (Stratagene, USA) at 65 °C for 10 min and subsequently placed on ice. The cDNA synthesis reactions were further assembled by adding 5x first strand buffer, 0.1 M DTT, RNase inhibitor, and 200 U Superscript II RNase H-reverse transcriptase (all from Invitrogen, Canada), to a total volume of 20 μl,

and incubated at 42 °C for 2 hours. The final cDNA solutions were diluted 20-fold in case of PBMC samples and 10-fold in case of blubber samples prior to quantification.

Aryl hydrocarbon receptor cloning

Polymerase chain reaction (PCR) primers for AhR expression were designed by using Primer Premier V4.1 (Premier Biosoft International, USA) software and the amino acid sequence of harbour seal AhR cDNA (Genbank AB056700; Kim and Hahn 2002), and synthesized by AlphaDNA (Canada). They consisted of the following sequence: 5'-GGCAATGAATTTCCAAGG-3' (up) and 5'-CGGGAGGAAAAGGACTGG-3' (down). The cDNA amplicon of a PCR reaction was separated on a 1.5% agarose gel accompanied by a 100 bp DNA ladder (New England BioLabs Inc., USA) and visualized with an ethidium bromide staining. Bands equal to the expected PCR product size of 563 base pairs were isolated, and exposed to three 5-minute cycles of freezing and thawing, on dry ice and in a 37°C waterbath, to elute the DNA. In order to obtain plasmid DNA for calibration curves used in quantitative PCR (QPCR) experiments and to produce sufficient quantities for sequencing, the DNA sample was inserted into a pCRII-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Canada) as per manufacturer's instructions. Plasmid DNA of the cloning reaction was purified using the QIAprep Spin Miniprep Kit (Qiagen, Canada), and sequenced in both directions. The sequence was compared by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) to sequences available in GenBank, to confirm the identity.

Aryl hydrocarbon receptor expression

AhR expression in PBMCs and blubber was quantified using a real-time QPCR assay on a Mx3000p system (Stratagene, USA). QPCR primers were identical to those

described for cloning in case of AhR, and of the following sequence for the internal control RPL8: 5'-GGTGTGGCTATGAATCCTGT-3' (up) and 5'-ACGACGAGCAGCAATAAGAC-3' (down)(Tabuchi et al., 2006). The QPCR method consisted of a 95°C/9 minute-denaturing cycle, followed by 40 cycles of denaturing at 95°C/15 seconds, annealing at 55°C/57 seconds (30 seconds for RPL8), and extension at 72°C/45 seconds, and a final extension of 10 minutes at 95°C. Each DNA amplification reaction (15 μl) included QPCR buffer (10 mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, and 40.000-fold diluted SYBR Green I), 10 pmol of each primer, 2 μl of 20-fold diluted cDNA template, 200 μM equimolar dNTPs (dATP, dCTP, dGTP, and dTTP; Invitrogen, Canada), 83.3 nM of ROX reference dye (Stratagene, USA) and 1.0 unit of platinum Taq DNA polymerase (Invitrogen, Canada). All samples were analysed in quadruplicate for AhR expression, and, in the case of blubber, in quadruplicate for RPL8 expression. A calibration curve of isolated AhR plasmid DNA in the QPCR method was used to quantify AhR expression in all samples, by plotting different plasmid DNA concentrations against their QPCR threshold cycle (Ct) values. Ct values represented the amplification cycles in which the fluorescence signals of the each of the samples reached a level significantly above the background fluorescence, taking place in the exponential phase of the amplication reaction.

PBMCs exposed *in vitro* were normalized to the amount (μg) of RNA isolated from the cells to adjust for cell proliferation and cell death. AhR expression on PBMCs from free-ranging seals was quantified as the number of transcripts in 1 μg of total RNA from 10^6 PBMCs. These procedures were applied to both types of PBMC samples to avoid biased normalisation by use of a normalizer gene. Although the latter procedure is often preferred because it represents an internal control, it requires that the gene of choice is constantly expressed in the tissue type investigated. In case of PBMCs, various cell

(sub)types are present. In addition, genes may be up- and/or downregulated during cell proliferation and differentiation, or as a result of contaminant exposure e.g. the stimulating effect of AhR on the assembly of new ribosomes accomponied by the upregulation of ribosomal transcripts and increased ubiquitination in the presence of liganded AhRs (Bas et al., 2004; 2002; Pollenz, 2002; Tijet et al., 2006). Normalization by use of the total amount of RNA present in a sample has been applied elsewhere (Crawford et al., 1997). Blubber was normalized over the expression of the normalizer gene RPL8 to adjust for tissue input/cell number, as previously validated in a study by Tabuchi *et al.* (Tabuchi et al., 2006).

A number of available harbour seal tissue types was investigated for the presence of AhR using RT-PCR, at the same thermocycle conditions as used during QPCR, followed by electrophoresis separation of the reaction products on a 1.5% agarose gel. In this reaction, RPL8 was used as a control for approximately equal cDNA input and quality among the tissue types. Ethidium bromide staining was used for visualization on an Eagle Eye system (Stratagene, USA).

Data analysis

Data are presented as the mean value plus/minus the SEM. All data was assessed for normality using the Shapiro-Wilk test and considered significant at $p<0.05$. In the comparison of groups of the same individuals receiving different doses of TCDD, the proliferative response (Stimulation Index or SI) and AhR expression of the control group data was considered equal to one, and exposed doses thereafter expressed relative to the control. *In vitro* exposure experiments were not normally distributed, and significant differences were assessed using the Friedman test for related samples, followed by Wilcoxon signed-rank test among each of the two dependent samples. Samples used in the *in vitro* exposure experiment concerning AhR expression, consisting of two sampling

locations (Sea and Gertrude Islands), were compared using a Student`s t-test assuming unequal variances, and treated as one since the groups did not significantly differ from each other.

In the comparison of free-ranging seals from different sampling locations, data did not exhibit a normal distribution; Kruskal Wallis one-way ANOVA for independent samples followed by Mann-Whitney U tests were applied. Associations among AhR expression in free-ranging seals and contaminant concentrations were investigated by Pearson correlation coefficients (R) if normally distributed and by Spearman's rho (R) in all other cases, using the Trent data set of PCB analysis (34 congeners) for comparability to the immunotoxicological study of seals.

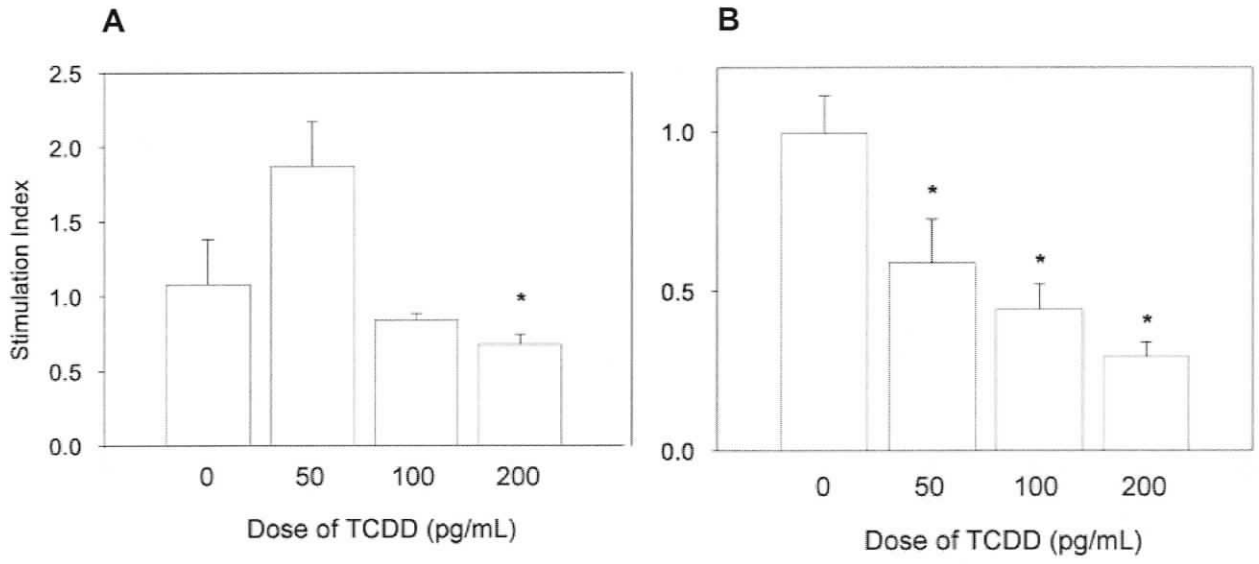
RESULTS & DISCUSSION

AhR ligands decrease lymphocyte function *in vitro*

In a study of free-ranging harbour seals, significant correlations between lymphocyte function and PCBs were observed (Chapter 3). According to *in vivo* and *in vitro* studies of laboratory rodents, the immunotoxicity of PCBs could be explained by their binding to AhR, and subsequent toxic effects. When seal PBMCs were modulated with the mitogen ConA to stimulate proliferation of T lymphocytes, in the presence of the most potent AhR ligand (TCDD), the dose of 200 pg/mL decreased cell proliferation significantly (Wilcoxon, $p=0.025$) compared to the control and two lower exposure doses (Figure 16A). However, at the lowest dose of TCDD (50 pg/mL), lymphocytes had a tendency to proliferate at a higher rate than unexposed cells. Although this difference was not significant, it was consistent with observations by others that TCDD and the structurally related PCBs may induce hormesis at low doses (Stebbing, 1982). In free-ranging loggerhead sea turtles (*Caretta caretta*), exposed to low contaminant concentrations

relative to wildlife species that feed at high trophic levels, mitogen-induced lymphocyte proliferation was significantly and positively correlated with PCB concentrations in blood. In addition, elevated proliferation of turtle PBMCs was observed following *in vitro* exposure to PCBs (Keller et al., 2006). In case of stimulation of harbour seal PBMCs with the immunopotentiating hormone Tα1 lymphocyte proliferation was significantly reduced at all exposure doses (50-200 pg/mL) compared to the control (Figure 16B).

Figure 16. TCDD exposure negatively affects lymphocyte proliferation *in vitro*.
Harbour seal (n=8) PBMCs were stimulated with 1 μg/mL of the T cell mitogen ConA (A) or 25 ng/mL of the immunopotentiating hormone Tα1 (B) exposed to a single dose of of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and their proliferation quantified by a BrdU incorporation ELISA. ConA-induced cell proliferation decreased significantly at the dose of 200 pg/mL (compared to 50 and 100 pg/mL) TCDD (p=0.025). Cells modulated with Tα1 showed negative effects of TCDD at all doses compared to the control (50 pg/mL p=0.017, 100 pg/mL p=0.012, 200 pg/mL p=0.012). Significant differences were assessed using the Friedman test, followed by Wilcoxon signed-rank test; significant results (p<0.05) are indicated with an asterisk.



This suggests that diminished immune function in response to TCDD exposure may be due to different mechanisms of action in presence of the two modulators. The *in vitro* exposure experiments using circulating white blood cells support the idea that AhR plays a role in immunotoxicity observed in harbour seals. It is generally viewed that the diminished proliferative response of lymphocytes results from developmental exposure to AhR ligands and the subsequent alteration of lymphocyte maturation (Schmidt & Bradfield, 1996). The experiments may therefore be reflective of past exposures of the seal pups during the thymic development of their lymphocytes. However, it has become apparent over recent years that AhR can also exert its influence on mature lymphocytes. In the latter case, effects take place through influences on cell-signalling pathways by alterations in the concentrations of cell signaling molecules including cyclins, intracellular Ca^{2+}, and cytokines (Holsapple et al., 1996; Jensen et al., 2003).

In case of stimulation with a mitogen, such as ConA, an increase followed by a decrease in cell proliferation of mature lymphocytes can be explained by the role that AhR plays in the cell cycle. In absence of ligand, AhR promotes progression of the cell cycle, whereas in presence of a ligand, it inhibits (G1) cell cycle progression and delays the G2→M phases (Puga et al., 2002). The latter process is mediated by complex formation between AhR and retinoblastoma protein (RB). RB represses E2F, and decreases the activity of cyclin-dependent kinase-2 (CDK-2), transcription factors which are both involved in progression of the cell cycle (Marlowe et al., 2004; Zhang, 1999). Therefore, if at a low TCDD exposure dose not all AhRs have been ligand-bound, this could exert a stimulating rather than inhibiting effect on cell proliferation. At high doses of TCDD, all AhRs are liganded and will interfere with cell cycle processes. When stimulated with Tα1, on the other hand, a process of IL-2 receptor expression and Il-2 secretion is stimulated, and a disruption of cytokine signalling would be responsible for

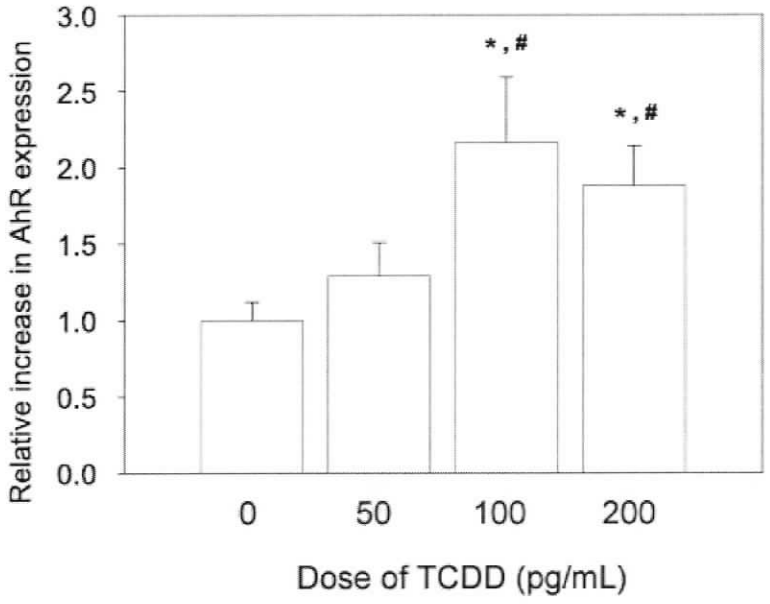
decreased proliferative response. The latter follows a dose-dependent response, and may therefore represent a more sensitive measure of immunotoxic effects.

AhR expression is upregulated by exposure to AhR ligands *in vitro*

Harbour seal PBMCs exhibited a significantly higher level of AhR expression upon exposure to a potent AhR ligand, TCDD, in vitro (Figure 17; Friedman test, $p=0.026$). Functional activation of AhR as well as upregulation of AhR expression by TCDD exposure has been previously documented in vivo and in vitro in lymphocytes of laboratory rodents (Doi et al., 2003; Franc et al., 2001). Elevated transcription following exposure takes place in response to chronic rather than acute exposure to TCDD (Franc et al., 2001). Upregulation of AhR expression is thought to represent a compensatory mechanism for elevated AhR protein degradation by the 26S proteasome, since, in the presence of ligands, the half life of AhR is decreased from up to 28 hours to less than 3 hours (Chang et al., 2005; Pollenz, 2002).

Figure 17. TCDD induces AhR expression on PBMCs *in vitro*.

Harbour seal (n=14) PBMCs were stimulated with 1 μg/mL of the T cell mitogen ConA and exposed to a single dose of 2,3,7,8-TCDD for 68 hours, after which AhR expression was quantified using QPCR, and expressed relative to that of an unexposed control. AhR expression was significantly elevated when cells were exposed to doses of 100 and 200 pg/mL TCDD, compared to the control and lowest dose (Friedman test-Wilcoxon signed rank test; significantly different from *control at $p \leq 0.05$ and $^{\#}$TCDD = 50 pg/mL at $p \leq 0.005$). AhR expression was not significantly different between the doses of 100 and 200 pg/mL ($p = 0.510$)



Relevance of *in vitro* experiments to free-ranging harbour seals

In vitro dosing experiments indicated significant reductions in lymphocyte proliferation and upregulation of AhR expression in harbour seal PBMCs. To assess how these experiments compare to the exposure that free-ranging harbour seals are experiencing, the doses (50-200 pg/mL of TCDD) were compared to values of EDIs of seals. According to Cullon et al. (Cullon et al., 2005), Strait of Georgia seal pups consume 1.2 ng TEQ per day (wet weight). Assuming that contaminants from dietary intake are absorbed efficiently during digestion, this would equal 0.47 ng TEQ per liter of blood (based on 125 mL of blood per kg body mass (Lenfant et al., 1969), and an average harbour seal pup body mass of 20.5 kg (Chapter 3). Therefore, our *in vitro* dosing experiment (where the dose in TCDD is equal to the dose in TEQ since the TEF is 1.0) exposed seal PBMCs at approximately 100 times higher doses than average free-ranging seal pups are experiencing through a marine diet.

However, the recently weaned free-ranging seal pups in the field study may have experienced (1) higher exposures to PCBs during their period of lactation, (2) PCB exposure coinciding with critical windows of immune system development, and (3) PCB mobilization from the blubber due to weaning (with our calculation underestimating blood concentrations). According to our comparison of lactating versus feeding harbour seal pups (Chapter 2), exposures may be up to 100 times higher in the former. The doses used in these *in vitro* experiments may therefore be relevant with concern to the adverse health effects of PCBs in pups during lactation. However, it is recommended that these experiments are expanded upon with a wider dose range for better understanding into mechanisms of toxicity, and the toxic effects that may take place at lower exposures.

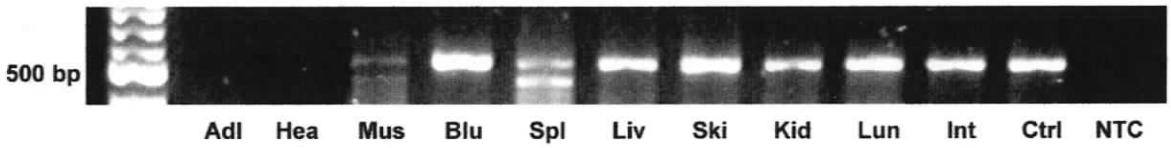
AhR characterization in harbour seal tissues

The results from *in vitro* experiments, as well as the fact that AhR is recognized as the primary mediator of PCB- and dioxin-associated toxic effects on the immune system, support that AhR expression levels could represent a relevant indicator of toxic exposure and effects in free-ranging seals. We therefore measured AhR expression in PBMCs and tissues of seals, to assess their potential usefulness in biomarker studies.

In harbour seals, AhR transcripts were present in PBMCs (results not shown) and many tissues, including the liver, kidney, lung and intestine, in low levels in the spleen, but undetectable or absent in the adrenal glands and heart (Figure 18). In the spleen of seals, the double-banded transcript implies the presence of a closely related protein to AhR, such as one of the other PAS proteins (e.g. ARNT, HIF-α); this secondary product was absent in all other tissue types, and was not further identified. Similarly to harbour seals, in the adult rat, AhR was found to be predominantly present in the lung, thymus, kidney and liver, and with lower levels in the heart and spleen (Carver et al., 1994). In adult humans, lung contains the highest level of AhR transcripts, but it was also high in spleen and intestine. Human liver, kidney and thymus were intermediate and heart and muscle low (Yamamoto et al., 2004). High AhR expression levels have been suggested to reflect the sensitivity of tissues to dioxin-like effects, including immunotoxicity. In this manner, the relatively high AhR expression levels in blubber and skin suggest that these tissues are sensitive to AhR-mediated effects, and such samples could represent a useful biomarker matrix when studying marine mammals.

Figure 18. AhR in harbour seal tissues.

Reverse transcriptase PCR validation of AhR expression in available tissues of harbour seals shows presence of the transcript (563 bp) in the muscle (Mus), blubber (Blu), spleen (Spl), liver (Liv), skin (Ski), kidney (Kid), lung (Lun), and intestine (Int). There was no evidence of AhR in the adrenal gland (Adl) and heart (Hea) of seals. Ctrl=positive control; NTC=No template control.



AhR expression in PBMCs of free-ranging harbour seals

AhR expression in PBMCs isolated from free-ranging seals was significantly correlated to the PCB concentration in their blubber (Figure 19A), suggesting a contaminant-related upregulation of AhR. This is consistent with the parallel *in vitro* exposure experiment, as well as an earlier study by Kim *et al* (2005) that showed that AhR expression in the liver of healthy Baikal seals was positively correlated to PCB concentrations as measured in blubber.

AhR expression levels in PBMCs isolated from free-ranging seals were elevated at all locations (Sea, Hornby, Smith, and Gertrude Islands) compared to the reference site (Queen Charlotte Strait), generally consistent with the degree of contamination of seals at these sites. However, seals from Gertrude Island had lower mean levels of AhR expression than expected from their blubber PCB concentrations (Figure 20). The accuracy of the Gertrude Island measurement was supported by the observation that Gertrude Island seals sampled during 2003 and 2004 were not significantly different in their AhR expression ($p=0.106$; t-test).

Although non-contaminant related factors may explain the deviation in AhR expression levels relative to their contaminant concentrations in Gertrude Island seals, it could also reflect a cellular regulatory response within the AhR signaling pathway. Ligand-bound

AhRs induce transcription of the negative regulator of AhR, the AhR repressor or AhRR. AhR upregulation can therefore only take place until a certain threshold dose, such that AhR expression may not be characterized by a linear dose-response curve. The *in vitro* experiments using harbour seal PBMCs supports this notion, by showing elevated AhR expression upon TCDD exposure up to the dose of 100 pg/mL but not thereafter.

Alternatively, different contaminant mixtures at each study location may influence AhR variably among sites. Gertrude Island seals are exposed to higher concentrations of AhR-ligands such as PCBs, PCDDs, and PCDFs compared to the other locations (Ross et al., 2004). This may have lead to the AhR-mediated upregulation of certain metabolizing enzymes that would decrease the half-time of the contaminants in these highly exposed seals, and therefore limit their effects. This hypothesis is supported by the comparison of PCB patterns of harbour seals compared to their prey items, that showed preferential metabolism/degradation in Gertrude Island seals (Cullon et al., 2005). In addition, the presence of high concentrations of PCBs with low affinity to AhR, compared to PCDDs and PCDFs, may be inhibitory rather than additive with concern to AhR expression.

To eliminate the uncertainty associated with the observations of free-ranging seals, future studies should be carried out. Experiments including a wider and more detailed spectrum of *in vitro* doses, including different compounds and contaminant mixtures as well as exposure times, and quantification of both AhR and AhRR expression and protein concentrations, may yield further insight into these observations and their importance.

AhR expression in blubber of free-ranging harbour seals

Blubber, with an average lipid content of 58-64% (based on Trent and RDL, respectively) in these seal pups, represents the primary site of PCB stores. However, in contrast to AhR expression in PBMCs, AhR expression in the blubber of seals was not significantly correlated with PCBs (Figure 19B). AhR expression levels may have been downregulated and may no longer reflect the elevated PCB concentrations in that tissue. The latter is consistent with our *in vitro* experiment. Alternatively, PCBs in blubber, stored in lipid droplets, may be separated from, and not necessarily exhibit toxic effects upon, cell signaling molecules within the cytoplasm such as AhR.

Figure 19. AhR expression in free-ranging harbour seals.
AhR expression was quantified in PBMCs (n=35) and in blubber (n=33) from free-ranging seals by quantitative RT-PCR. AhR expression in PBMCs correlated significantly with PCB concentrations as determined in blubber (A). However, AhR expression in blubber was not related to contaminant concentrations. R represents in both cases the Spearman correlation coefficient.

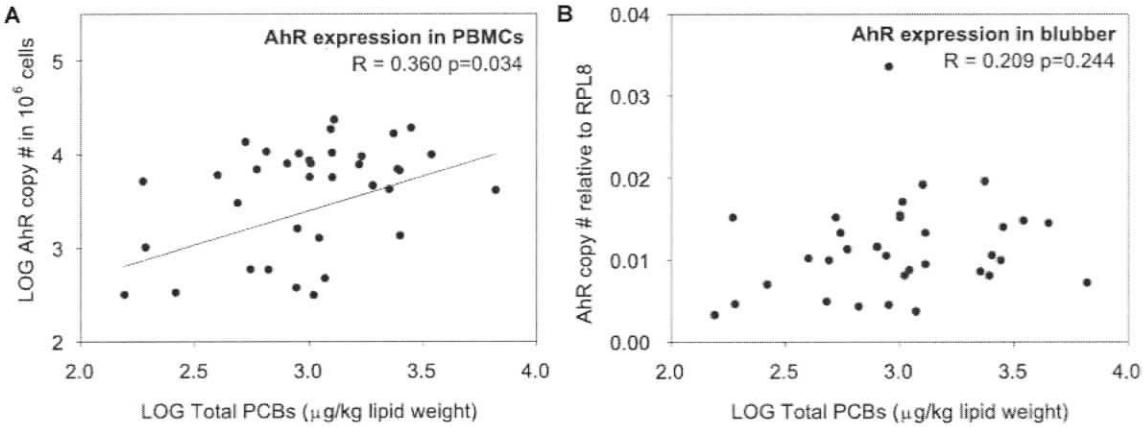
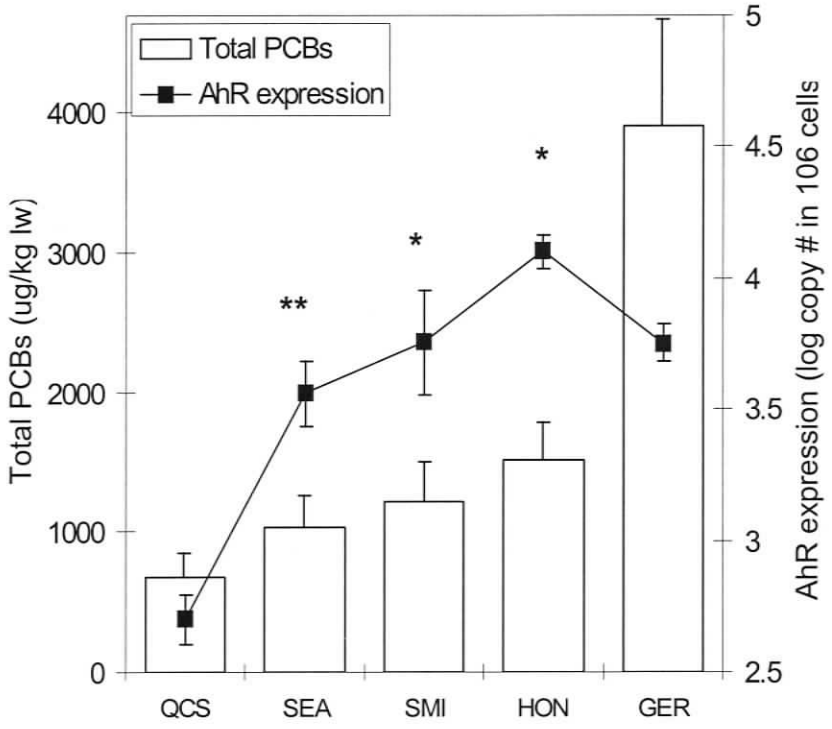


Figure 20. AhR expression is upregulated in seals from contaminated sites.
AhR expression (squares) in seal PBMCs was elevated at all sites compared to the reference location, QCS (n=7; p≤0.005) consistent with higher PCB concentrations in blubber (bars). AhR was upregulated in a dose-dependent manner in seals from QCS to Sea Island (n=9; p=0.001), Sea to Smith Island (n=6; p=0.026), and Smith to Hornby Island (n=8; p=0.029). Gertrude Island seals (n=5) had lower AhR expression than expected from PCB concentrations. P-values represent the two-sided level of significance (Mann-Whitney U test) with ** p≤0.001 and * p≤0.05.



Conclusions

The data from *in vitro* experiments using harbour seal PBMCs indicates that AhR ligands reduce lymphocyte proliferation and increase AhR expression. In addition, correlative evidence of a contaminant-associated upregulation of AhR expression in white blood cells of free-ranging harbour seals, combined with the earlier presented observations of diminished lymphocyte function in free-ranging seals from contaminated areas (Chapter 3), suggest a role for AhR in mediating immunotoxicity in free-ranging seals.

CHAPTER 5

A preliminary investigation of (pro)thymosin-α1 as a biomarker of immunotoxicity in harbour seals

INTRODUCTION

In addition to being the primary lymphoid organ, responsible for T lymphocyte development, the thymus is an endocrine tissue that secretes a number of hormones, including the family of polypeptide hormones known as thymosins. Thymosins were first discovered in the 1970s as part of a purified thymus extract referred to as thymosin fraction V (TF5). TF5 contained several similar polypeptides of which thymosin-α1 (Tα1) was present in the highest concentrations. This 28 amino acid polypeptide is the most acidic molecule within the body, and the product of a gene family of five prothymosin-α1 (proTα1) genes (in humans) of which only one gene is transcribed (Piñeiro et al., 2000). From the proTα1 gene product, two thymosins, Tα1 and thymosin-α11, are produced (Sarandeses et al., 2003). (Pro) Tα1 is highly conserved among vertebrate species, and likely serves important functions, especially in the regulation of the immune system (Goldstein & Badamchian, 2004; Sarandeses et al., 2003).

Tα1 has been identified as a biological response modifier within the immune system. It mainly acts on thymocytes and mature T lymphocytes, which are affected by the central role of Tα1 in thymocyte maturation, in maintaining a stable pool of circulating T lymphocytes, as well as in regulating their sensitivity to antigens and production of cytokines. Tα1 increases MHC-I expression in cells, and induces interleukin-2 synthesis and high affinity IL-2R expression (Baxevanis et al., 1990b; Leichtling et al., 1990; Baxevanis et al., 1990a). Furthermore, Tα1 stimulates and restores adaptive immune responses (Maric et al., 1991), enhances natural killer cell activity (Cordero et al., 1992), and induces tumor-specific T lymphocyte responses (Baxevanis et al., 1995). These are all properties that have been used clinically, in human lung cancer, melanoma, leukemia and Hepatitis C treatments (Goldstein & Badamchian, 2004).

Whereas the clinical use of Tα1 has been widely accepted since the 1990s, few studies have examined a possible relationship between thymosins and immunotoxic compounds, such as the widespread PCBs and PCDDs. Diminished levels of Tα1 were observed in humans living, working, or recreating in a TCDD-contaminated residential area versus persons at a reference site. Tα1 concentrations showed a significantly decreasing trend with years of residence in the contaminated group (Stehr-Green et al., 1989). Although the latter study suggested that TCDD affects Tα1 concentrations, the biological significance was unclear, since no association with other measures of immune function could be found (Stehr-Green et al., 1989). In contrast with that study of humans, a significant dose-related increase in circulatory Tα1 was observed in rhesus monkeys (*Macaca mulatta*) chronically exposed to low level mixtures of PCBs (Aroclor 1254). The observed effect was attributed to mild inflammatory processes following PCB-induced liver necrosis. Despite the elevated Tα1 concentrations, general immunosuppression persisted in these monkeys (Tryphonas et al., 1991b).

Marine mammals are exposed to high levels of immunotoxic PCBs in many industrial coastal areas around the world. Whereas a number of adverse health effects have been documented in these animals, including reproductive failure (Reijnders, 1986), endocrine disruption (Brouwer et al., 1989; Tabuchi et al., 2006), and developmental abnormalities (Bergman et al., 1992), the immune system in particular is believed to be extremely sensitive to PCB exposure. A number of studies have established a correlative link between PCBs and decreased immune function in marine mammals (Chapter 3, De Swart et al., 1994; Lahvis et al., 1995). However, immunological studies of marine mammals are often difficult, and limited to the use of alternative methods. Biomarkers of exposure and effect that can be measured in tissues that can be obtained using minimally invasive methods, but are relevant to immune function, should therefore be

developed. The endocrine products of the thymus could potentially serve such a function.

Among the many thymic hormones that have been described and characterised over the last decade, only one, thymulin, has been previously described in a marine mammal (Kendall et al., 1992). However, thymulin is only present at low levels in circulation and in the thymus (Spangelo, 1995). Tα1, on the other hand, is thought to be the thymic hormone with the largest immunoregulatory potential (Goldstein et al., 1977), and has been studied in the widest range of species, including humans, rodents, pigs and monkeys (McClure et al., 1981; Stehr-Green et al., 1989; Tryphonas et al., 1991b; Goya et al., 1990; Wise, 1992; Tsitsiloni et al., 1993; Palaszynski et al., 1983). The highly conserved nature of (pro) Tα1, its wide distribution in the body, its up to a thousand fold higher concentrations than thymulin (Franco et al., 1992), and its important function in immune status imply that this small hormone would represent a more suitable biomarker candidate for immunotoxicological studies of marine mammals.

In harbour seals, the conserved immunomodulatory actions of this small hormone were applied as a tool in the study of AhR-mediated effects on lymphocyte function *in vivo* and *in vitro* (Chapter 3 and 4). However, little is known about the physiology of this hormone in seals. Such information would benefit the validation of Tα1 as a potential biomarker of immunotoxicity in these marine mammals. In this first effort to characterize Tα1 in a marine mammal, we investigated the function, distribution, and expression of (pro)Tα1 in the harbour seal. Furthermore, Tα1 concentrations were related to PCB exposure in a group of free-ranging harbour seal pups that was previously studied with concern to contaminant exposure (Chapter 2), and the associated adverse effects on the immune system and AhR expression (Chapter 3 and 4).

MATERIALS & METHODS

Seal sampling

Blood, blubber, and skin samples from seal pups were obtained during the 2003 field season following a procedure of live-capture and release, as described in Chapter 2. Blubber and skin biopsies were wrapped in hexane-rinsed aluminium foil, and frozen in liquid nitrogen directly following sampling, and, upon return in the laboratory, moved to -80°C until further processing. Tissue samples of important internal organs were obtained during necropsies of two seal pups that had died accidental deaths but were otherwise healthy, and had been stored at -80°C. All research described has been carried out in conformity with the applicable guidelines for studies involving wild animals.

Tissue thymosin-α1 analyses

Blubber (approximately 200 mg) and skin (50-100 mg) biopsies of free-ranging (Sea and Gertrude Island) seals were washed twice in cold phosphate buffered saline (PBS; pH=7.4) to remove blood and residues, and homogenized in 1.0 mL demineralized water (DMQ) using a PowerGen 125 (Model FTH115, Fisher-Scientific, Canada). ProTα1 and Tα1 were extracted from the homogenates according to the method of Moody et al. (Moody et al., 1996). Briefly, homogenates were boiled for 5 minutes, cooled to 4°C, vortexed for 20 seconds, and centrifuged for 5 minutes at 1500 x g at 4°C. The supernatant, containing proTα1, was moved into a new 5 mL cryovial. The pellet was subsequently dissolved in 1.5 mL acetic acid (0.5 M) by vortexing for 20 seconds. The acidic homogenate was boiled and centrifuged for a second time, and the supernatant, containing Tα1, combined with the previous one. Extracts were stored at -80°C until freeze-drying. Freeze-dried extracts were dissolved in a radioimmunoassay (RIA) buffer consisting of PBS containing 0.1% bovine serum albumine (BSA), Tα1 antiserum

(1:1000) and ^{125}I-Tyr0- Tα1 (5000cpm). After 16 hours at 4°C, normal rabbit serum (1:100), goat anti-rabbit serum (1:10), and 12% polyethylene glycol were added. The samples were vortexed, cetrifuged at 3000 x g for 10 minutes, and the supernatant (containing free peptide) removed. The pellet (containing bound peptide) was subsequently quantified using a LKB gamma counter. Protein content of the tissue samples, quantified using the Laurie assay, was used to express Tα1 concentrations on a protein content basis, avoiding influences of extraction efficiency among a range of sample sizes. Tα1 concentrations were not quantified in serum samples of free-ranging seals due to cross-reactivity of the antibodies with serum proteins (T.H. Moody, personal communication).

Prothymosin-α1 expression

Primers to amplify harbour seal proTα1 were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) against the conserved regions of human (BC066905), mouse (BC083135), and rat (BC092569) proTα1 obtained from Genbank (http//www.ncbi.nlm.nih.gov). Sequences of the forward and reverse primers used in the PCR were as follows 5'-GAGGCAGAGAATGGAAGAGA-3' and 5'-TTCTTGGTGTCCACATCGT-3', with an expected product size of 236 base pairs.

Harbour seal cDNA was prepared from freshly isolated PBMCs dissolved in RNAlater™ reagent (Ambion, USA), whereas tissue samples from earlier necropsies were removed from storage and thawed in RNAlater™ Ice (Ambion, USA) according to the manufacturers protocol. Total RNA was subsequently prepared from all samples using TRIzol™ and reverse transcribed into cDNA as described elsewhere (Chapter 4). Samples of cDNA were used in 15 μl PCR reactions under the following thermocycle conditions: 9 minutes/95°C, 40 cycles of 15 seconds/95°C, 30 seconds/56°C, and 45

seconds/72°C, followed by a final extension of 10 minutes/72°C, using a PerkinElmer 9600 (PerkinElmer Biosystems, USA).

PCR products were separated by electrophoresis on a 1.0% agarose gel, visualized by use of an ethidium bromide staining, and the photo stored on a Epichemi Darkroom system (Stratagene, USA). PCR products of the expected size of 236 base pairs were isolated from the agarose gel, cloned using the TOPO II Cloning kit per manufacturer's instructions (Invitrogen, Canada), and sequenced to confirm the identity. A 233 base pair sequence resulted, which was trimmed of its primers. The identity of the clone was confirmed using BLASTn (http://www.ncbi.nlm.nih.gov/BLASTn), followed by alignment to similar sequences using ClustalW (http://clustalw.genome.ad.jp). The multiple sequence alignment was shaded in areas of similarity among species using Boxshade v3.33C (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade-simple.html). In addition, the nucleotide sequence as translated in all possible reading frames was compared to a protein sequence database using BLASTx. The matching protein sequences were used to deduce protein similarity percentages (identies).

Data analysis

Data are presented as the mean value of the population plus/minus the SEM. Data was assessed for normality, using the Shapiro-Wilk test ($p<0.05$) if further analysis was carried out. In the comparison of blubber and skin samples of seals from the two locations, data exhibited a normal distribution, and a Student's t-test assuming unequal variances was applied. PCB concentrations in the blubber of each animal were based on the Trent analysis (See Chapter 2 for details).

RESULTS & DISCUSSION

Molecular characterisation of proTα1 in harbour seals

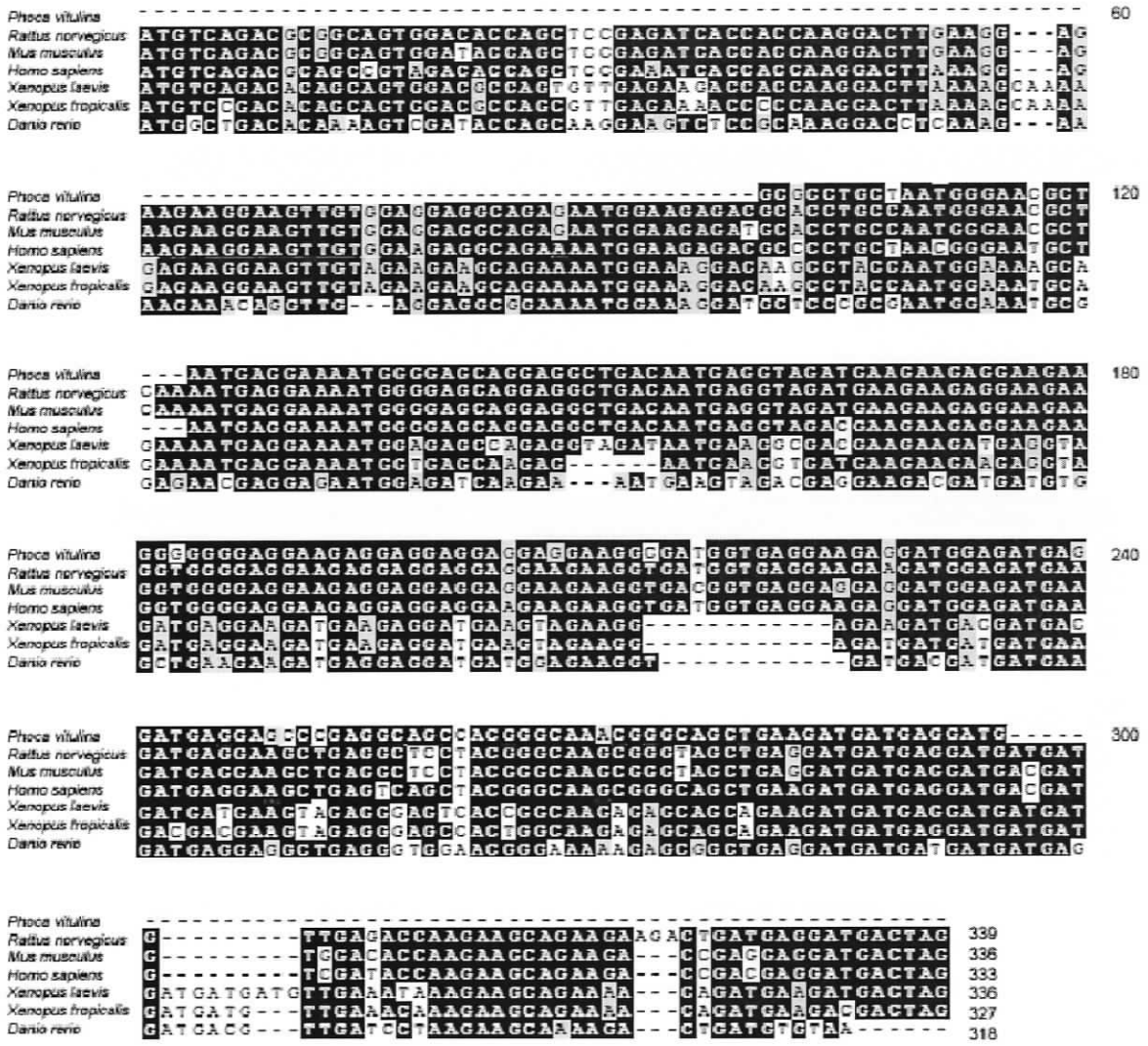
The molecular structure of the precursor molecule of Tα1, proTα1, was firstly characterized. A partial cDNA sequence of 194 base pairs was obtained. This sequence represented the middle portion of the proTα1 nucleotide sequence and did not include the sequence coding for Tα1 (Figure 21).

The partial sequence was compared to six available complete cDNA sequences of species representative of the groups of mammals, amphibians, and fish (humans, mice, rats, frogs, and zebrafish). A high level of conservation was documented among mammals, with seals being 93% similar to humans, 90% similar to mice, and 89% similar to rats. Seal proTα1 was only approximately 60% similar to two species of frogs and one species of fish. The large differences between mammals and others could be attributed to a number of deletions and insertions that proTα1 has undergone.

At the protein level, harbour seal proTα1 was only one amino acid different from human proTα1, consisting of a serine to alanine substitution at position 83. This same non-conservative substitution is found in cows (Piñeiro et al., 2000), but its biological significance is unknown.

Figure 21. Prothymosin-α1 nucleotide sequence of seals and other species.

Comparisons of the obtained partial harbour seal nucleotide sequence (194 bp) for proTα1 to other species shows a high level of conservation, especially among mammal species, however, with some significant species-specific deletions and/or insertions. For example, humans and seals share a 3 base pair deletion (bp 121-123) whereas a three base pair insertion (bp 56-58) characterises two species of frogs. The sequence of Tα1 itself, represented by the first 28 amino acids of the proTα1 molecule (bp 4-88 in this alignment plot) was not obtained for seals, but seems to consist of the more conserved portion of the proTα1 molecule.



Pro Tα1 expression in harbour seal tissues

The primers that were developed for the cloning of harbour seal proTα1 were used with ten tissue types of seals to investigate the tissue-specific synthesis of proTα1. ProTα1 cDNA was present in liver, kidney and spleen, consistent with previous studies of other species (Franco et al., 1992). ProTα1 was also produced in the blubber and skin of harbour seals, tissues that have not been previously investigated for the presence of this hormone in any of the species studied (Figure 22). The multiple sites of proTα1 synthesis suggest an independence of the thymus as a primary producer of this thymic hormone for many tissues, and possibly, a tissue type-dependent regulatory mechanism.

Figure 22. Prothymosin cDNA expression in seals.
Reverse transcriptase PCR using proTα1 specific primers was applied to cDNA of PBMCs and tissues from harbour seals to investigate tissue-specific expression. ProTα1 transcripts (233 bp) were present in PBMCs and all available tissues except heart and muscle. The presence of the transcript suggest that proTα1 (and therefore Tα1) is ubiquitously produced in seals.

ADL=adrenal gland, SPL=spleen, HEA=heart, MUS=muscle, BLU=blubber, LIV=liver, SKI=skin, KID=kidney, LUN=lung, INT=intestine, NTC=no template control.

Tα1 distribution in harbour seal tissues

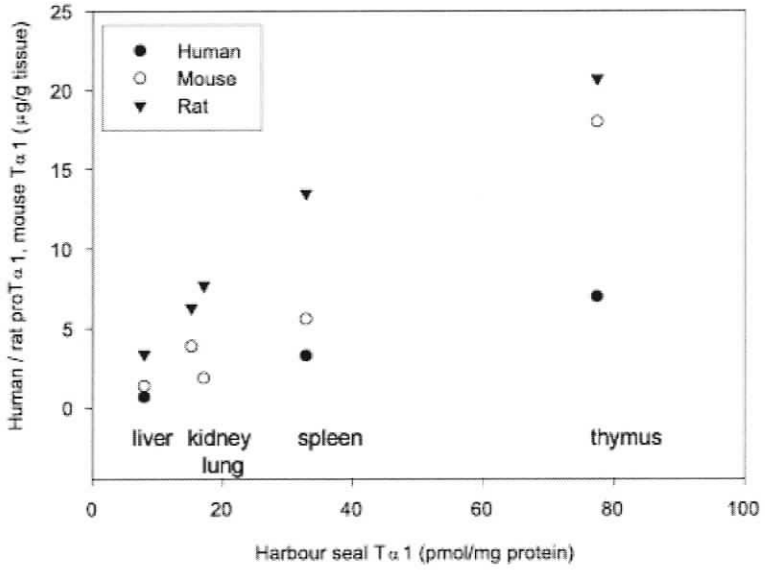
Of ten tissue types obtained from seal necropsies, all contained quantifiable concentrations of Tα1 protein. Tα1 protein concentrations were the highest in seal thymus, and the lowest in the liver (Table 13). The overall distribution of Tα1 among tissue types was similar in seals as previously reported for humans, mice and rats (Haritos et al., 1984; Tsitsiloni et al., 1993; Badamchian et al., 1997; Figure 23). Although Tα1 is not found in fat tissue of mice, seal blubber did contain relatively high concentrations of Tα1, underlining the uniqueness of this tissue in seals. Surprisingly, the adrenal gland and intestines also contained high concentrations of Tα1, which was inconsistent with the low proTα1 mRNA levels found in these two tissues. This implies that certain tissues within the body may supplement their local production of Tα1 with that released into circulation by the thymus. Muscle, heart, brain, and ovaries of seals were not available for this study, but contain low to no Tα1 according to studies of rodents (Haritos et al., 1984; Tsitsiloni et al., 1993).

Table 13. Thymosin-α1 protein concentrations in seals.

Tissue	n	Concentration (pmol mg protein^{-1})
liver	3	11.3 ± 4.1
kidney	1	15.2
lung	1	17.1
spleen	1	32.8
pancreas	1	34.2
skin	2	46.7
blubber	2	47.6
adrenals	1	49.2
intestine	1	54.3
thymus	1	77.4

Figure 23. Thymosin-α1 protein concentrations in seals and other species.

When tissue concentrations of thymosin-α1 (Tα1) as quantified in harbour seals were compared with those derived from the literature for other species (humans and rodents), an exactly similar tissue distribution was observed, with thymus being the highest and liver being the lowest of the tissue types for which data was available. Harbour seal Tα1 concentrations were overall higher than those of other species, possibly due to their young age.



Tα1 concentrations in blubber and skin biopsies of free-ranging seals

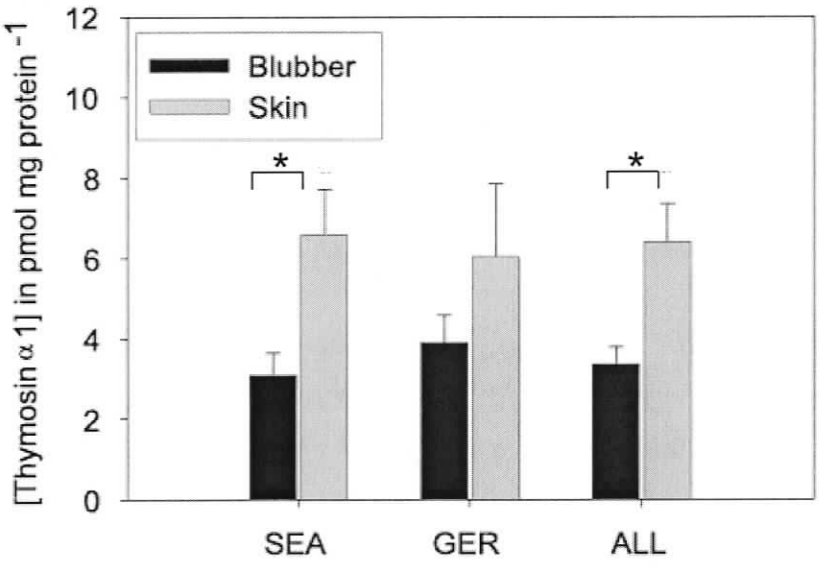
In an approach to assess its potential as a biomarker of PCB-associated immunotoxicity, Tα1 concentrations were investigated in the blubber and skin of two groups of seal pups, namely those from the Sea Island and Gertrude Island sampling locations. These seals are characterised by a 3.5-fold difference in the concentrations of PCBs found in their blubber (1034 ± 258 versus 3614 ± 573 μg kg-1 lipid weight; Trent method), allowing the investigation of a contaminant-related effect on Tα1 concentrations.

There were no significant differences in the Tα1 concentrations of skin or blubber between the two locations (Figure 24). These results are consistent with those from the Tα1 concentration-based analysis and the presence of proTα1 transcripts in blubber and skin, that suggested independent regulation of (pro)Tα1 in these compartments, and therefore uncertainty about the mechanism of action that dioxin-like compounds could exert in these tissues.

Interestingly, Tα1 concentrations were higher in skin than in the blubber. If the presence of Tα1 is linked to the immune system, this implies Tα1-regulated activity of immune components in seal skin (e.g. $^{\gamma\delta}$T lymphocytes). However, although many experts still believe that Tα1 has a primary role in immune function, alternative hypotheses have arisen over recent years that could explain high skin Tα1, based on a hypothesis of molecular mimicry. Based on its characteristic of no secondary structure in solution, it has been suggested that Tα1 uses structural homology to interfer with other proteins (Piñeiro et al., 2000). Four of such homologies have been described, and related to a potential function of Tα1 as an acceptor and carrier of histone 1 (H1) released from the chromatin (thereby facilitating decondensation during processes of transcription and replication), as a shuttle protein, and as a regulator of IFN-α2 and

vasoactive intestinal peptide (VIP) receptors (Piñeiro et al., 2000). In consideration of those alternative hypotheses, high concentrations of this hormone in skin could simply reflect the Tα1-H1 interactions that support the proliferative and differentiating rate of renewing skin compared to the relative steady state of the blubber tissue. The exact physiological role of Tα1 remains elusive and under scientific discussion, which determines the indecisive role of this small hormone as a biomarker of immunotoxicity.

Figure 24. Thymosin-α1 concentrations in free-ranging harbour seals.
Tα1 concentrations were determined in blubber and skin samples of free-ranging harbour seal pups from Sea Island (SEA; n=12) and Gertrude Island (GER; n=7) using a radioimmunoassay. Concentrations of Tα1 were significantly higher in skin than blubber in the whole group, as well as at the Sea Island location (both p=0.01). There was no significant difference between Sea Island and Gertrude Island seals in Tα1 concentrations of skin and blubber.



Conclusions

The experiments described in this chapter represented the first effort in characterizing (pro)Tα1 in a marine mammal, namely harbour seals. Although (pro)Tα1 is not commonly applied in *in vitro* assessments of lymphocyte function in any species, our immunotoxicological study underscored the sensitivity of this thymic hormone in the detection of AhR-mediated immunotoxicity. However, (pro)Tα1 concentrations is tissues were not reflective of *in vivo* exposure to dioxin-like contaminants, but likely a result of physiological processes in which it plays a role not yet completely understood.

CHAPTER 6

PCB-associated disruption of vitamin A and its receptor (Retinoic acid receptor-*α*) in free-ranging harbour seals

Portions of this chapter have been submitted for publication as follows:

Mos L, Tabuchi M, Dangerfield N, Jeffries SJ, Koop BF, Ross PS (2006). PCB-associated disruption of vitamin A and its receptor (RARα) in free-ranging harbour seals.

INTRODUCTION

PCBs have been associated with a number of toxic effects in marine mammals, such as endocrine disruption and immunotoxicity that, in turn, are widely thought to have contributed to population level impacts including reproductive failure and outbreaks of disease (Reijnders, 1986; Tabuchi et al., 2006; Brouwer et al., 1989; Ross et al., 1996a). Since the direct association between contaminant exposure and associated health effects remains difficult to investigate in free-ranging wildlife, biomarkers have been used as an alternative measure to assess adverse health effects in wildlife species. Vitamin A represents one biomarker that has been widely applied to a variety of samples (including plasma, liver, and yolk), and has been commonly accepted as a sensitive indicator of PCB-associated toxic effects in marine mammals, birds, and fish (reviewed in Rolland, 2000; Simms & Ross, 2001).

Vitamin A is a collective name for a group of lipophilic compounds, also referred to as retinoids, that are essential to growth, development, reproduction, and immune function (Blomhoff, 1994). In all mammals, the physiology of this dietary hormone is highly regulated within the body to ensure constant availability during fluctuating intake. Circulatory concentrations of vitamin A are kept within close ranges by a transport complex shared with thyroid hormone, consisting of a retinol-binding protein (RBP) and transthyretin (TTR), and excess amounts stored in the liver. In target tissues, where vitamin A is required for physiological functions, cellular retinol-binding proteins (CRBP and CRABP) prevent metabolism, and present the hormone to their nuclear receptors (Retinoic Acid Receptors or RARs). Upon binding of vitamin A to RARs, they dimerize with another nuclear receptor, the Retinoid X Receptor (RXR). This further initiates transcription by displacement of the negative regulator Silent Mediator of Retinoic Acid and Thyroid Hormones (SMRT) from retinoid responsive genes and binding of a

coactivator complex. Marine mammals are thought to share many of these basic regulatory processes for vitamin A with other mammals, which has enabled an extrapolation of our understanding of natural and contaminant-related influences on its physiology. However, some species-specific alterations of vitamin A regulation have been documented in seals (Simms & Ross, 2000; Debier et al., 2004; Debier et al., 1999; Schweigert et al., 1996). For example, two primary sites of storage can be recognised in young and adult marine mammals, namely the liver and the blubber (Mos & Ross, 2002; Kakela et al., 1997). The fact that adipose tissue plays an active role in vitamin A physiology in all mammals (Bonet et al., 2003), has led to a ready access to tissue samples in studies of free-ranging marine mammals using minimally-invasive biopsy techniques (Mos & Ross, 2002).

Although vitamin A physiology is strictly regulated in all mammals, PCBs, PCDDs, and PCDFs interfere with vitamin A transport, storage and metabolism, promoting more rapid excretion (Zile, 1992). The physiological importance of vitamin A and its response to PCB exposure in several species, has made vitamin A an attractive biomarker of contaminant-related effects in wildlife species. In addition to the effects on hormone concentrations, more recently, PCBs have also been shown to decrease the level of expression of a number of the nuclear receptors, including the thyroid hormone, progesterone and estrogen receptors (Yamada-Okabe et al., 2004; Kuil et al., 1998; Safe & Wormke, 2003). Accumulating evidence suggests that RARs could also be sensitive to the effects of PCBs. Receptor expression levels may therefore be useful as additional measures of PCB-associated effects on vitamin A physiology. RARs are present in tissues throughout the body, which is beneficial in the limited availability of tissues from marine mammals.

In this study, we assessed the applicability of vitamin A concentrations and the expression levels of one of the vitamin A receptors, RARα, as biomarkers of PCB-

associated toxic effects, in the harbour seal. These harbour seal pups were previously documented to exhibit contaminant-related effects on immune function (Chapter 3), AhR expression (Chapter 4), and disruption of thyroid hormone physiology (Tabuchi et al., 2006).

MATERIALS & METHODS

Seal sampling

Harbour seal pups were sampled in at three locations in BC and WA, namely Queen Charlotte Strait (BC), Smith Island (WA), and Gertrude Island (WA), as described in Chapter 2. Blood was collected from the extradural vein, using a 3.2-cm 19-gauge needle, into plasma-separation and heparin-containing vacutainers (Becton-Dickinson, USA), and kept at 4 °C until processing. Blubber/skin was sampled using biopsy punches (Acuderm, USA). A first biopsy, 3.5-mm in diameter, was kept in a 1.5 mL Rnase-free microcentrifuge tube containing RNAlater reagent, whereas a second biopsy, 8-mm in diameter was immediately frozen in liquid nitrogen.

Upon return in the laboratory, plasma was collected from plasma separation tubes, and frozen at -80°C. PBMCs were isolated from heparinized whole blood as described in Chapter 3. A volume equal to 10 million PBMCs was dissolved into RNAlater™ (Ambion, Canada) for RNA isolation as described in Chapter 4. These PBMC samples, together with the blubber biopsy in RNAlater, were stored at –20 degrees until further analysis. Blubber/skin biopsies, frozen in liquid nitrogen, were separated into subsamples taken over the full length of the biopsy for vitamin A analysis (consisting of approximately 40 mg of blubber and 40 mg of skin) and contaminant analysis (100 mg) under yellow light while kept frozen.

Vitamin A analyses

Skin and blubber samples were saponified to aid the efficient extraction of retinoids from small biopsy samples, as described elsewhere (Mos & Ross, 2002). Briefly, samples were added to tubes containing an ethanolic KOH solution (10 mL ethanol and 1.6 g of KOH per gram of sample; Sigma-Aldrich, Canada), 0.1% butyl-hydroxytoluene (BHT) to prevent oxidative degradation (Sigma-Aldrich, Canada), and the synthetic retinol isomer all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-OH; Hoffman-La Roche, Switzerland) as an internal standard. The tubes were incubated for 30 minutes in an 80°C water bath, with a brief mixing step at 15 minutes. After the incubation, the tubes were checked for the presence of solid tissue remains, cooled, and an equal amount of water was added. Retinol was subsequently extracted in two steps using n-hexane (VWR Canlab, Canada) and vigorous shaking for 3 minutes. A known volume of the organic layer was gently evaporated. The residue was dissolved in methanol–dichloromethane (9:1; VWR Canlab, Canada) and immediately used for HPLC analysis.

The high performance liquid chromatography (HPLC) method consisted of a reversed-phase Beckman System Gold Solvent Module 126 equipped with a Beckman System Gold Detection Module 166 for ultraviolet detection. A Zorbax Rx C8 column of 0.46 x 25, 5 μm and guard column (Chromatographic Specialties, Canada) were used to elute retinol isocratically with a 100% methanol (HPLC-grade, VWR Canlab, Canada) mobile phase at a flow rate of 1.0 mL min^{-1} at 325 nm. Calibration curves were obtained using an all-trans retinol (Sigma-Aldrich, Canada) standard solution, that was quantified by absorption spectroscopy, and serial diluted to obtain at least five known concentrations for subsequent HPLC analysis. All work was performed under yellow light, using HPLC-grade solvents during extraction and analysis. Blood plasma samples were analyzed for

all-trans retinol and retinol-palmitate by a clinical laboratory according to their established laboratory procedures (Central Lab for Veterinarians, Langley, B.C.).

RARα cloning

PCR primers for the harbour seal RARα were designed using Primer Premier v4.1 software (Premier Biosoft International, USA) against the conserved regions of human, rat, mouse and zebrafish RARα (Table 15), and synthesized by AlphaDNA (Montreal, Canada). The primer sequences are 5'-CAGTACTGCCGRCTGCAGAA-3' (forward) and 5'-GCATCATCCATCTCCAGGG-3' (reverse). DNA amplification was performed on a Gene Amp®PCR System 9700 (PerkinElmer Biosystems, USA). The PCR thermocycle consisted of 95°C/9 minutes, followed by 40 cycles of 95°C/15 seconds, 55°C/30 seconds, and 72°C/45 seconds.

PCR products were separated on a 1.5% agarose gel and visualized with an ethidium bromide staining. Bands equal to the expected PCR product size of 551 base pairs were isolated, and exposed to three 5-minute cycles of freezing and thawing, on dry ice and in a 37°C waterbath, to elute the cDNA. Amplified cDNA sample was cloned using a pCR2.1-TOPO vector and the TOPO TA Cloning Kit (Invitrogen, Canada). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Canada), and sequenced in both directions. The cloning reaction and following sequencing were performed twice on separate samples to assure accuracy. The obtained nucleotide sequence was compared by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST) to other RAR sequences available in GenBank, to confirm the identity, and aligned to these sequences using ClustalW (http://clustalw.genome.ad.jp), for comparison to other species. The alignment was shaded in areas of similarity using Boxshade v3.33C (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade-simple.html). Six-frame translation was used to obtain all possible

protein sequences, and the protein sequence that matched that of the other RAR proteins was used to deduce protein similarity percentages (identies) by BLASTp.

Real-time quantitative polymerase chain reaction (QPCR) assay

Total RNA was isolated from PBMCs and blubber samples and a cDNA sample produced as described in Chapter 4. RARα expression in PBMCs and blubber was quantified using a QPCR assay performed on a MX3000p system (Stratagene, USA). QPCR primers were identical to those used during cloning, but with an altered thermocycle consisting of a 95°C/9 minutes enzyme activation step, 40 cycles of denaturing at 95°C/15 seconds, annealing at 58°C/57 seconds, and extension at 72°C/45 seconds, followed by a final extension step of 10 minutes at 72°C. Each DNA amplification reaction (15 μl) included QPCR buffer (10 mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, and 40.000-fold diluted SYBR Green I), 10 pmol of each primer, 2μl of 20-fold diluted cDNA template, 200 μM equimolar dNTPs (dATP, dCTP, dGTP, and dTTP; Invitrogen, Canada), 83.3 nM ROX reference dye (Stratagene, USA) and 1.0 units of platinum Taq DNA polymerase (Invitrogen, Canada Inc). PBMC samples were quantified in quadruplicate and blubber samples in duplicate, and the values averaged. Plasmid DNA was used to obtain a calibration curve that allowed the conversion of threshold cycle numbers into copy numbers. RARα expression values for PBMCs were normalized using the amount of total RNA obtained from 10^6 cells, whereas RARα expression values for blubber samples were normalized to those of the expression of the ribosomal protein L8 internal control, as described elsewhere (Tabuchi et al., 2006).

Data analysis

Data of vitamin A concentrations and RARα were explored for outliers, and significant outliers (average +/- two times the standard deviation) were removed. Differences among locations were assessed using ANOVA, followed by Tukey`s post-hoc test in case of a normal distribution and homogeneity of variance, and by a Kruskal-Wallis and Mann-Whitney test of significance in all other cases. PCB concentrations (based on RDL analyses, for comparability to a study of thyroid hormones in these seals (Tabuchi et al., 2006) were log transformed to obtain a normal distribution. For the investigation of correlations in the whole group of seals, log transformation of vitamin A concentrations in blubber and skin, and RARα expression levels in blubber (times 100) and PBMCs, was used to optimise normality. Vitamin A concentrations in plasma were not log normalized, because untransformed data followed the normal distribution more closely. Parameters were subsequently assessed for associations with each other, body weight, lipid concentrations (in blubber), and (the log of) contaminant concentrations using Pearson correlation coefficients. Results were considered significant at $p<0.05$.

RESULTS

Vitamin A concentrations in harbour seals

Among the three sampling locations, a total of 22 seals were sampled for this study. This group consisted of an equal distribution among sexes of 11 females and 11 males. The average body weight of seal pups was 20.4 ± 0.7 kg, and did not differ among the three locations ($p=0.100$; ANOVA). All seal pups were in good health as assessed by size (body weight-length-girth), the absence of lesions on skin, eyes, and mouth, and hematology. One seal, that represented a significant outlier in vitamin A concentrations of both blubber and skin, was removed from the data set. Gertrude Island seals had

significantly lower circulatory concentrations of retinol and lower concentrations of total retinol in blubber than their counterparts from the reference site, Queen Charlotte Strait. There were no significant differences between the reference site and the two sampling locations in circulatory retinyl palmitate concentrations and concentrations of total retinol in skin (Table 14).

Table 14. Vitamin A concentrations in plasma and tissues of seals.
Average vitamin A concentrations (± SEM) in plasma, blubber and skin of harbour seal (*Phoca vitulina*) pups live-captured at three sites in coastal British Columbia, Canada (Queen Charlotte Strait) and Washington State, USA (Smith and Gertrude Islands). Significant differences between the sampling locations were tested using ANOVA, and, where significant, Tukey's honestly significant difference test was applied post-hoc, to determine which site(s) differed from the reference site, Queen Charlotte Strait.

		n	All	Queen Charlotte Strait	Smith Island	Gertrude Island
Plasma Retinol	(μg/L)	22	290.2 ± 24.4	345.1 ± 34.5	301.7 ± 36.0 N.S.	187.3 ± 28.1 p=0.014*
Plasma Retinyl-palmitate	(μg/L)	22	204.1 ± 33.6	267.7 ± 41.1	208.2 ± 70.8 N.S.	94.0 ± 55.3 N.S.
Blubber Total retinol[1]	(μg/g)	16	3.36 ± 0.52	4.63 ± 1.01	3.97 ± 0.46 N.S.	1.69 ± 0.38 p=0.026*
Skin Total Retinol[1]	(μg/g)	16	2.19 ± 0.23	2.13 ± 0.33	-	2.26 ± 0.31 N.S.

[1] Total retinol in blubber and skin represents the total of retinol and retinol-esters.

The harbour seal RARα

A partial RARα cDNA sequence from harbour seals was obtained and deposited into Genbank with accession number DQ767970 (Figure 25). The cloned DNA sequence (512 base pairs trimmed of primer sequences) is approximately 40% of the total RARα cDNA sequence (1398 base pairs in the mouse). The sequence overlaps with parts of the DNA binding domain, ligand binding domain, and the intermediate sequence. The harbour seal RARα cDNA sequence reveals conservation to many other vertebrates

(Table 15). The nucleotide sequence of the seal RARα was most similar to that of dogs, and was also highly similar to humans and rodents. This high level of conservation was not just reflective of the partial sequence obtained for harbour seals, but seems to be a property of the complete receptor sequence (Figure 26). At the protein level, seals were most similar to humans and rodents (Table 15).

Figure 25. RAR*α* nucleotide and putative amino acid sequence of seals.
A partial harbour seal RAR*α* nucleotide sequence was obtained during this study and submitted to GenBank (http://www.ncbi.nlm.nih.gov) under the accession number DQ767970. The shaded regions represent the areas of primer annealing.

```
     Q  Y  C  R  L  Q  K  C  F  E  V  G  M  S  K  E  S  V  R  N
1    cagtactgccggctgcagaagtgcttcgaagtgggcatgtccaaggagtctgtgaggaat  60
     D  R  N  K  K  K  K  E  A  P  K  P  E  C  S  E  S  Y  T  L
61   gacagaaacaagaagaagaaggaggcgcccaagcccgagtgctccgagagctacacgctg  120
     T  P  E  V  G  E  L  I  E  K  V  R  K  A  H  Q  E  T  F  P
121  acgcccgaggtgggggagctcatcgagaaggtgcgcaaagcgcaccaggaaaccttccct  180
     A  L  C  Q  L  G  K  Y  T  T  N  N  S  S  E  Q  R  V  S  L
181  gccctctgtcagctgggcaaatacactacgaacaacagctcagagcaacgtgtctctctg  240
     D  I  D  L  W  D  K  F  S  E  L  S  T  K  C  I  I  K  T  V
241  gacattgacctctgggacaagttcagtgaactctccaccaagtgcatcattaagactgtg  300
     E  F  A  K  Q  L  P  G  F  T  T  L  T  I  A  D  Q  I  T  L
301  gagttcgccaagcaactgcccggcttcaccaccctcaccattgctgaccagatcaccctc  360
     L  K  A  A  C  L  D  I  L  I  L  R  I  C  T  R  Y  T  P  E
361  ctcaaggctgcctgcctggacatcctgatcctgcggatctgcacgcggtacacgcccgag  420
     Q  D  T  M  T  F  S  D  G  L  T  L  N  R  T  Q  M  H  N  A
421  caggacacaatgaccttctccgatgggctgaccctgaaccggacccagatgcacaatgcc  480
     G  F  G  P  L  T  D  L  V  F  A  F  A  N  Q  L  L  P  L  E
481  ggcttcggccccctcacggacctggtcttcgccttcgccaaccagctgctgcccctggag  540
     M  D  D
541  atggatgatgc  551
```

Figure 26. RAR*α* (partial) nucleotide sequence of seals and other species.

A comparison of the partial harbour seal RARα nucleotide sequence with those earlier reported for human, mouse, and newt, show a high level of conservation among vertebrates in both the area characterized for the harbour seal and other regions of this receptor.

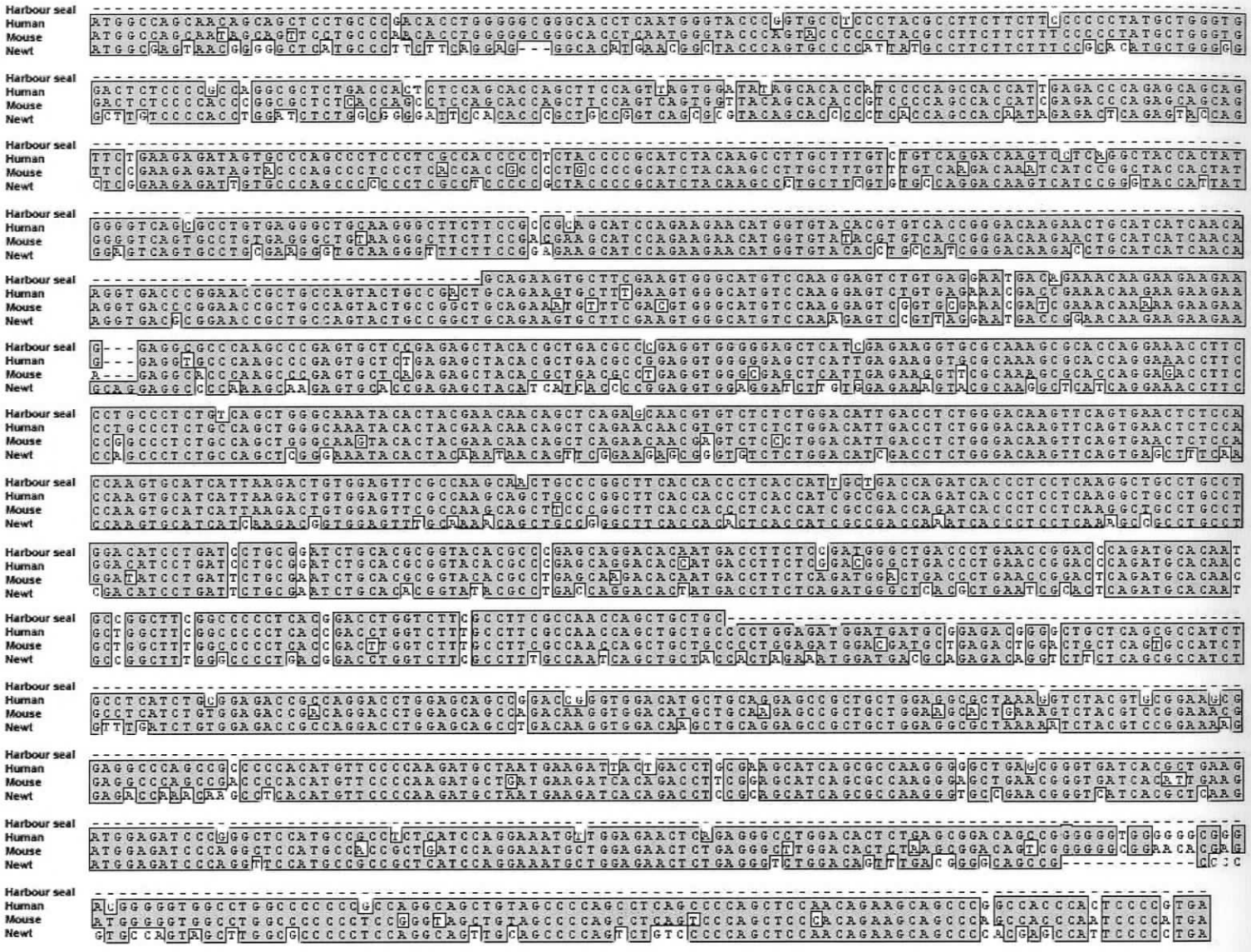


Table 15. RARα sequence comparison of seals and other species.

The cloned and sequenced cDNA nucleotide and putative protein sequence of the harbour seal (Genbank accession number DQ767970) were compared with sequences available for other species in GenBank (http://www.ncbi.nlm.nih.gov).

		Phoca vitulina RARα	
	Genbank accession #	Nucleotide (512 bp)[1]	Amino acid (170 aa)[1]
Dog (*Canis familiaris*)	AB179779	96	98
Human (*Homo sapiens*)	BC008727	96	99
Mouse (*Mus musculus*)	BC010216	92	97
Rat (Rattus *norvegicus*)	BC099830	92	99
Hamster (*Mesocricetus auratus*)	AY046943	90	99
Newt (*Notophthalmus viridescens*)	X17585	84	94
Zebrafish RARα-b (*Danio rerio*)	BC049301	83	86
Zebrafish RAR α-a (*Danio rerio*)	BC092692	81	89

1 Values represent the percentage identity for the comparable nucleotide and putative amino acid sequences.

Correlations among vitamin A endpoints

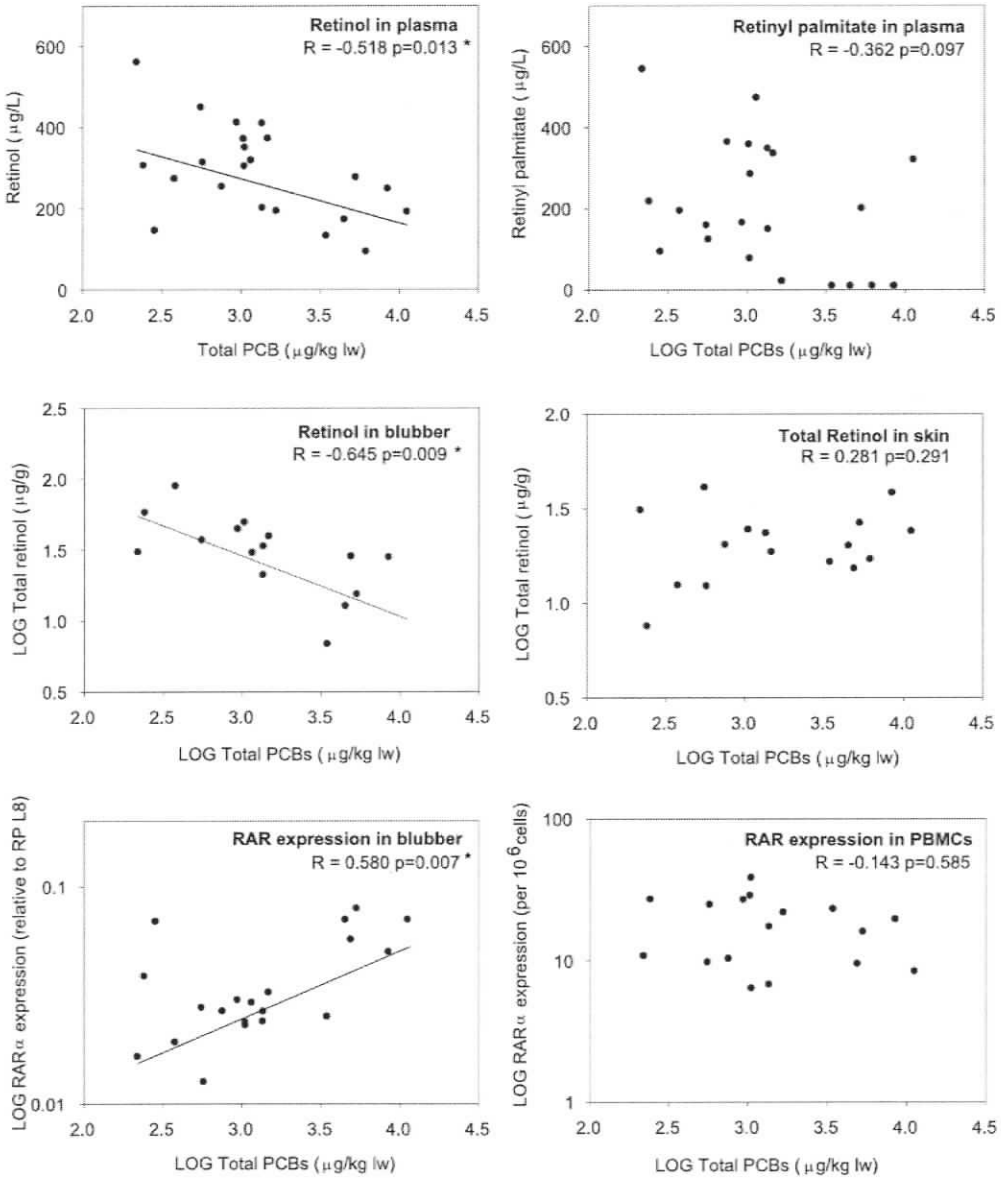
Among the six parameters of vitamin A physiology measured, three showed significant correlations with each other. The two forms of vitamin A found in plasma, retinol and retinyl palmitate, were positively correlated (R= 0.663, p=0.001; Pearson). Circulatory retinol also correlated to RARα expression in blubber (R= -0.507, p=0.027; Pearson). Circulatory retinyl palmitate was correlated to RARα expression on circulatory white blood cells (PBMC; R= -0.548, p=0.028; Pearson). Vitamin A concentrations did not correlate among tissue compartments. Vitamin A concentrations and RARα expression levels were not associated with seal body weights ($p \geq 0.20$), and blubber vitamin A concentrations showed no relationship to blubber lipid percentages p=0.142).

PCB-associated effects on vitamin A concentrations and RAR expression

Total PCB concentrations in the seals averaged 2506 ± 594 μg/kg lipid weight in blubber, and were lowest at Queen Charlotte Strait (683 ± 137 μg/kg lipid weight), intermediate at Smith Island (1190 ± 110 μg/kg lipid weight), and highest at Gertrude Island (6237 ± 1008 μg/kg lipid weight). Average PCB concentrations at Smith and Gertrude Islands were significantly higher than that of the reference site, Queen Charlotte Strait (p=0.05 and p=0.001, respectively; Mann-Whitney). PCB concentrations were negatively associated with vitamin A concentrations in plasma (retinol) and in blubber, and positively associated with the relative RARα expression of blubber (Figure 27). The concentration of retinyl palmitate in plasma, vitamin A in skin, and RARα expression in PBMCs were not correlated to PCB concentrations. The log ratio of vitamin A concentrations in blubber to skin, that reflects the amounts of vitamin A in (blubber) storage relative to that available for direct use in the skin, also correlated significantly to the concentration of PCBs (R=-0.613, p=0.045; Pearson).

Figure 27. Associations between vitamin A and PCBs in seals.

Correlation analyis of vitamin A concentrations, RARα expression levels, and PCB concentrations in harbour seals indicated that vitamin A transport and storage, as well as its receptor expression are affected by these contaminants, whereas skin and circulatory cells remain unaffected.



DISCUSSION

Vitamin A appears to be highly regulated in seals

Vitamin A is an essential dietary component for mammals, which is reflected in its highly regulated transport, storage, and metabolic processes. Marine mammals consume a diet that is considered to be rich in vitamin A, such that they may not be at risk of vitamin A deficiencies under natural conditions. However, young marine mammals such as harbour seal pups rely on the amounts of vitamin A transferred from its mother's stores to milk for their early developmental processes (Debier & Larondelle, 2005).

The short duration of the lactation period in many species of seals requires the pup to cope with high concentrations of dietary vitamin A, and efficiently deposits of the hormone for later use (e.g. during weaning). Some of the evolutionary adaptations that may be involved in the latter include the presence of three forms of circulatory vitamin A, namely the highly regulated amounts of retinol bound to a carrier complex (TTR-RBP, similar to humans and rodents), but also free retinol and retinol esters. Furthermore, seal pups are able to deal with elevated circulatory concentrations of vitamin A which would be considered toxic in most other mammals (Simms & Ross, 2000). Lastly, seals use blubber as an important storage compartment in addition to the liver (approximately 66% of the vitamin A body burden is stored in the blubber of seal pups (Mos & Ross, 2002). In this study, we documented another potential adaptive mechanism, consisting of what appears to be a vitamin A-dependent downregulation of RARα on both circulatory cells and in the blubber. Decreased expression of the receptor in cells and/or tissues that are being exposed to high concentrations of vitamin A could prevent that toxic effects take place.

Although certain regulatory mechanisms of vitamin A physiology in seals may have evolved differently from other mammals, the structure of one of its receptors, RARα, on the other hand, was highly similar to that of other mammals, and did not reflect the high

dietary vitamin A concentrations in the harbour seal food chain. The harbour seal RARα nucleotide sequence was most similar to that of its closest evolutionary counterpart studied, the domestic dog, and decreased in its similarity to other species in general accordance with the taxonomic tree of species evolution. This pattern was somewhat different at the protein level, but reflected an even higher degree of conservation.

PCB-associated disruptions of vitamin A physiology

Vitamin A concentrations of retinol and retinyl palmitate correlated in the study seals, likely reflecting their common origin in the diet. Plasma retinol, however, but not retinyl palmitate, also significantly correlated to the PCB concentrations in blubber. This suggests that a reduced dietary intake does not underlie this observation, but rather that a contaminant-related disruption of the retinol transporting protein complex (TTR-RBP) is taking place.

PCB metabolites (hydroxylated PCBs) are known to effectively compete for the thyroid hormone binding site on TTR, reducing the affinity of TTR for RBP, and eventually resulting in the dissociation and glomerular excretion of RBP-retinol (Brouwer et al., 1986). Decreased concentrations of circulatory retinol have been observed in wildlife species exposed to PCBs and structurally related compounds including polar bears, sea lions, mink, and herring gulls (Debier et al., 2005; Grasman et al., 1996; Braathen et al., 2004; Kakela et al., 1999), as well as harbour seals (Jenssen et al., 1995; Brouwer et al., 1989). Disruption of circulatory thyroid hormones (T4), which would presumably accompany a PCB metabolite related disruption of vitamin A, was also observed in the study seals (Tabuchi et al., 2006), and provides further support that this mechanism of toxicity likely underlies our observations.

The ability of PCBs to affect vitamin A stores in blubber could originate at multiple levels. Firstly, the observed disruption of circulatory vitamin A could result in a lower amount available for storage in the blubber of seals. Secondly, PCBs are known to increase the release of vitamin A from liver storage, and may exert similar effects on blubber stores. Although the vitamin A esterification enzymes (retinol acyltransferases and retinyl ester hydrolases) that are a primary target of PCBs in the liver have not been characterized in seal blubber, elevated mobilization from storage may also result from contaminant-related upregulation of other enzymes. Thirdly, an increased demand from target tissues could have increased mobilization from storage to sites of use. A higher rate of vitamin A excretion, and subsequent higher demand, is believed to originate from the contaminant-associated induction of metabolizing enzymes (e.g. cytochrome P450 1A) that also play a role in vitamin A homeostasis.

The hypothesis that icreased demands from a target tissue such as skin could result into increased mobilization from vitamin A blubber stores is supported by an earlier observed equilibrium between vitamin A concentrations in blubber and skin in relatively uncontaminated seals (Mos & Ross, 2002), that we did not observe in this study of seals spanning a wide range of contaminant concentrations (0.22-11.1 mg/kg lipid weight). Constant vitamin A concentrations in skin, a major site of use adjacent to the blubber, implies that its levels are maintained in tissues where a physiological need exists for the hormone. The ratio of vitamin A in blubber to skin decreased at the higher contaminant concentrations, suggesting that stored vitamin A may be readily delivered to target tissues to compensate and help prevent adverse health effects such as diminished growth and development.

Elevated RAR expression in blubber could reflect an upregulation caused by reduced concentrations of vitamin A present in this tissue compartment, or a direct PCB-associated effect at the molecular level. A limited number of studies have investigated

the effects of contaminant exposure on RARs. RARα and RARβ expression levels were found to decrease as a result of contaminant exposure (Weston et al., 1995; Krig et al., 2002). These effects were related to interference of retinoic acid-RAR binding by the AhR. In addition, a contrary mechanism of action underlying effects at the receptor level was recently elucidated, explaining an increase in RAR expression by AhR mediated sequestration of SMRT (Widerak et al., 2006). This would relieve RARs from the suppressive actions that SMRT exerts upon them in the absence of ligand. Our study showed the potential utility of RARα expression measurements as a sensitive biomarker of contaminant-associated effects in harbour seals, and further studies into the molecular mechanisms of action would therefore benefit its application.

Vitamin A and PCBs behave in similar manners in the blubber of seals

Vitamin A and PCBs share many features within seals. Both vitamin A and PCBs associate with lipid, accumulate with age, and dilute with growth. This dual relationship in which PCBs and body weight are correlated with vitamin A in seals highlights the potential for confounding influences to complicate wildlife toxicology studies. In this study, confounding effects of natural factors were eliminated or carefully controlled by using animals of known sex and age, with no differences in body weight or condition, identifying multiple potentially contaminant-related effects on vitamin A physiology in seals. Although it is possible that multiple mechanisms of action may underlie our observations, we suggest that lower (circulatory and stored) concentrations of vitamin A and elevated receptor expression, are likely related to a single mechanism of action, e.g. disruption of vitamin A transport.

Conclusions

This study documented a disruption of vitamin A physiology in harbour seals, suggesting that contaminants are adversely affecting this dietary hormone in free-ranging marine mammals. Furthermore, observations of reproductive impairment (Reijnders, 1986), skeletal abnormalities (Zakharov and Yablokov, 1990), and immunotoxicity (Ross et al., 1996; De Swart et al., 1994) in contaminated marine mammals represent symptoms that have also been observed in malnutrition-associated vitamin A deficiency in humans (Belamarich, 1998; Hofmann and Eichele, 1994; Eskild and Hansson, 1994; West et al., 1991). While the mechanisms of action underlying the marine mammal and human observations differ (i.e. contaminants vs malnutrition), it is evident that disruption of vitamin A represents a serious threat to the health of the individual. Therefore, vitamin A represents a meaningful biomarker of high sensitivity in toxicological studies of marine mammals. Our combination of biopsy-based techniques, coupled with the characterization of the molecular structure of the harbour seal RARα, provides a basis for further minimally-invasive biomarker studies of contaminant-associated toxicity in marine mammals.

CHAPTER 7

Canine Distemper Virus in river otters in British Columbia as an emergent risk for coastal pinnipeds

Portions of this chapter first appeared in the following publication:

Mos L, Ross PS, McIntosh D, Raverty S (2003). Canine Distemper Virus in river otters in British Columbia as an emergent risk for coastal pinnipeds (Short communication). Veterinary Record **152**, 237-239.

The spill-over of terrestrial pathogens into marine mammal populations has been recognised as one of the primary factors in the world-wide occurrence of mass mortalities among them. While members of the genus *Morbillivirus* (Paramyxoviridae) appear to be enzootic in some marine mammal species (Ross et al., 1992; Visser et al., 1993), others have emerged following interspecies transmission resulting in several mass mortalities over recent decades (for review see (Osterhaus et al., 1995). For example, an outbreak of phocine distemper virus-1 (PDV-1) in 1988 killed up to 60 percent of the harbour seals (*Phoca vitulina*) in northwestern Europe (Osterhaus & Vedder, 1988). Migrating harp seals (*P. groenlandica*) were believed to have introduced the virus into harbour seal populations (Dietz et al., 1989a; Ross et al., 1992). Canine distemper virus (CDV) epizootics among Baikal seals (*P. sibirica*) in 1987-88 (Visser et al., 1990) and Caspian seals (*P. caspica*) in 2000 (Kennedy et al., 2000) were attributed to domestic dogs (Grachev et al., 1989; Kennedy et al., 2000). Additionally, a mortality among Crabeater seals (*Lobodon carcinophagus*) coincided with an introduction of sled dogs into Antarctica in 1955 (Laws & Taylor, 1957). Retrospective analysis identified CDV as the putative agent responsible (Bengtson et al., 1991).

Since morbilliviruses commonly infect both marine and terrestrial species (Osterhaus et al., 1995; Deem et al., 2000; Williams, 2001), animals with a semi-aquatic existence may serve as vectors and facilitate disease transmission between species of the marine and terrestrial environment. River otters (*Lutra canadensis*), for example, are found along the coast and in the estuaries of British Columbia (BC), Canada. In 1999, two river otter pups were removed from beneath a human dwelling on one of the coastal islands of southern British Columbia, Canada, and admitted into a rehabilitation facility. One individual (male) died without clear indications as to the cause of death; no necropsy was performed. The other individual, a female, was anorexic after admission, developed

a serous nasal and mucopurulent ocular exudate, and died 5 days later. The clinical signs were consistent with CDV in mustelids (Williams, 2001). A field post mortem was conducted and representative tissues were forwarded to the Animal Health Center (Abbottsford, BC) for diagnostic evaluation. Histopathology revealed a subacute broncho-interstitial pneumonia with type II pneumocyte hyperplasia, scattered alveolar syncytia, and rare intracytoplasmic eosinophilic inclusions. Based on the microscopic lesions, a presumptive diagnosis of morbillivirus infection was investigated by PCR (Shin et al., 1995) and immunohistochemistry (IHC) specific for canine distemper. Suspensions of homogenized lung tissue were innoculated on Marbin Dawby and Vero cell lines for viral culture. Although no virus was successfully isolated from tissues, both the PCR and IHC were positive for CDV.

Review of previous case reports on file did not indicate that CDV is enzootic in BC river otter populations (S. Raverty, unpublished data). However, CDV has been reported in free-ranging river otters from Alaska (n=1; R. Zarnke, Director Division of Wildlife Conservation, Alaska Department of Fish & Wildlife; personal communication) and New York, U.S.A. (n=3; Kimber, 2000), as well as captive otters (Bronx Zoo, New York, U.S.A.; N. Duplaix, WWF Giant Otter Program; personal communication), confirming the susceptibility of this species to the virus in question. Post mortem examination of 110 harbour seals from 1993-2002 has failed to detect any evidence of morbillivirus infection in this species in BC (S. Raverty, unpublished data). In addition, no seropositive harbour seals have been identified following virus neutralization screening of archived plasma samples (P.S. Ross and A.D.M.E. Osterhaus, unpublished data), suggesting that this species represents an unlikely source of CDV for river otters. Previous documentation of a number of CDV cases in BC raccoons (*Procyon lotor*) and coyotes (*Canis latrans*) (Table 16), as well as domestic dogs (11 cases in the period of 1995-2000; S. Raverty,

unpublished data), suggests that these river otters may have contracted CDV from a terrestrial host species.

Table 16. Cases of CDV in terrestrial wildlife in British Columbia, Canada.
Besides the prevalence of CDV in an aquatic mammal sharing its environment and often its food with seals, previous cases of CDV in terrestrial wildlife in British Columbia identify a number of additional potential vectors that could introduce CDV into harbour seal populations. Coyotes are known to prey on seal pups, while racoons commonly scavenge seal haul-out sites. The prevalence of CDV, in combination with the increasing interactions between humans, domestic pets, and wildlife, and the elevated abundance of scavenging species such as coyotes and racoons nearby human agglomerations, indicates an increasing risk of CDV introduction into the naïve BC harbour seal population. PCR=polymerase chain reaction; FAT=fluorescent antibody titer.

Species	Number of cases	Age class	Location	Date	Diagnostic modality
Coyote	1	Pup	Salmon Arm	26/07/1996	Histopathology
Raccoon	2	Yearlings	Vancouver	12/05/1998	Histopathology / PCR
Raccoon	1	Yearling	Vancouver	15/05/1998	Histopathology / FAT
Raccoon	1	Yearling	Vancouver	22/05/1998	Histopathology / FAT
Raccoon	1	10 weeks	Victoria	25/06/1998	Histopathology / PCR
Raccoon	> 8	Adults/yearlings	Vancouver	13/11/1998	Histopathology / PCR

Since morbilliviruses are enzootic in some populations of marine mammals, infection with morbilliviruses may only lead to mass mortalities in immunologically naive (Neel et al., 1970; Harder et al., 1995) or highly susceptible (Harder & Osterhaus, 1997) populations. The immunologically naive populations of Pacific pinnipeds may therefore be vulnerable to infection by CDV or other morbilliviruses. In addition, the high levels of immunotoxic POPs often found in marine mammals are thought to have exacerbated previous morbillivirus-related mass mortalities (De Swart et al., 1994; Ross et al., 1996a;

Ross et al., 1996c; Ross, 2002). Correlative evidence of immunotoxicity and endocrine disruption associated with PCB exposure, as described in this thesis (Chapter 3, 4, 6), suggest that environmental contaminants are adversely affecting the health of harbour seals in this region.

In BC, there are more than 108.000 harbour seals (Olesiuk et al., 1990) and between 15.000 and 30.000 river otters, most of which reside in the marine environment (M.J. Badry, BC Ministry of Water, Lands, and Parks; personal communication). We report here the emergence of CDV in an aquatic mammal that frequents coastal BC. Such an occurrence may not be an isolated episode; interactions between an infected river otter and seronegative harbour seals or other pinnipeds could trigger a catastrophic epizootic. While harbour seal haul-outs provide an ideal environment for intra- and interspecies viral transmission, the rehabilitation of often susceptible animals of multiple species at animal care facilities may provide an additional, and potentially significant, mechanism for interspecies transmission. Anthropogenic impacts on coastal ecosystems and the increasing interactions between humans, domestic animals and wildlife (Osterhaus, 2001) are likely to facilitate the emergence of infectious diseases in marine mammals in BC and elsewhere.

CHAPTER 8

Discussion

Mass mortalities among marine mammals represent complex events

Of the several mass mortality events that have taken place among marine mammals (as described in Chapter 3 and 7), the die-offs of harbour seals in Northwestern Europe in 1988 and 2002 have been the most extensively studied. An at the time novel Morbillivirus, PDV was identified as the primary cause of the mortality (Mahy et al., 1988; Cosby et al., 1988), but this did not end the speculation about other probable causes of both a natural and anthropogenic kind. Natural circumstances included low food abundance with consequent harp seal migration southwards, introducing a novel virus into the naïve harbour and grey seal populations, and abnormal warm temperatures leading to increased seal haul-out behaviour, facilitating disease transmission (Heide-Jörgensen et al., 1992). Anthropogenic influences may have been associated both with prey abundance (overfishing) as well as environmental contamination with immunotoxic substances (Dietz et al., 1989a; Ross, 2002). Although the latter hypothesis was later confirmed in a semi-field approach with harbour seals, direct evidence of immunotoxicity in free-ranging seal populations has been scarcely documented.

In this study, a comprehensive evaluation of immunotoxicology in free-ranging marine mammals was carried out, in which challenges were overcome in obtaining samples from healthy seals using minimally-invasive techniques, minimising confounding factors, processing the samples immediately and appropriately in the field, transporting the samples rapidly to a cell culture facility, and conducting functional assays. By evaluating the responses and concentrations of several white blood cell populations, and comparing these to the concentrations of PCBs measured in blubber biopsies, it was revealed that more contaminated seals had compromised immune function. By combining the single measures of immune function in a PCA model immunological

profiles of individual seals provided further evidence of a PCB-associated influence on the immune system (Chapter 3).

Immunotoxic effects in seals are of concern since their immune system represents a critical barrier against pathogens, and when adversely affected, the immune system may be less able to respond to pathogens. Therefore, the low immunocompetence associated with PCB exposure may be affecting free-ranging harbour seals in BC and WA by predisposing them to disease (Chapter 3).

The combination of immunotoxicity and emerging pathogens

In addition to the importance of the PCA model in illustrating the effects of PCBs on the complex immune system, the obtained immunological profiles of harbour seals also reflected elevated pathogen exposure in urban coastal regions. Observations of sewage-related bacteria and protozoa, and pathogens typical of terrestrial wildlife species, in urban seals furthermore suggested that biological pollution may be influencing the types and numbers of pathogens marine mammals are exposed to (Chapter 3).

Pathogen spill-over has recently been recognized as one of the putative causes of mass mortality events in Northwestern Europe, explaining the occurrence and the scale of the PDV epidemics. Grey seals have been suggested to act as reservoirs for PDV and/or act as subclinical infected carriers of the virus between Arctic and North Sea populations of pinniped species. Grey seals may be responsible for the initial introduction of PDV into European harbour seal populations. Subsequently, the disease was only able to spread with geographical jumps to other (non-migratory) harbour seal populations by further species crossover. It is likely that grey seals, which show long-distance movements, facilitated this process, by acting as a vector species (Harkonen et al., 2006).

Although pathogen spill-over may be occurring naturally, human activities may increase the prevalence of such events, for example by the current practice of wildlife rehabilitation in Canada that allows multiple species, including terrestrial and marine mammals, within one facility. The documented case of a (semi-)aquatic species infected with a morbillivirus closely related to PDV in BC illustrates the risk such an isolated case may represent. These risks extend to marine mammals within the rehabilitation centre, and free-ranging marine mammals in the region (Chapter 7). The continued use of antibiotics and live vaccines in rehabilitation, pets, and zoos, are other human activities that may facilitate spill-over of pathogens into susceptible marine mammals.

Human activities in coastal regions are believed to have played an important role in the increase in emerging pathogens and the number of documented diseases among marine organisms at many levels of the ecosystem, ranging from coral reef micro-organisms to marine mammals (Daszak et al., 2000; Harvell et al., 1999). National and international regulatory offices have recognised this increasing threat of land-based activities to marine ecosystems. During an intergovernmental conference to adopt a global programme of action, both POPs and sewage were among the significant threats listed with a need for action (United Nations Environment Programme, 1995).

Although it is recommended that a study specifically designed to assess possible impacts of biological pollution in urban seals will be carried out, this current study underscores that the combination of both chemical and biological pollution, in particular, may represent a risk to the health of marine mammals, by introducing (new) pathogens into susceptible animals.

Contributions to the weight of evidence of immunotoxicity in marine mammals

This study set out to contribute to the weight of evidence in support of the notion that environmental contaminants are immunotoxic to marine mammals by supplying evidence of immunotoxicity in free-ranging healthy harbour seals while controlling for confounding factors. For additional lines of evidence, the immunotoxicological study was combined with a biomarker approach. Biomarkers, in particular, could be powerful tools in the study of marine mammals, because they often require less invasive methods than traditionally used immunological assays, and are therefore applicable to a wider range of samples and species.

Table 17. Overview of PCB-associated toxic effects in seals.
A number of parameters were investigated during the 2003 field study of harbour seals described in this thesis and elsewhere (Tabuchi et al., 2006), documenting effects of PCBs on the immune and endocrine systems of seals. Abbreviations as used elsewhere; TT4 = total thyroxine; TR = thyroid receptor.

Immunotoxicity	T Lymphocyte function	↓	R = -0.400 p = 0.028	Chapter 3
	T Lymphocyte signaling	↓	R = -0.412 p = 0.025	Chapter 3
	Total lymphocyte count	↓	R = -0.539 p = 0.002	Chapter 3
	Phagocytosis	↓	R = -0.548 p = 0.002	Chapter 3
	Respiratory burst	↑	R = 0.671 p < 0.001	Chapter 3
	AhR expression on PBMCs	↑	R = 0.360 p = 0.034	Chapter 4
Endocrine disruption	Circulatory vitamin A (retinol)	↓	R = -0.518 p = 0.013	Chapter 6
	Blubber vitamin A (total retinol)	↓	R = -0.645 p = 0.009	Chapter 6
	Blubber RARα expression	↑	R = 0.580 p = 0.007	Chapter 6
	Circulatory TT4	↓	R = -0.711 p < 0.001	Tabuchi et al., 2006
	Blubber TRα expression	↑	R = 0.679 p < 0.001	Tabuchi et al., 2006

An overview of the adverse health effects and their associations with PCB concentrations can be found in Table 17. Besides the immunotoxic effects described in this study, AhR expression on circulatory white blood cells (Chapter 4), and vitamin A concentrations in plasma and blubber as well as retinoic acid receptor expression in blubber (Chapter 6) were reflective of contaminant exposure in free-ranging harbour seals. Furthermore, a disruption of thyroid hormones and thyroid receptor (TR) expression in these same seals was described elsewhere (Tabuchi et al., 2006). Combining this toxicological evidence of harbour seals suggests that PCBs are adversely affecting the health of British Columbia and Washington State seals at the immunological and endocrine level. This information is especially relevant from a point of view that BC seals are considered low to moderately contaminated compared to harbour seal populations elsewhere, and other marine mammal species worldwide (Chapter 2).

A critical evaluation of biomarkers

Biomarkers are identified as easily quantifiable endpoints at the molecular, biochemical, or physiological level, which can be related to exposure to a specific contaminant. Biomarkers should therefore ideally be (1) relevant to the adverse health effect studied, (2) sensitive (i.e. affected at low doses), and (3) specific (not affected by multiple stressors and/or natural factors). However, this is rarely the case. For example, although AhR expression was correlated to PCB exposure, another important group of environmental contaminants (polycyclic aromatic hydrocarbons, PAHs) can also induce AhR, although with lower affinity. In case of vitamin A, the dietary origin of this small hormone introduces probable effects of differences in diet among seal populations into the study. Therefore, although the biomarkers applied in this study of seals were quantifiable in the available tissues from marine mammals, and were both relevant and sensitive, their specificity may be questioned.

Continuation of wildlife toxicology studies may eventually result in the identification of a biological endpoint that complies with all biomarker requirements. In such studies of marine mammals, especially blubber may represent an important tissue sample for both contaminant analyses and biological endpoints, as evidenced by studies of vitamin A and thyroid hormone receptors in this tissue compartment. Although blubber has been frequently viewed as primarily a thermoregulatory and streamlining tissue in marine mammals, the presence of hormones and hormone receptors, as well as contaminant-related responses of the former and latter, suggest blubber is a living and dynamic tissue. However, the biomarkers in blubber may benefit from (1) further physiological studies of the compounds in question in harbour seals, and (2) identification of the responsible mechanism of toxicity. For example, decreased hormone concentrations in blubber may simply reflect the disruption taking place in circulation rather than locally within the tissue. This may explain why hormones did reflect contaminant exposure in the blubber, whereas cell signalling molecules such as AhR and thymosin-α1, that are locally produced and serve a local function, did not. The latter furthermore suggests that the high concentrations of PCBs stored in the blubber may be largely inaccessible to, and unable to affect, cell machinery.

New biomarker approaches should continue to be developed and validated, as illustrated by the investigation of thymosin-α1 in this study. Although thymosin-α1 concentrations in seal tissues did not supply us with a biomarker tool, this first investigation of its use as a stimulatory agent in the assessment of lymphocyte function *in vitro* provided a novel sensitive and accurate indicator of AhR-mediated effects of PCBs. Other biologically active molecules that may be potentially relevant to, and useful in, immunotoxicological studies could include cytokine concentrations and expression

levels, as well as hormones and nuclear receptor expression other than those described in this study (e.g. estrogen receptor-α).

Whereas biomarkers for wildlife toxicology are commonly selected based on *in vitro* and *in vivo* exposure studies of laboratory rodents, micro-arrays are an upcoming technology of interest that could rapidly screen for, and identify, molecular biomarkers of toxicity. For such an approach, the validation of the cross-species use of existing arrays would represent an important first step, in making the technique available to wildlife species.

Established reference values underestimate immunotoxicity in marine mammals

Besides the use of physiological and molecular endpoints as tools in the assessment of the health of marine mammals directly, they may serve in the establishment of adverse effect levels that could provide indications of potential health risks in other marine mammal species. The evidence presented in this study including both immunological parameters and biomarkers suggests that the current toxicological thresholds underestimate the risk of adverse health effects of PCBs to marine mammals.

A threshold for immunotoxicity in marine mammals was established by Ross *et al* (Ross et al., 1996a) based on the results of a captive feeding study with harbour seals. In this study, where two groups of seals were feeding on a diet with a difference in PCB concentrations, a NOAEL based on the exposure of the reference group, and LOAEL based on the exposure of the more contaminated group were suggested. When comparing these two groups with our least contaminated (Queen Charlotte Strait) and most contaminated (Gertrude Island) study groups (Table 18), both are characterised by lower contaminant burdens than the reference group of Ross *et al.* In addition, seals in this study were at risk of adverse health effects according to CCME guidelines for

aquatic wildlife, as well as rodent-based thresholds if considering lactating seals. Therefore, our study population suggests that the NOAEL of PCB-associated immunotoxic effects may be magnitudes lower than previously believed. Based on the contamination of the Queen Charlotte Strait reference group of seals in this study, it is suggested that a NOAEL of 15 ng TEQ/kg lipid weight or 0.68 mg PCB/kg lipid weight in blubber is adapted in studies of marine mammals.

Table 18. Comparison of this study to a captive study of immunotoxicity in seals.
Total TEQ concentrations in blubber of the seals described in this study were compared to the seals from the semi-field study of seals (reviewed in Ross et al., 1996), on which current risk assessment threshold values (NOAEL and LOAEL) and the associated descisions are often based.

	Low dose	High dose
Semi-field study	90 ± 6 ng/kg lipid weight	286 ± 17 ng/kg lipid weight
This study	15 ± 3 ng/kg lipid weight	70 ± 14 ng/kg lipid weight

Harbour seals as sentinels of marine ecosystem health

Observations of immunotoxicity in harbour seals in this study, considering the low level PCB concentrations these animals are exposed to, indicate that many other marine mammal species, often exposed to similar or higher concentrations of these compounds, may be at risk. These may include the many harbour seal populations inhabiting industrial coastal areas elsewhere, as well as other pinniped and cetacean species. Pacific killer whales (*Orcinus orca*), for example, frequent the same coastal region as the seals in this study, but have been shown to represent one of the most PCB-contaminated marine mammals in the world (Ross et al., 2000a), with a PCB body burden at least 150 times higher than seals.

Besides the risk that PCBs may pose to marine mammal health, a number of high trophic fish-consuming, terrestrial wildlife species (e.g. river otters, bald eagles, and salmon-eating grizzly bears) as well as certain human populations that rely heavily on marine resources may be exposed to harmful concentrations of PCBs. In the case of humans, cohorts that depend on traditional food gathering and hunting, and are involved in sportsfishing, have been shown to be especially at risk of PCB-associated toxicity due to their elevated intake of marine (aquatic) foods.

Within Canada, many subsistence-oriented peoples continue to consume in traditional manners, of which the Sencoten First Nation, occupying coastal villages at the Saanich Peninsula, BC, within our seal study area, consist of a representative example. In a preliminary assessment of consumption habits among the Sencoten, the intake of marine foods among them was 4-10 times higher than in average Canadians (Mos et al., 2004; Table 19). In the high reliance of the Sencoten on marine foods, they occupy a niche in the marine food web much similar to that of harbour seals. This indicates an emerging need for human toxicological and epidemiological research in Pacific coastal areas, and underlines the relevance and need to integrate human and ecological risk assessments in this region and elsewhere.

Table 19. Annual intake of aquatic foods and the associated PCB exposure in humans.
Whereas the adverse health effects of PCBs on marine mammals have been extensively assessed in this study, there is increasing concern for humans in this region, especially those that occupy similar trophic levels in the ecosystem, i.e. those consuming diets rich in marine foods. A comparison of Canadians, including average consumers (such as those from southern Ontario) as well as subsistence-oriented First Nations in BC, shows that the large variation among them makes risk assessments difficult. However, considering that the effects of PCBs in human diets rich in marine foods have been documented to be harmful in some of these populations, as well as others worldwide, the potentially harmful effects of PCBs on humans in British Columbia should be addressed.

Consumer group	Annual intake of aquatic foods (kg)	Annual intake of PCBs (μg/kg BW)	References
Average (southern) Canadians	4.4	2.9	(Conacher & Mes, 1993)
Mohawk First Nation, southern QC	8.4	10.0	(Chan et al., 1999)
Sencoten First Nation, southern coastal BC	24.5 (14.8 - 41.9)	?	(Mos et al., 2004)
Subsistence fishermen, Gulf of St. Lawrence, northern QC	51.1	?	(Dewailly et al., 1994)
Inuit, Arctic QC	109.5	110	(Dewailly et al., 1994; Ayotte et al., 1995)

Taken from Mos et al., 2004.

The continued relevance of toxicological research of POPs

POPs have become spread worldwide, and by the many mechanisms of toxicity they are able to affect the health of humans and wildlife alike it remains a challenge to assess the risks of these anthropogenic contaminants. The use of a sentinel species, such as the harbour seal, can prove a valuable alternative as shown in this study. Harbour seals can provide indications of a hazard, and provide us with a real world measure of exposure and the associated effects, that may help to characterise the risk for other consumers including ourselves. However, many marine organisms are exposed to

POPs, and, whereas the emphasis is often laid upon humans and mammals, organisms such as marine invertebrates may also be affected. The continued characterization of risks associated with legacy POPs in all creatures great and small, as well as increased vigilance in the design and application of new emergent POPs, will therefore remain an important investment in the future.

Bibliography

1. Anonymous (1996). *Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals*. World Health Organization. Geneva, Switzerland.

2. Glare,E.M., Divjak,M., Bailey,M.J., & Walters,E.H. (2002). β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax*, 57, 765-770.

3. Addison,R.F. & Brodie,P.F. (1987). Transfer of organochlorine residues from blubber through the circulatory system in milk in the lactating grey seal, *Halichoerus grypus*. *Canadian Journal of Fisheries and Aquatic Sciences*, 44, 782-786.

4. Addison,R.F. & Stobo,W.T. (1993). Organochlorine residue concentrations and burdens in grey seal (*Halichoerus grypus*) blubber during the first year of life. *Journal of Zoology*, 230, 443-450.

5. Agency for Toxic Substances and Disease Registry (2000). *Toxicological profile for polychlorinated biphenyls (Update)*. US Department of Health and Human Services.

6. Aguilar,A. & Borrell,A. (1994). Abnormally high polychlorinated biphenyl levels in striped dolphins (*Stenella coeruleoalba*) affected by the 1990-1992 Mediterranean epizootic. *Science of the Total Environment*, 154, 237-247.

7. Allende,L.M., Corell,A., Madrono,A., Gongora,R., Rodriguez-Gallego,C., Lopez-Goyanes,A., Rosal,M., & Asnaiz-Villena,A. (1997). Retinol (vitamin A) is a cofactor in CD3-induced human T-lymphocyte activation. *Immunology*, 90, 388-396.

8. Ayotte,P., Dewailly,E., Bruneau,S., Careau,H., & Vezina,A. (1995). Arctic air pollution and human health: what effects should be expected? *Science of the Total Environment*, 160/161, 529-537.

9. Badamchian,M., Mora,C.A., Baumann,C.A., Paino,J.E., & Goldstein,A.L. (1997). Biodistribution of synthetic thymosin α1 in the serum, urine, and major organs of mice. *International Society for Immunopharmacology*, 19(2), 59-66.

10. Bas,A., Forsberg,G., Hammarstrom,S., & Hammerstrom,M.-L. (2004). Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scandinavian Journal of Immunology*, 59, 566-573.

11. Basu,N., Scheuhammer,A.M., Bursian,S.J., Elliot,J., Rouvinen-Watt,K., Chan,H.M. (2006). Mink as a sentinel species in environmental health. *Environmental Research*, In press.

12. Baxevanis,C.N., Anastasopoulos,E., Reclos,G.J., & Papamichail,M. (1990a). Prothymosin-α enhances HLA-DR antigen expression on monocytes from patients with multiple sclerosis. *Journal of Neuroimmunology*, 27, 141-148.

13. Baxevanis,C.N., Gritzapis,A.D., Dedoussis,G.V.Z., Papadopoulos,N.G., Tsolas,O., & Papamichail,M. (1990b). Enhancement of human T lymphocyte function by prothymosin alpha: increased production of IL-2 and expression of IL-2 receptors on normal human peripheral blood T lymphocytes. *Immunopharmacology and Immunotoxicology*, 12, 595-617.

14. Baxevanis,C.N., Gritzapis,A.D., Spanakos,G., Tsitsilonis,O.E., & Papamichail,M. (1995). Induction of tumor-specific T lymphocyte responses in vivo by prothymosin alpha. *Cancer Immunology and Immunotherapy*, 40, 410-418.

15. Bengtson,J.L., Boveng,P., Franzen,U., Have,P., Heide-Jörgensen,M.-P., & Härkönen,T. (1991). Antibodies to canine distemper virus in antarctic seals. *Marine Mammal Science*, 7, 85-87.

16. Bergman,A., Olsson,M., & Reiland,S. (1992). Skull-bone lesions in the Baltic grey seal (*Halichoerus grypus*). *Ambio*, 21, 517-519.

17. Birnbaum,L. (1995). Workshop on perinatal exposure to dioxin-like compounds. V. Immunologic effects. *Environmental Health Perspectives Supplements*, 103, 135-142.

18. Bishop,C.A., Mahony,N.A., Trudeau,S., & Pettit,K.E. (1999). Reproductive success and biochemical effects in tree swallows (*Tachycineta bicolor*) exposed to chlorinated hydrocarbon contaminants in wetlands of the Great Lakes and St. Lawrence River basin, USA and Canada. *Environmental Toxicology and Chemistry*, 18, 263-271.

19. Blaylock,B.L., Holladay,S.D., Comment,C.E., Heindel,J.J., & Luster,M.I. (1992). Exposure to tetrachlorodibenzo-p-dioxin (TCDD) alters fetal thymocyte maturation. *Toxicology and Applied Pharmacology*, 112, 207-213.

20. Blomhoff,R. (1994). Overview of vitamin A metabolism and function. InR.Blomhoff (Ed.), *Vitamin A in health and disease* (pp. 1-35). New York: Marcel Dekker.

21. Bonet,M.L., Ribot,J., Felipe,F., & Palou,A. (2003). Vitamin A and regulation of fat reserves. *Cellular and Molecular Life Sciences*, 60, 1311-1321.

22. Boon,J.P., Oostingh,I., Van der Meer,J., & Hillebrand,M.T.J. (1994). A model for the bioaccumulation of chlorobiphenyl congeners in marine mammals. *European Journal of Pharmacology - Environmental Toxicology*, 270, 237-251.

23. Boon,J.P., Van Arnhem,E., & Jansen,S. (1992). The toxicokinetics of PCBs in marine mammals with special reference to possible interactions of individual congeners with the cytochrome P450-dependent monooxygenase system: an overview. InC.H.Walker & D.R.Livingstone (Eds.), *Persistent pollutants in marine ecosystems* (pp. 119-159). New York: Pergamon Press.

24. Boon,J.P., Van der Meer,J., Allchin,C.R., Law,R.J., Klungsoyr,J., Leonards,P.E.G., Spliid,H., Storr-Hansen,E., Mckenzie,C., & Wells,D.E. (1997). Concentration-dependent changes of PCB patterns in fish-eating mammals: Structural evidence for induction of cytochrome P450. *Archives of Environmental Contamination and Toxicology*, 33, 298-311.

25. Boutros,P.C., Moffat,I.D., Franc,M.A., Tijet,N., Tuomisto,J., Pohjanvirta,R., & Okey,A.B. (2004). Dioxin-responsive AHRE-II gene battery: identification by phylogenetic footprinting. *Biochemical and Biophysical Research Communications*, 321, 707-715.

26. Boverhof,D.R., Tam,E., Harney,A.S., Crawford,R.B., Kaminski,N.E., & Zacharewski,T.R. (2004). 2,3,7,8-tetrachlorodibenzo-p-dioxin induces suppressor of cytokine signalling 2 in murine B cells. *Molecular Pharmacology*, 66, 1662-1670.

27. Braathen,M., Derocher,A.E., Wiig,O., Sormo,E.G., Lie,E., Skaare,J.U., & Jenssen,B.M. (2004). Relationships between PCBs and thyroid hormones and retinol in female and male polar bears. *Environmental Health Perspectives*, 112, 826-833.

28. Brouwer,A., Morse,D.C., Lans,M.C., Schuur,A.G., Murk,A.J., Klasson,W.E., Bergman,A., & Visser,T.J. (1998). Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. *Toxicology and Industrial Health*, 14, 59-84.

29. Brouwer,A., Reijnders,P.J.H., & Koeman,J.H. (1989). Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquatic Toxicology*, 15, 99-106.

30. Brouwer,A., Van den Berg,K.J., Blaner,W.S., & Goodman,D.S. (1986). Transthyretin (prealbumin) binding of PCBs, a model for the mechanism of interference with vitamin A and thyroid hormone metabolism. *Chemosphere*, 15, 1699-1706.

31. Canadian Council of Ministers of the Environment (1999). Canadian tissue residue guidelines for the protection of wildlife consumers of aquatic biota: polychlorinated biphenyls (PCBs). *Canadian environmental quality guidelines* (pp. 1-9). Winnipeg: Canadian Council of Ministers of the Environment.

32. Carson,R. (1962). *Silent Spring*. Boston: Houghton-Mifflin.

33. Carver,L.A., Hogenesch,J.B., & Bradfield,C.A. (1994). Tissue specific expression of the rat Ah-receptor and ARNT mRNAs. *Nucleic Acid Research*, 22, 3038-3044.

34. CCME (1999). Canadian tissue residue guidelines for the protection of wildlife consumers of aquatic biota: Polychlorinated biphenyls. Canadian Committee of Ministers of the Environment. Winnipeg, Manitoba, Canada.

35. CCME (1997). A framework for ecological risk assessment: Technical appendices. CCME Subcommittee on Environmental Quality Criteria for Contaminated Sites. The National Contaminated Sites Remediation Program. Winnipeg, Manitoba, Canada.

36. CCREM (1987). *PCB action plan: Final report.* Ottawa.

37. Chan,H.M., Trifonopoulos,M., Ing,A., Receveur,O., & Johnson,E. (1999). Consumption of freshwater fish in Kahnawake: risks and benefits. *Environmental Research*, 80, S213-S222.

38. Chang,H., Wang,Y.J., Chang,L.W., & Lin,P. (2005). A histochemical and pathological study on the interrelationships between TCDD-induced AhR expression, AhR activation, and hepatotoxicity in mice. *Journal of Toxicology and Environmental Health*, 68, 1567-1579.

39. Chang,K.-J., Hsiung,K.H., Lee,T.P., Tang,S.Y., & Tung,T.C. (1981). Immunological evaluation of patients with polychlorinated biphenyl poisoning: determination of lymphocyte subpopulations. *Toxicology and Applied Pharmacology*, 61, 58-63.

40. Chao,W.Y., Hsu,C.C., & Guo,Y.L. (1997). Middle-ear disease in children exposed prenatally to polychlorinated biphenyls and polychlorinated dibenzofurans. *Archives of Environmental Health*, 52, 257-262.

41. Collins,S.J. (2002). The role of retinoids and retinoic acid receptors in normal hematopoiesis. *Leukemia*, 16, 1896-1905.

42. Conacher,H. & Mes,J. (1993). Assessment of human exposure to chemical contaminants in food. *Food Additives and Contaminants*, 10, 5-15.

43. Cordero,O.J., Sarandeses,C., Lopez,J.L., & Nogueira,M. (1992). Prothymosin alpha enhances human natural killer cell cytotoxicity: role in mediating signals for NK activity. *Lymphokine and Cytokine Research*, 11, 277-285.

44. Cosby,S.L., McQuaid,S., Duffy,N., Lyons,C., Rima,B.K., Allan,G.M., McCullough,S.J., Kennedy,S., Smyth,J.A., McNeilly,F., Craig,C., & Örvell,C. (1988). Characterization of a seal morbillivirus. *Nature*, 336, 115-116.

45. Cottrell,P.E., Jeffries,S.J., Beck,B., & Ross,P.S. (2002). Growth and development in free-ranging harbour seal (*Phoca vitulina*) pups from southern British Columbia. *Marine Mammal Science*, 18, 721-733.

46. Crawford,R.B., Holsapple,M.P., & Kaminski,N.E. (1997). Leukocyte activation induces aryl hydrocarbon receptor upregulation, DNA binding, and increased *Cyp1a1* expression in the absence of exogenous ligand. *Molecular Pharmacology*, 52, 921-927.

47. Cullon,D., Jeffries,S., & Ross,P.S. (2005). Persistent organic pollutants in the diet of harbor seals (*Phoca vitulina*) inhabiting Puget Sound, Washington (USA), and

the Strait of Georgia, British Columbia (Canada): A food basket approach. *Environmental Toxicology & Chemistry*, 24, 2562-2572.

48. Dallaire,F., Dewailly,E., Muckle,G., Vezina,C., Jacobson,S.W., Jacobson,J.L., & Ayotte,P. (2004). Acute infections and environmental exposure to organochlorines in Inuit infants from Nunavik. *Environmental Health Perspectives*, 112, 1359-1364.

49. Dalton,T.P., Puga,A., & Shertzer,H.G. (2002). Induction of cellular oxidative stress by aryl hydrocarbon receptor activation. *Chemico-Biological Interactions*, 141, 77-95.

50. Daszak,P., Cunningham,A.A., & Hyatt,A.D. (2000). Emerging infectious diseases of wildlife-Threats to biodiversity and human health. *Science*, 287, 443-449.

51. De Guise,S., Flipo,D., Boehm,J.R., Martineau,D., Béland,P., & Fournier,M. (1995). Immune functions in beluga whales (*Delphinapterus leucas*): Evaluation of phagocytosis and respiratory burst with peripheral blood leukocytes using flow cytometry. *Veterinary Immunology and Immunopathology*, 47, 351-362.

52. De Guise,S., Lagacé,A., & Béland,P. (1994). Tumors in St Lawrence beluga whales (Delphinapterus leucas). *Veterinary Pathology*, 31, 444-449.

53. De Guise,S., Martineau,D., Beland,P., & Fournier,M. (1998). Effects of *in vitro* exposure of beluga whale leukocytes to selected organochlorines. *Journal of Toxicology and Environmental Health*, 55, 479-493.

54. De Krey,G.K., Hollingshead,N.C., Kerkvliet,N.I., & Smith,B.B. (1994). Suppression of prolactin and cytotoxic T-lymphocyte activity in PCB-treated mice. *International Society for Immunopharmacology*, 16, 251-257.

55. De Swart,R.L., Kluten,R.M.G., Huizing,C.J., Vedder,L.J., Reijnders,P.J.H., Visser,I.K.G., UytdeHaag,F.G.C.M., & Osterhaus,A.D.M.E. (1993). Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). *Veterinary Immunology and Immunopathology*, 37, 217-230.

56. De Swart,R.L., Ross,P.S., Timmerman,H.H., Vos,H.W., Reijnders,P.J.H., Vos,J.G., & Osterhaus,A.D.M.E. (1995). Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. *Clinical and Experimental Immunology*, 101, 480-486.

57. De Swart,R.L., Ross,P.S., Vedder,L.J., Timmerman,H.H., Heisterkamp,S.H., Van Loveren,H., Vos,J.G., Reijnders,P.J.H., & Osterhaus,A.D.M.E. (1994). Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. *Ambio*, 23, 155-159.

58. De Swart,R.L., Ross,P.S., Vos,J.G., & Osterhaus,A.D.M.E. (1996). Impaired immunity in harbour seals (*Phoca vitulina*) exposed to bioaccumulated environmental contaminants: review of a long-term study. *Environmental Health Perspectives*, 104 (suppl. 4), 823-828.

59. De Waal,E.J., Schuurman,H.J., Loeber,J.G., Van Loveren,H., & Vos,J.G. (1992). Alterations in the cortical thymic epithelium of rats after in vivo exposure to 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) - an (immuno)histological study. *Toxicology and Applied Pharmacology*, 115, 80-88.

60. Debier,C., Kovacs,K.M., Lydersen,C., Mignolet,E., & Larondelle,Y. (1999). Vitamin E and vitamin A contents, fatty acid profiles, and gross composition of harp and hooded seal milk through lactation. *Canadian Journal of Zoology*, 77, 952-958.

61. Debier, C. and Larondelle, Y. Vitamins A and E: metabolism, roles, and transfer to offspring. British Journal of Nutrition 93, 153-174. 2005.

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

63. Debier,C., Pomeroy,P.P., Thome,J.-P., Mignolet,E., de Tilesse,T., & Larondelle,Y. (2004). An unexpected parallelism between vitamin A and PCBs in seal milk. *Aquatic Toxicology*, 68, 179-183.

64. Debier,C., Ylitalo,G.M., Weise,M., Gulland,F., Costa,D.P., Le Boeuf,B.J., de Tilesse,T., & Larondelle,Y. (2005). PCBs and DDT in serum of juvenile California sea lions: associations with vitamins A and E and thyroid hormones. *Environmental Pollution*, 134, 323-332.

65. Deem,S.L., Spelman,L.H., Yates,R.A., & Montali,R.J. (2000). Canine distemper in terrestrial carnivores: a review. *Journal of Zoo and Wildlife Medicine*, 31(4), 441-451.

66. Denison,M.S. & Nagy,S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annual Review of Pharmacology and Toxicology*, 43, 309-334.

67. Dewailly,E., Ayotte,P., Bruneau,S., Gingras,S., Belles-Iles,M., & Roy,R. (2000). Susceptibility to infections and immune status in Inuit infants exposed to organochlorines. *Environmental Health Perspectives*, 108, 205-211.

68. Dewailly,E., Ryan,J.J., Laliberte,C., Bruneau,S., Weber,J.P., Gingras,S., & Carrier,G. (1994). Exposure of remote maritime populations to coplanar PCBs. *Environmental Health Perspectives*, 102, 205-209.

69. Dietz,R., Ansen,C.T., & Have,P. (1989a). Clue to seal epizootic? *Nature*, 338, 627.

70. Dietz,R., Heide-Jörgensen,M.-P., & Härkönen,T. (1989b). Mass deaths of harbor seals (*Phoca vitulina*) in Europe. *Ambio*, 18, 258-264.

71. Doi,H., Baba,T., Tohyama,C., & Nohara,K. (2003). Functional activation of aryl hydrocarbon receptor (AhR) in primary T cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Chemosphere*, 52, 655-662.

72. Elliott,J.E., Wilson,L.K., Henny,C.J., Trudeau,S.F., Leighton,F.A., Kennedy,S.W., & Cheng,K.M. (2001). Assessment of biological effects of polychlorinated hydrocarbons in osprey chicks. *Environmental Toxicology and Chemistry*, 20(4), 866-877.

73. Ertesvag,A., Engedal,N., Naderi,S., & Blomhoff,H.K. (2002). Retinoic acid stimulates the cell cycle machinery in normal T cells: involvement of retinoic acid receptor-mediated IL-2 secretion. *Journal of Immunology*, 169, 5555-5563.

74. Fernandez-Salguero,P., Pineau,T., Hilbert,D.M., McPhail,T., Lee,S.S.T., Kimura,S., Nebert,D.W., Rudikoff,S., Ward,J.M., & Gonzalez,F.J. (1995). Aryl hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicology and Applied Pharmacology*, 140, 173-179.

75. Fine,J.S., Silverstone,A.E., & Gasiewicz,T.A. (1990). Impairment of prothymocyte activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Journal of Immunology*, 144, 1169-1176.

76. Franc,M.A., Pohjanvirta,R., Tuomisto,J., & et al (2001). In vivo upregulation of aryl hydrocarbon receptor expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in a dioxin-resistant rat model. *Biochemical Pharmacology*, 62, 1565-1578.

77. Franco,F.J., Diaz,C., Barcia,M., & Freire,M. (1992). Thymosin alpha 1 is a native peptide in several tissues. *Biochimica et Biophysica Acta*, 1120(1), 43-48.

78. Funseth,E. & Ilbäck,N.-G. (1992). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on blood and spleen natural killer (NK) cell activity in the mouse. *Toxicology Letters*, 60, 247-256.

79. Ganey,P.E., Sirois,J.E., Denison,M., Robinson,J.P., & Roth,R.A. (1993). Neutrophil function after exposure to polychlorinated biphenyls in vitro. *Environmental Health Perspectives*, 101, 430-434.

80. Goldsby,R.A., Kindt,T.J., & Osborne,B.A. (2000). *Kuby Immunology*. New York: W.H. Freeman and Company.

81. Goldstein,A.L. & Badamchian,M. (2004). Thymosins: chemistry and biological properties in health and disease. *Expert Opinion in Biological Therapy*, 4, 559-573.

82. Goldstein,A.L., Low,T.L.K., McAdoo,M., McClure,J., Thurman,G.B., Rossio,J., Chun,Y.L., Chang,D., Su,S.W., Harvey,C., Ramel,A.H., & Meienhofer,J. (1977). Thymosin α_1: isolation and sequence analysis of an immunologically active thymic polypeptide. *Proceedings of the National Academy of Sciences of the United States of America*, 74(2), 725-729.

83. Goya,R.G., Naylor,P.H., Goldstein,A.L., & Meites,J. (1990). Changes in circulating levels of neuroendocrine and thymic hormones during aging in rats: A correlation study. *Experimental Gerontology*, 25, 149-157.

84. Grachev,M.A., Kumarev,V.P., Mamaev,L.V., Zorin,V.L., Baranova,L.V., Denikjna,N.N., Belikov,S.I., Petrov,E.A., Kolesnik,V.S., Kolesnik,R.S., Dorofeev,V.M., Beim,A.M., Kudelin,V.N., Nagieva,F.G., & Sidorov,V.N. (1989). Distemper virus in Baikal seals. *Nature*, 338, 209.

85. Grant,S.C.H. & Ross,P.S. (2002). Southern resident killer whales at risk: Toxic chemicals in the British Columbia and Washington environment. *Canadian Technical Reports of Fisheries and Aquatic Sciences*, 2412.

86. Grasman,K.A., Fox,G.A., Scanlon,P.F., & Ludwig,J.P. (1996). Organochlorine-associated immunosuppression in prefledgling Caspian Terns and Herring gulls from the Great Lakes: An ecoepidemiological study. *Environmental Health Perspectives Supplements*, 104, 829-842.

87. Gu,Y.Z., Hogenesch,J.B., & Bradfield,C.A. (2000). The PAS superfamily: sensors of environmental and developmental signals. *Annual Review of Pharmacology and Toxicology*, 40, 519-561.

88. Gulland,F.M., Koski,M., Lowenstine,L.J., Colagross,A., Morgan,L., & Spraker,T.R. (1996). Leptospirosis in California sea lions (Zalophus californianus) stranded along the central California coast, 1981-1994. *Journal of Wildlife Diseases*, 32, 572-580.

89. Hahn,M.E. (2002). Aryl hydrocarbon receptors: diversity and evolution. *Chemico-Biological Interactions*, 141, 131-160.

90. Hammond,J.A., Hall,A.J., & Dyrynda,E.A. (2005). Comparison of polychlorinated biphenyl (PCB) induced effects on innate immune functions in harbour and grey seals. *Aquatic Toxicology*, 74, 126-138.

91. Hankinson,O. (1995). The Aryl hydrocarbon receptor complex. *Annual Review of Pharmacology and Toxicology*, 35, 307-340.

92. Harder,T.C., Kenter,M., Appel,M.J.G., Roelke-Parker,M.E., Barrett,T., & Osterhaus,A.D.M.E. (1995). Phylogenetic evidence of canine distemper virus in Serengeti's lions. *Vaccine*, 13, 521-523.

93. Harder,T.C. & Osterhaus,A.D.M.E. (1997). Canine distemper virus - a morbillivirus in search of new hosts? *Trends in Microbiology*, 5, 120-124.

94. Harding,K.C., Harkonen,T., & Caswell,H. (2002). The 2002 European seal plague: epidemiology and population consequences. *Ecology Letters*, 5, 727-732.

95. Harding,L.E., Harris,M.L., Stephen,C.R., & Elliott,J.E. (1999). Reproductive and morphological condition of wild mink (*Mustela vison*) and river otters (*Lutra*

canadensis) in relation to chlorinated hydrocarbon contamination. *Environmental Health Perspectives*, 107(2), 141-147.

96. Haritos,A.A., Tsolas,O., & Horecker,B.L. (1984). Distribution of prothymosin alpha in rat tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 81, 1391-1393.

97. Harkonen,T., Dietz,R., Reijnders,P.J.H., Teilmann,J., Harding,K., Hall,A., Brasseur,S., Siebert,U., Goodman,S.J., Jepson,P.D., Dau Rasmussen,T., & Thompson,P. (2006). The 1988 and 2002 phocine distemper virus epidemics in European harbour seals. *Diseases of Aquatic Organisms*, 68, 115-130.

98. Harvell,C.D., Kim,K., Burkholder,J.M., Colwell,R.R., Epstein,P.R., Grimes,D.J., Hofmann,E.E., Lipp,E.K., Osterhaus,A.D.M.E., Overstreet,R.M., Porter,J.W., Smith,G.W., & Vasta,G.R. (1999). Emerging marine diseases - climate links and anthropogenic factors. *Science*, 285, 1505-1510.

99. Heide-Jörgensen,M.-P., Härkönen,T., Dietz,R., & Thompson,P.M. (1992). Retrospective of the 1988 European seal epizootic. *Diseases of Aquatic Organisms*, 13, 37-62.

100. Hickie,B.E., Muir,D.C.G., Addison,R.F., & Hoekstra,P.F. (2005). Development and application of bioaccumulation models to assess persistent organic pollutant temporal trends in arctic ringed seal (*Phoca hispida*) populations. *Science of the Total Environment*, 351-352, 413-426.

101. Hoffman,D.J., Melancon,M.J., Klein,P.N., Rice,C.P., Eisemann,J.D., Hines,R.K., Spann,J.W., & Pendleton,G.W. (1996). Developmental toxicity of PCB 126 (3,3',4,4',5-pentachlorobiphenyl) in nesting American kestrels (*Falco sparverius*). *Fundamental and Applied Toxicology*, 34, 188-200.

102. Holladay,S.D. & Luster,M.I. (1994). Developmental immunotoxicology. InC.Kimmel & J.Bucksam (Eds.), *Developmental toxicology* (pp. 93-118). New York: Raven Press.

103. Holsapple,M.P., Karras,J.G., Ledbetter,J.A., Schieven,G.L., Burchiel,S.W., Davila,D.R., Schatz,A.R., & Kaminski,N.E. (1996). Molecular mechanisms of toxicant-induced immunosuppression: Role of second messengers. *Annual Review of Pharmacology and Toxicology*, 36, 131-159.

104. Holsapple,M.P., Paustenbach,D.J., Charnley,G., West,L.J., Luster,M.I., Dietert,R.R., & Burns-Naas,L.A. (2004). Symposium summary: Children's health risk - What`s so special about the developing immune system? *Toxicology and Applied Pharmacology*, 199, 61-70.

105. Holsapple,M.P., Snyder,N.K., Wood,S.C., & Morris,D.L. (1991). A review of 2,3,7,8,-tetrachlorodibenzo-p-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology*, 69, 219-255.

106. House,R.V., Lauer,L.D., Murray,M.J., Thomas,P.T., Ehrlich,J.P., Burleson,G.R., & Dean,J.H. (1990). Examination of immune parameters and host resistance

mechanisms in B6C3F1 mice following adult exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Journal of Toxicology and Environmental Health*, 31, 203-215.

107. Humphrey,H.E.B. (1987). The human population - An ultimate receptor for aquatic contaminants. *Hydrobiologica*, 149, 75-80.

108. Hundeiker,C., Pineau,T., Cassar,G., Betensky,R.A., Gleichmann,E., & Esser,C. (1999). Thymocyte development in Ah-receptor-deficient mice is refractory to TCDD-inducible changes. *International Journal of Immunopharmacology*, 21, 841-859.

109. Hunt,G. & Hunt,M. (1977). Female-female pairing in western gulls (*Larus occidentalis*) in Southern California. *Science*, 196, 1466-1467.

110. Hutchinson,J.D. & Simmonds,M.P. (1994). Organochlorine contamination in pinnipeds. *Reviews of Environmental Contamination and Toxicology*, 136, 123-168.

111. Huuskonen,H., Unkila,M., Pohjanvirta,R., & Tuomisto,J. (1994). Developmental toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the most TCDD-resistant and -susceptible rat strains. *Toxicology and Applied Pharmacology*, 124, 174-180.

112. Ikonomou,M.G., Fraser,T., Crewe,N., Fischer,M.B., Rogers,I.H., He,T., Sather,P., & Lamb,R. (2001). A comprehensive multiresidue ultra-trace analytical method, based on HRGC/HRMS, for the determination of PCDDs, PCDFs, PCBs, PBDEs, PCDEs, and organochlorine pesticides in six different environmental matrices. *Canadian Technical Reports of Fisheries and Aquatic Sciences*, 2389, 1-95.

113. Jeffries,S.J., Brown,R.F., & Harvey,J.T. (1993). Techniques for capturing, handling and marking harbour seals. *Aquatic Mammals*, 19.1, 21-25.

114. Jeffries,S.J., Gearin,P.J., Huber,H.R., Saul,D.L., & Pruett,D.A. (2000). *Atlas of seal and sea lion haulout sites in Washington*. Washington Department of Fish and Wildlife.

115. Jensen,B.A., Leeman,R.J., Schlezinger,J.J., & Sherr,D.H. (2003). Aryl hydrocarbon receptor (AhR) agonists suppress interleukin-6 expression by bone marrow stromal cells: an immunotoxicology study. *Environmental Health*, 2, 16.

116. Jenssen,B.M., Skaare,J.U., Woldstad,S., Nastad,A.T., Haugen,O., Kloven,B., & Sormo,E.G. (1995). Biomarkers in blood to assess effects of polychlorinated biphenyls in free-living grey seal pups. InA.S.Blix, L.Walløe, & Ø.Ulltang (Eds.), *Whales, seals, fish and man* (pp. 607-615). Amsterdam: Elsevier Science BV.

117. Jepson,P.D., Bennett,P.M., Allchin,C.R., Law,R.J., Kuiken,T., Baker,J.R., Rogan,E., & Kirkwood,J.K. (1999). Investigating potential associations between chronic exposure to polychlorinated biphenyls and infectious disease mortality in harbour porpoises from England and Wales. *Science of the Total Environment*, 244, 339-348.

118. Jiang,X.L., Everson,M.P., & Lamon,E.W. (1993). A mechanism of retinoid potentiation of murine T-cell responses: early upregulation of interleukin-2 receptors. *International Society for Immunopharmacology*, 15, 309-317.

119. Kakela,R., Hyvarinen,H., & Kakela,A. (1997). Vitamins A1 (retinol), A2 (3,4-didehydroretinol) and E (alpha-tocopherol) in the liver and blubber of lacustrine and marine ringed seals (*Phoca hispida* sp.). *Comparative Biochemistry and Physiology B*, 116B, 27-33.

120. Kakela,R., Kakela,A., Hyvarinen,H., Asikainen,J., & Dahl,S.-K. (1999). Vitamins A1, A2 and E in minks exposed to polychlorinated biphenyls (Aroclor 1242) and copper, via diet based on freshwater or marine fish. *Environmental Toxicology and Chemistry*, 18(11), 2595-2599.

121. Karol,M.H. (1998). Target organs and systems: methodologies to assess immune system function. *Environmental Health Perspectives*, 106(Suppl 2), 533-540.

122. Keller,J.M., McClennan-Green,P.D., Kucklick,J.R., Keil,D.E., & Peden-Adams,M.M. (2006). Effects of organochlorine contaminants on loggerhead sea turtle immunity: Comparison of a correlative field study and *in vitro* exposure experiments. *Environmental Health Perspectives*, 114(1), 70-76.

123. Kendall,M.D., Safieh,B., Harwood,J., & Pomeroy,P.P. (1992). Plasma thymulin concentrations, the thymus and organochlorine contaminant levels in seals infected with phocine distemper virus. *Science of the Total Environment*, 115, 133-144.

124. Kennedy,S., Kuiken,T., Jepson,P.D., Deaville,R., Forsyth,M., Barrett,T., Van de Bildt,M.W.G., Osterhaus,A.D.M.E., Eybatov,T., Duck,C., Kydyrmanov,A., Mitrofanov,I., & Wilson,S. (2000). Mass die-off of Caspian seals caused by canine distemper virus. *Emerging Infectious Diseases*, 6(6), 637-639.

125. Kerkvliet,N.I., Baecher Steppan,L., Smith,B.B., Youngberg,J.A., Henderson,M.C., & Buhler,D.R. (1990). Role of the Ah locus in suppression of cytotoxic T lymphocyte activity by halogenated aromatic hydrocarbons (PCBs and TCDD): Structure-activity relationships and effects in C57Bl/6 mice congenic at the Ah locus. *Fundamental and Applied Toxicology*, 14, 532-541.

126. Kerkvliet,N.I., Brauner,J.A., & Matlock,J.P. (1985). Humoral immunotoxicity of polychlorinated diphenyl ethers, phenoxyphenols, dioxins, and furans present as contaminants of technical grade pentachlorophenol. *Toxicology*, 36, 307-324.

127. Kerkvliet,N.I. & Burleson,G.R. (1994). Immunotoxicity of TCDD and related halogenated aromatic hydrocarbons. InJ.H.Dean, M.I.Luster, A.E.Munson, & I.Kimber (Eds.), *Immunotoxicology and Immunopharmacology* (pp. 97-121). New York, NY: Raven Press.

128. Kewley,R.J., Whitelaw,M.L., & Chapman-Smith,A. (2004). The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *International Journal of Biochemistry and Cell Biology*, 36, 189-204.

129. Kim,E.-Y. & Hahn,M.E. (2002). cDNA cloning and characterization of an aryl hydrocarbon receptor from the harbor seal (*Phoca vitulina*): a biomarker of dioxin susceptibility? *Aquatic Toxicology*, 58, 57-73.

130. Kim,E.-Y., Iwata,H., Suda,T., Tanabe,S., Amano,M., Miyazaki,N., & Petrov,E.A. (2005). Aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) expression in Baikal seal (*Pusa sibirica*) and association with 2,3,7,8-TCDD toxic equivalents and CYP1 expression levels. *Comparative Biochemistry and Physiology*, 141, 281-291.

131. Kimber,K. (2000). Serologic survey of selected viral agents in recently captured wild North American river otters. *Journal of Zoo and Wildlife Medicine*, 31, 168-175.

132. King,D.P. & Stott,J.L. (2002). The cytokines of marine mammals: biological properties and molecular analysis. In: Pfeiffer,C.J. (ed.) Molecular and Cell Biology of Marine Mammals. Krieger Publishing. Malabar, Florida.

133. Klaassen,C.D. (2001). *Casarett & Doull's Toxicology. The basic science of poisons*. New York: McGraw-Hill.

134. Krig,S.R., Roshantha,A.S., Chang,M.J., Wu,R., & Rice,R.H. (2002). Gene-specific TCDD suppression of RARα- and RXR-mediated induction of tissue transglutaminase. *Toxicological Sciences*, 68, 102-108.

135. Kuil,C.W., Brouwer,A., Van der Saag,P.T., & Van der Burg,B. (1998). Interference between progesterone and dioxin signal transduction pathways. *Journal of Biological Chemistry*, 273, 8829-8834.

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

137. Lambourn,D.M., Jeffries,S.J., & Dubey,J.P. (2001). Seroprevalence of *Toxoplasma gondii* in harbor seals (*Phoca vitulina)* in Southern Puget Sound. *Journal of Parasitology*, 87(1196), 1197.

138. Laws,R.J. & Taylor,R.J.F. (1957). A mass dying of crabeater seals, *Lobodon carcinophagus* (Gray). *Proceedings of the Zoological Society of London*, 129, 315-324.

139. Leichtling,K.D., Serrate,S.A., & Sztein,M.B. (1990). Thymosin alpha 1 modulates the expression of high affinity interleukin-2 receptors on normal human lymphocytes. *International Journal of Immunopharmacology*, 12(1), 19-29.

140. Levin,M., De Guise,S., & Ross,P.S. (2005). Lymphocyte proliferation is associated with PCBs in free-ranging harbor seal (*Phoca vitulina*) pups from British Columbia, Canada. *Environmental Toxicology and Chemistry*, In press.

141. Levin,M., Morsey,B., Mori,C., & De Guise,S. (2004). Specific non-coplanar PCB-mediated modulation of bottlenose dolphin and beluga whale phagocytosis upon in vitro exposure. *Journal of Toxicology and Environmental Health*, 67, 1517-1535.

142. Lu,Y.-C. & Wu,Y.-C. (1985). Clinical findings and immunological abnormalities in Yu-Cheng patients. *Environmental Health Perspectives*, 59, 17-29.

143. Luebke,R.W., Copeland,C.B., Diliberto,J.J., Akubue,P.I., Andrews,D.L., Riddle,M.M., Williams,W.C., & Birnbaum,L. (1994). Assessment of host resistance to *Trichinella spiralis* in mice following preinfection exposure to 2,3,7,8-TCDD. *Toxicology and Applied Pharmacology*, 125, 7-16.

144. Lundholm,C.E. (1997). DDE-induced eggshell thinning in birds: effects of p,p'-DDE on the calcium and prostaglandin metabolism of the eggshell gland. *Comparative Biochemistry and Physiology B*, 118C, 113-128.

145. Mahy,B.W.J., Barrett,T., Evans,S., Anderson,E.C., & Bostock,C.J. (1988). Characterization of a seal morbillivirus. *Nature*, 336, 115-116.

146. Maric,D., Veljic,J., Ranin,J., & Jankovic,B.D. (1991). In vivo effect of prothymosin-α1 on humoral and cell-mediated immune responses in the young rat. *International Journal of Neuroscience*, 59, 135-142.

147. Marlowe,J.L., Knudsen,E.S., Schwemberger,S., & Puga,A. (2004). The aryl hydrocarbon receptor displaces p300 from E2F-dependent promoters and represses S-phase specific gene expression. *Journal of Biological Chemistry*.

148. McClure,J.E., Lameris,N., Wara,D.W., & Goldstein,A.L. (1981). Immunochemical studies on thymosin: Radioimmunoassay of thymosin alpha1. *Journal of Immunology*, 368-375.

149. McConkey,D.J., Hartzell,P., Duddy,S.K., Hakansson,H., & Orrenius,S. (1988). 2,3,7,8-tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca2+-mediated endonuclease activation. *Science*, 242, 256-259.

150. McGinn,A. (2000). Why poison ourselves? A precautionary approach to synthetic chemicals. *Worldwatch Paper*, 153.

151. Mehta,K. (2002). Retinoic acid-a player that rules the game of life and death in neutrophils. *Indian Journal of Experimental Biology*, 40, 874-881.

152. Mellish,J.E., Iverson,S.J., Bowen,W.D., & Hammill,M.O. (1999). Fat transfer and energetics during lactation in the hooded seal: the roles of tissue lipoprotein lipase in milk fat secretion and pup blubber deposition. *Journal of Comparative Physiology B*, 169, 377-390.

153. Metcalfe,C.D., Koenig,B.G., Metcalfe,T.L., Paterson,G., & Sears,R. (2004). Intra- and inter-species differences in persistent organic contaminants in the blubber of blue whales and humpback whales from the Gulf of St. Lawrence, Canada. *Marine Environmental Research*, 57, 245-260.

154. Metcalfe,T.L. & Metcalfe,C.D. (1997). The trophodynamics of PCBs, including mono- and non-ortho congeners, in the food web of North-Central Lake Ontario. *Science of the Total Environment*, 201, 245-272.

155. Moody,T.W., Fagarasan,M., & Gozes,M. (1996). Inhibition of non-small cell lung cancer growth by thymosin α1 analogues. InH.R.Mauer, A.L.Goldstein, & E.D.Hager (Eds.), *Thymic peptides in preclinical and clinical medicine* (pp. 77-88). Munchen: W. Zuckschwerdt Verlag.

156. Mori,C., Morsey,B., Levin,M., Nambiar,P.R., & De Guise,S. (2005). Immunomodulatory effects of *in vitro* exposure to organochlorines on T cell proliferation in marine mammals and mice. *Journal of Toxicology and Environmental Health*, In press.

157. Mortensen,P., Bergman,A., Bignert,A., Hansen,H.-J., Härkönen,T., & Olsson,M. (1992). Prevalence of skull lesions in harbor seals (*Phoca vitulina*) in Swedish and Danish museum collections: 1835 - 1988. *Ambio*, 21, 520-524.

158. Mos,L., Jack,J., Cullon,D., Montour,L., Alleyne,C., & Ross,P.S. (2004). The importance of marine foods to a near-urban First Nations community in coastal British Columbia, Canada: Toward a risk-benefit assessment. *Journal of Toxicology and Environmental Health, Part A*, 67, 791-808.

159. Mos,L. & Ross,P.S. (2002). Vitamin A physiology in the precocious harbour seal (*Phoca vitulina)*: A tissue-based biomarker approach. *Canadian Journal of Zoology*, 80, 1511-1519.

160. Murk,A.J., Leonards,P.E.G., Van Hattum,B., Luit,R., Van der Weiden,M.E.J., & Smit,M. (1998). Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). *Environmental Toxicology and Pharmacology*, 6, 91-102.

161. Narayanan,P.K., Carter,W.O., Ganey,P.E., Roth,R.A., Voytik-Harbin,S.L., & Robinson,J.P. (1998). Impairment of human neutrophil oxidative burst by polychlorinated biphenyls: inhibition of superoxide dismutase activity. *Journal of Leukocyte Biology*, 63, 216-224.

162. Neale,J.C.C., Kenny,T.P., Tjeerdema,R.S., & Gershwin,M.E. (2005). PAH- and PCB-induced alterations of protein tyrosine kinase and cytokine gene transcription in harbor seal (*Phoca vitulina*) PBMC. *Clinical and Developmental Immunology*, 12, 91-97.

163. Neel,J.V., Centerwall,W.R., Chagnon,N.A., & Casey,H.L. (1970). Notes on the effect of measles in virgin-soil population of South American Indians. *American Journal of Epidemiology*, 91, 418-429.

164. Nohara,K., Fujimaki,H., Tsukumo,S., Inouye,K., Sone,H., & Tohyama,C. (2002). Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on T cell-derived cytokine production in ovalbumin (OVA)-immunized C57Bl/6 mice. *Toxicology*, 172, 49-58.

165. Nohara,K., Pan,X., Tsukumo,S., Hida,A., Ito,T., Nagai,H., Inouye,K., Motohashi,H., Yamamoto,M., Fujii-Kuriyama,Y., & Tohyama,C. (2005). Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage causes thymus involution and suppresses the immunization-induced increase in splenocytes. *Journal of Immunology*, 174, 2770-2777.

166. Nyman,M., Bergknut,M., Fant,M.L., Raunio,H., Jestoi,M., Bengs,C., Murk,A., Koistinen,J., Backman,C., Pelkonen,O., Tysklind,M., Hirvi,T., & Helle,E. (2003). Contaminant exposure and effects in Baltic ringed and grey seals as assessed by biomarkers. *Marine Environmental Research*, 55(73), 99.

167. Ohtake,F., Takeyama,K., Matsumoto,T., Kitagawa,H., Yamamoto,Y., Nohara,K., Tohyama,C., Krust,A., Mimura,J., Chambon,P., Yanagisawa,J., Fujii-Kuriyama,Y., & Kato,S. (2003). Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature*, 423, 545-550.

168. Olesiuk,P.F. (1999). *An assessment of the status if harbour seals (Phoca vitulina) in British Columbia*.

169. Olesiuk,P.F., Bigg,M.A., & Ellis,G.M. (1990). Recent trends in the abundance of harbour seals, *Phoca vitulina*, in British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences*, 47, 992-1003.

170. Olson,J.R. & Mcgarrigle,B.P. (1992). Comparative developmental toxicity of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD). *Chemosphere*, 25, 71-74.

171. Orris,P., Kaatz Chary,L., Perry,K., & Asbury,J. (2000). *Persistent Organic Pollutants and Human Health*. Washington DC: World Federation of Public Health Associations.

172. Osterhaus,A.D.M.E. (2001). Catastrophes after crossing spcies barriers. *Philos.Trans.R.Soc.Lond.B.Biol.Sci.*, 356, 791-793.

173. Osterhaus,A.D.M.E., De Swart,R.L., Vos,H.W., Ross,P.S., Kenter,M.J.H., & Barrett,T. (1995). Morbillivirus infections of aquatic mammals: newly identified members of the genus. *Veterinary Microbiology*, 44, 219-227.

174. Osterhaus,A.D.M.E. & Vedder,E.J. (1988). Identification of virus causing recent seal deaths. *Nature*, 335, 20.

175. Palace,V.P., Allen-Gil,S.M., Brown,S.B., Evans,R.E., Metner,D.A., Landers,D.H., & et al. (2001). Vitamin and thyroid status in arctic grayling (*Thymallus arcticus*) exposed to doses of 3,3',4,4'-tetrachlorobiphenyl that induce the phase I enzyme system. *Chemosphere*, 45(2), 185-193.

176. Palaszynski,E.W., Moody,T.W., O'Donohue,T.L., & Goldstein,A.L. (1983). Thymosin α_1-Like Peptides: Localization and Biochemical Characterization in the Rat Brain and Pituitary Gland. *Peptides*, 4, 463-467.

177. Peterson,R.E., Theobald,H.M., & Kimmel,G.L. (1993). Developmental and reproductive toxicity of dioxins and related compounds: cross-species comparisons. *Critical Reviews in Toxicology*, 23, 283-335.

178. Piñeiro,A., Cordero,O.J., & Nogueira,M. (2000). Fifteen years of prothymosin alpha: contradictory past and new horizons. *Peptides*,(21), -1433.

179. Poland,A. & Knutson,J.C. (1982). 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanisms of toxicity. *Annual Review of Pharmacology and Toxicology*, 22, 517-554.

180. Poland, A., Palen, D., and Glover, E. Analysis of the four alleles of the murine aryl hydrocarbon receptor. *Molecular Pharmacology,* 46, 915-921. 1991.

181. Pollenz,R.S. (2002). The mechanism of AH receptor protein downregulation (degradation) and its impact on AH receptor-mediated gene regulation. *Chemical and Biological Interactions*, 141, 41-61.

182. Puga,A., Xia,Y., & Elferink,C. (2002). Role of the aryl hydrocarbon receptor in cell cycle regulation. *Chemico-Biological Interactions*, 141, 117-130.

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

184. Rogan,W.J., Gladen,B.C., Hung,K.-L., Koong,S.-L., Shih,L.-Y., Taylor,J.S., Wu,Y.-C., Yang,D., Ragan,N.B., & Hsu,C.-C. (1988). Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. *Science*, 241, 334-336.

185. Roitt,I. (1990). *Essential Immunology*. Oxford: Blackwell Scientific Publications.

186. Rolland,R.M. (2000). A review of chemically-induced alterations in thyroid and vitamin A status from field studies of wildlife and fish. *Journal of Wildlife Diseases*, 36(4), 615-635.

187. Ross,A.C. & Hämmerling,U.G. (1994). Retinoids and the immune system. InM.B.Sporn, A.B.Roberts, & D.S.Goodman (Eds.), *The retinoids: biology, chemistry, and medicine* (pp. 521-543). New York: Raven Press, Ltd.

188. Ross,A.C. & Stephensen,C.B. (1996). Vitamin A and retinoids in antiviral responses. *Federation of American Societies for Experimental Biology Journal*, 10, 979-985.

189. Ross,P.S. (2000). Marine mammals as sentinels in ecological risk assessment. *Human and Ecological Risk Assessment*, 6, 29-46.

190. Ross,P.S. (2002). The role of immunotoxic environmental contaminants in facilitating the emergence of infectious diseases in marine mammals. *Human and Ecological Risk Assessment*, 8, 277-292.

191. Ross,P.S. & Birnbaum,L.S. (2002). Integrated human and ecological risk assessment. Case study package I: persistent organic pollutants (POPs) in humans and wildlife. *Human and Ecological Risk Assessment*, 9, 303-324.

192. Ross,P.S. & De Guise,S. (2005). Marine mammal immunotoxicology.

193. Ross,P.S., De Swart,R.L., Addison,R.F., Van Loveren,H., Vos,J.G., & Osterhaus,A.D.M.E. (1996a). Contaminant-induced immunotoxicity in harbour seals: wildlife at risk? *Toxicology*, 112, 157-169.

194. Ross,P.S., De Swart,R.L., Reijnders,P.J.H., Van Loveren,H., Vos,J.G., & Osterhaus,A.D.M.E. (1995). Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals fed herring from the Baltic Sea. *Environmental Health Perspectives*, 103, 162-167.

195. Ross,P.S., De Swart,R.L., Timmerman,H.H., Reijnders,P.J.H., Vos,J.G., Van Loveren,H., & Osterhaus,A.D.M.E. (1996b). Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. *Aquatic Toxicology*, 34, 71-84.

196. Ross,P.S., De Swart,R.L., Van der Vliet,H., Willemsen,L., De Klerk,A., Van Amerongen,G., Groen,J., Brouwer,A., Schipholt,I., Morse,D.C., Van Loveren,H., Osterhaus,A.D.M.E., & Vos,J.G. (1997). Impaired cellular immune response in rats exposed perinatally to Baltic Sea herring oil or 2,3,7,8-TCDD. *Archives of Toxicology*, 17, 563-574.

197. Ross,P.S., De Swart,R.L., Van Loveren,H., Osterhaus,A.D.M.E., & Vos,J.G. (1996c). The immunotoxicity of environmental contaminants to marine wildlife: A review. *Annual Review of Fish Diseases*, 6, 151-165.

198. Ross,P.S., De Swart,R.L., Visser,I.K.G., Murk,W., Bowen,W.D., & Osterhaus,A.D.M.E. (1994). Relative immunocompetence of the newborn harbour seal, *Phoca vitulina*. *Veterinary Immunology and Immunopathology*, 42, 331-348.

199. Ross,P.S., Ellis,G.M., Ikonomou,M.G., Barrett-Lennard,L.G., & Addison,R.F. (2000a). High PCB concentrations in free-ranging Pacific killer whales, *Orcinus orca*: effects of age, sex and dietary preference. *Marine Pollution Bulletin*, 40, 504-515.

200. Ross,P.S., Jeffries,S.J., Yunker,M.B., Addison,R.F., Ikonomou,M.G., & Calambokidis,J.C. (2004). Harbor seals (*Phoca vitulina*) in British Columbia, Canada, and Washington State, USA, reveal a combination of local and global polychlorinated biphenyl, dioxin, and furan signals. *Environmental Toxicology and Chemistry*, 23, 157-165.

201. Ross,P.S., Pohajdak,B., Bowen,W.D., & Addison,R.F. (1993). Immune function in free-ranging harbor seal (*Phoca vitulina*) mothers and their pups during lactation. *Journal of Wildlife Diseases*, 29, 21-29.

202. Ross,P.S. & Troisi,G.M. (2001). Pinnipeds. InR.F.Shore & B.A.Rattner (Eds.), *Ecotoxicology of wild mammals* New York: John Wiley & Sons.

203. Ross,P.S., Van Loveren,H., De Swart,R.L., Van der Vliet,H., De Klerk,A., Timmerman,H.H., Van Binnendijk,R.S., Brouwer,A., Vos,J.G., & Osterhaus,A.D.M.E. (1996d). Host resistance to rat cytomegalovirus (RCMV) and immune function in adult PVG rats fed herring from the contaminated Baltic Sea. *Archives of Toxicology*, 70, 661-671.

204. Ross,P.S., Visser,I.K.G., Broeders,H.W.J., Van de Bildt,M.W.G., Bowen,W.D., & Osterhaus,A.D.M.E. (1992). Antibodies to phocine distemper virus in Canadian seals. *Veterinary Record*, 130, 514-516.

205. Ross,P.S., Vos,J.G., Birnbaum,L.S., & Osterhaus,A.D.M.E. (2000b). PCBs are a health risk for humans and wildlife. *Science*, 289, 1878-1879.

206. Safe,S. & Wormke,M. (2003). Inhibitory aryl hydrocarbon receptor-estrogen receptor α crosstalk and mechanisms of action. *Chemical Research in Toxicology*, 16, 807-816.

207. Sagerup,K., Henriksen,E.O., Skorping,A., Skaare,J.U., & Gabrielsen,G.W. (2000). Intensity of parasitic nematodes increases with organochlorine levels in the glaucous gull. *Journal of Applied Ecology*, 37, 532.

208. Sarandeses,C.S., Covelo,G., Diaz-Jullien,C., & Freire,M. (2003). Prothymosin α is processed to thymosin α_1 and thymosin α_{11} by a lysosomal asparaginyl endopeptidase. *Journal of Biological Chemistry*, 278, 13286-13293.

209. Schmidt,J.V. & Bradfield,C.A. (1996). Ah receptor signalling pathways. *Annual Review of Cell and Developmental Biology*, 12, 55-89.

210. Schweigert,F.J., Ryder,O.A., Rambeck,W.A., & Zucker,H. (1996). The majority of vitamin A is transported as retinyl esters in the blood of most carnivores. *Comparative Biochemistry and Physiology A*, 95, 573-578.

211. Sharma,R.P., Kociba,R.J., & Gehring,P.J. (1978). Immunotoxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals. *Toxicology and Applied Pharmacology*, 45, 333.

212. Shaw,S.D. (1998). *Organochlorines and biomarkers of immune and endocrine effects in Pacific harbor seal and Northern elephant seal pups*. New York, NY: PhD.Thesis.

213. Shin,Y., Mori,T., Okita,M., Gemma,T., Kai,C., & Mikami,T. (1995). Detection of canine distemper virus nucleocapsid protein gene in canine peripheral blood mononuclear cells by RT-PCR. *Journal of Veterinary Medical Science*, 57(3), 439-445.

214. Silkworth,J.B., Antrim,L., & Kaminsky,L.S. (1984). Correlations between polychlorinated biphenyl immunotoxicity, the aromatic hydrocarbon locus, and

liver microsomal enzyme induction in C57BL/6 and DBA/2 mice. *Toxicology and Applied Pharmacology*, 75, 156-165.

215. Silkworth,J.B., Antrim,L.A., & Sack,G. (1986). *Ah* receptor mediated suppression of the antibody response in mice is primarily dependent on the *Ah* phenotype of lymphoid tissue. *Toxicology and Applied Pharmacology*, 86, 380-390.

216. Silkworth,J.B. & Grabstein,E.M. (1982). Polychlorinated biphenyl immunotoxicity: dependence on isomer planarity and the *Ah* gene complex. *Toxicology and Applied Pharmacology*, 65, 109-115.

217. Simms,W., Jeffries,S.J., Ikonomou,M.G., & Ross,P.S. (2000). Contaminant-related disruption of vitamin A dynamics in free-ranging harbor seal (*Phoca vitulina*) pups from British Columbia, Canada and Washington State, USA. *Environmental Toxicology and Chemistry*, 19(11), 2844-2849.

218. Simms,W. & Ross,P.S. (2000). Developmental changes in circulatory vitamin A (retinol) and its transport proteins in free-ranging harbour seal (*Phoca vitulina*) pups. *Canadian Journal of Zoology*, 78(10), 1862-1868.

219. Simms,W. & Ross,P.S. (2001). Vitamin A physiology and its application as a biomarker of contaminant-related toxicity in marine mammals: a review. *Toxicology and Industrial Health*, 16(6), 291-302.

220. Smialowicz,R.J., Burleson,G.R., & Kerkvliet,N.I. (2000). Immunotoxicity. *Health assessment document for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds* Washington, D.C.: Office of Health and Environmental Assessment. US EPA.

221. Spangelo,B.L. (1995). The thymic-endocrine connection. *Journal of Endocrinology*, 147, 5-10.

222. Spear,P.A., Bourbonnais,D.H., Peakall,D.B., & Moon,T.W. (1989). Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3',4,4'-tetrachlorobiphenyl. *Canadian Journal of Zoology*, 67, 908-913.

223. Spear,P.A., Moon,T.W., & Peakall,D.B. (1986). Liver retinoid concentrations in natural populations of herring gulls (*Larus argentatus*) contaminated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and in ring doves (*Streptopelia risoria*) injected with a dioxin analogue. *Canadian Journal of Zoology*, 64, 204-208.

224. Stebbing,A.R.D. (1982). Hormesis: the stimulation of growth by low levels of inhibitors. *Science of the Total Environment*, 22, 213-234.

225. Stehr-Green,P.A., Naylor,P.H., & Hoffman,R.E. (1989). Diminished thymosin-alpha-1 levels in persons exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Journal of Toxicology & Environmental Health*, 28, 285-295.

226. Subramanian,A.N., Tanabe,S., & Tatsukawa,R. (1988). Use of organochlorines as chemical tracers in determining some reproductive parameters in Dalli-type

Dall's porpoise Phocoenoides dalli. *Marine Environmental Research*, 25, 161-174.

227. Subramanian,A.N., Tanabe,S., Tatsukawa,R., Saito,S., & Miyazaki,N. (1987). Reduction in the testosterone levels by PCBs and DDE in Dall's porpoises of northwestern North Pacific. *Marine Pollution Bulletin*, 18, 643-646.

228. Tabuchi,M., Veldhoen,N., Dangerfield,N., Helbing,C.C., & Ross,P.S. (2006). PCB-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbor seals (*Phoca vitulina*). *Environmental Health Perspectives*, In press.

229. Tanabe,S., Iwata,H., & Tatsukawa,R. (1994). Global contamination by persistent organochlorines and their ecotoxicological impact on marine mammals. *Science of the Total Environment*, 154, 163-177.

230. Tanabe,S. & Tatsukawa,R. (1992). Chemical modernization and vulnerability of cetaceans: increasing toxic threat of organochlorine contaminants. InC.H.Walker & D.R.Livingstone (Eds.), *Persistent pollutants in marine ecosystems* (pp. 161-177). New York: Pergamon Press.

231. Tanabe,S., Tatsukawa,R., Maruyama,K., & Miyazaki,N. (1982). Transplacental transfer of PCBs and chlorinated hydrocarbon pesticides from the pregnant striped dolphin (*Stenella coeruleoalba*) to her fetus. *Agricultural and Biological Chemistry*, 46, 1249-1254.

232. Thigpen,J.E., Faith,R.E., McConnell,E.E., & Moore,J.A. (1975). Increased susceptibility to bacterial infection as a sequela of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Infection and Immunity*, 12, 1319-1324.

233. Tijet,N., Boutros,P.C., Moffat,I.D., Okey,A.B., Tuomisto,J., & Pohjanvirta,R. (2006). Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. *Molecular Pharmacology*, 69, 140-153.

234. Tryphonas,H., Luster,M.I., Schiffman,G., Dawson,L.-L., Hodgen,M., Germolec,D., Hayward,S., Bryce,F., Loo,J.C.K., Mandy,F., & Arnold,D.L. (1991a). Effect of chronic exposure of PCB (Aroclor 1254) on specific and nonspecific immune parameters in the Rhesus (*Macaca mulatta*) monkey. *Fundamental and Applied Toxicology*, 16, 773-786.

235. Tryphonas,H., Luster,M.I., White,K.L., Jr., Naylor,P.H., Erdos,M.R., Burleson,G.R., Germolec,D., Hodgen,M., Hayward,S., & Arnold,D.L. (1991b). Effects of PCB (Aroclor 1254) on non-specific immune parameters in rhesus (*Macaca mulatta*) monkeys. *International Society for Immunopharmacology*, 13, 639-648.

236. Tsitsiloni,O.E., Stiakakis,J., Koutselinis,A., Gogas,J., Markopoulos,C., Yialouris,P.P., Bekris,S., Panoussopoulos,D., Kiortsis,V., Voelter,W., & Haritos,A.A. (1993). Expression of α-thymosins in human tissues in normal and abnormal growth. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 9504-9507.

237. Tucker,A.N., Vore,S.J., & Luster,M.I. (1986). Suppression of B cell differentiation by 2,3,7,8- tetrachlorodibenzo-p-dioxin. *Molecular Pharmacology*, 29, 372-377.

238. United Nations Environment Programme (1995). *Global Programme of Action for the Protection of the Marine Environment from Land-Based Activities.* Washington DC.

239. Van den Berg,M., Birnbaum,L., Bosveld,A.T.C., Brunstrom,B., Cook,P., Feeley,M., Giesy,J.P., Hanberg,A., Hasegawa,R., Kennedy,S.W., Kubiak,T., Larsen,J.C., Van Leeuwen,F.X.R., Liem,A.K., Nolt,C., Peterson,R.E., Poellinger,L., Safe,S.H., Schrenk,D., Tillitt,D.E., Tysklind,M., Younes,M., Waern,F., & Zacharewski,T.R. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environmental Health Perspectives*, 106, 775-792.

240. Vecchi,A., Sironi,M., Canegrati,M.A., Recchia,M., & Garrattini,S. (1983). Immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in strains of mice with different susceptibility to induction of aryl hydrocarbon hydroxylase. *Toxicology and Applied Pharmacology*, 68, 434-441.

241. Veldhoen,N. & Helbing,C.C. (2001). Detection of environmental endocrine-disruptor effects on gene expression in live *Rana catesbeiana* tadpoles using a tail fin biopsy technique. *Environmental Toxicology & Chemistry*, 20(12), 2704-2708.

242. Vethaak,A.D., Jol,J.G., Meijboom,A., Eggens,M.L., Rheinallt,T., Wester,P.W., De Zande,T., Bergman,A., Dankers,N., Ariese,F., Baan,R.A., Everts,J.M., Opperhuizen,A., & Marquenie,J.M. (1996). Skin and liver diseases induced in flounder (*Platichthys flesus*) long-term exposure to contaminated sediments in large-scale mesocosms. *Environmental Health Perspectives*, 104(11), 1218-1229.

243. Visser,I.K.G., Kumarev,V.P., Örvell,C., De Vries,P., Broeders,H.W.J., Van de Bildt,M.W.G., Groen,J., Teppema,J.S., Burger,M.C., UytdeHaag,F.G.C.M., & Osterhaus,A.D.M.E. (1990). Comparison of two morbilliviruses isolated from seals during outbreaks of distemper in North West Europe and Siberia. *Archives of Virology*, 111, 149-164.

244. Visser,I.K.G., Van Bressem,M.F., De Swart,R.L., Van de Bildt,M.W.G., Vos,H.W., Van der Heijden,R.W.J., Saliki,J.T., Örvell,C., Kitching,P., Kuiken,T., Barrett,T., & Osterhaus,A.D.M.E. (1993). Characterization of morbilliviruses isolated from dolphins and porpoises in Europe. *Journal of General Virology*, 74, 631-641.

245. Voie,Ø.A., Tysklind,M., Andersson,P.L., & Fonnum,F. (2000). Activation of respiratory burst in human granulocytes by polychlorinated biphenyls: A structure-activity study. *Toxicology and Applied Pharmacology*, 167, 118-124.

246. Von Boehmer,H. (1988). The developmental biology of T lymphocytes. *Annual Reviews in Immunology*, 6, 309-326.

247. Vorderstrasse,B.A., Steppan,L.B., Silverstone,A.E., & Kerkvliet,N.I. (2001). Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. *Toxicology and Applied Pharmacology*, 171, 157-164.

248. Vos,J.G., Moore,J.A., & Zinkl,J.G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Environmental Health Perspectives*, 5, 149-162.

249. Weisglas-Kuperus,N., Patandin,S., Berbers,G.U.M., Sas,T.C., Mulder,P.G.H., Sauer,P.J.J., & Hooijkaas,H. (2000). Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. *Environmental Health Perspectives*, 108(12), 1203-1207.

250. Weisglas-Kuperus,N., Sas,T.C., Koopman-Esseboom,C., Van der Zwan,C.W., De,R.M.A., Beishuizen,A., Hooijkaas,H., & Sauer,P.J.J. (1995). Immunologic effects of background prenatal and postnatal exposure to dioxins and polychlorinated biphenyls in Dutch infants. *Pediatric Research*, 38, 404-410.

251. Weston,W.M., Nugent,P., & Greene,R.M. (1995). Inhibition of retinoic acid-induced gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochem Biophys Res Comm*, 207, 690-694.

252. Widerak,M., Ghoneim,C., Dumontier,M.-F., Quesne,M., Corvol,M.T., & Savouret,J.-F. (2006). The aryl hydrocarbon receptor activates the retinoic acid receptor α through SMRT antagonism. *Biochimie*, In press.

253. Williams,E.S. (2001). Canine distemper. InE.S.Williams & I.K.Barker (Eds.), *Infectious diseases of wild mammals* (pp. 50-59). Iowa State University Press.

254. Wise,T.H. (1992). Developmental changes of serum thymosin α1 and β4 in male and male castrated pigs: Modulation by testosterone and human chorionic gonadotropin. *Biology of Reproduction*, 46, 892-897.

255. Wolkers,H., Lydersen,C., & Kovacs,K.M. (2004). Accumulation and lactational transfer of PCBs and pesticides in harbor seals (*Phoca vitulina*) from Svalbard, Norway. *Science of the Total Environment*, 319, 137-146.

256. WWF (2005). *Stockholm Convention: "New POPs". Screening additional POPs candidates*. Washington D.C.: WWF Global Toxics Programme.

257. Yamada-Okabe,T., Aono,T., Sakai,H., Kashima,Y., & Yamada-Okabe,H. (2004). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin augments the modulation of gene expression mediated by the thyroid hormone receptor. *Toxicology and Applied Pharmacology*, 194, 201-210.

258. Yamamoto,J., Ihara,K., Nakayama,H., Hikino,S., Satoh,K., Kubo,N., Lida,T., Fujii,Y., & Hara,T. (2004). Characteristic expression of aryl hydrocarbon receptor repressor gene in human tissues: organ-specific distribution and variable induction patterns in mononuclear cells. *Life Sciences*, 74, 1039-1049.

259. Yu,M.-L., Hsin,J.-W., Chan,W.C., & Guo,Y.L. (1998). The immunological evaluation of the Yucheng children. *Chemosphere*, 37, 1855-1865.

260. Yunker,M.B., Belicka,L.L., Harvey,H.R., & Macdonald,R.W. (2005). Tracing the inputs and fate of marine and terrigenous organic matter in Arctic Ocean sediments: A multivariate analysis of lipid biomarkers. *Deep Sea Research II*, In press.

261. Zhang,P. (1999). The cell cycle and development: redundant roles of cell cycle regulators. *Current Opions in Cell Biology*, 11, 655-662.

262. Zile,M.H. (1992). Vitamin-A homeostasis endangered by environmental pollutants. *Proceedings of the Society for experimental biology and medicine*, 201, 141-153.