Thyroid hormones and their receptor gene expression as biomarkers of endocrine disruption in harbour seals (*Phoca vitulina*)

by

Maki Tabuchi
B.Sc, Florida State University, 2001

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In the Department of Biochemistry and Microbiology

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Abstract

Persistent organic pollutants (POPs) are ubiquitous environmental contaminants that are lipophilic, slow to degrade, bioaccumulate in aquatic food webs and threaten the health of both humans and wildlife. Predatory marine mammals, such as harbour seals (*Phoca vitulina*), are at particularly risk for the accumulation of high POP concentrations and resultant increased risk of toxic effects. Elevated levels of certain POPs have been implicated in the disruption of the endocrine system in marine mammals. The main purpose of this study was to assess whether current levels of POP are affecting thyroid hormone physiology of free-ranging harbour seals (*Phoca vitulina*) in British Columbia (BC), Canada and Washington State (WA), U.S.A. TH functions mainly by binding to nuclear thyroid hormone receptors (TRs) in target tissues and modulating specific gene expression programs. TR isoforms α (TRα) and β (TRβ) from harbour seals were isolated and quantified in internal and external organs. Harbour seals inhabiting industrialized regions exhibited a contaminant-related increase in blubber TRα and a decrease in

circulating total thyroxine (TT_4) concentrations. Our TRα expression results provide evidence of contaminant-related disruption of TH action at the level of regulation of gene expression. Our findings of a metabolically active blubber layer, and a contaminant–related disruption of blubber TRα expression, suggest that, in addition to disruption of normal development, contaminant exposure could have important implications for lipid metabolism in seals. Consequently, the disruption of blubber TRα expression could influence such critical life processes as energy storage, thermoregulation, and buoyancy in marine mammals. In this study, the use of gene expression biomarkers in combination with a biopsy-based sampling approach was successfully applied to a small marine mammal (i.e. harbour seal) and demonstrates great promise for investigations in other sentinel species (i.e. cetaceans).

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List of Abbreviations

AhR	aryl hydrocarbon receptor
BC	British Columbia
BMR	basal metabolic rate
bp	base pairs
cDNA	complementary DNA
CYP	cytochrome p450 oxidase
DBD	DNA binding domains
DDD	dichlorodiphenyldichloromethane
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
ELISA	enzyme linked immunosorbent assay
FT_3	free triiodothyronine
FT_4	free thyroxine
GI	Gertrude Island
GPC	gel permeation chromatography
HAT	histone acetyltransferaes
HCB	hexachlorobenzene
HCH	hexachlorohexane
HDACs	histone deacetylases
HRP	horseradish peroxidase
hTRβ	human thyroid hormone receptor beta
ID	iodothyronine deiodinase
L8	ribosomal protein L8
LBD	ligand binding domain
lw	lipid weight
NCoR	nuclear receptor corepressor
OC	organochlorine
OH-PCB	hydroxyl polychlorinated biphenyl
PBDEs	polybrominated diphenylethers
PCB	polychlorinated biphenyl

PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
POP	persistent organic pollutant
PS	Puget Sound
PUFA	polyunsaturated fatty acid
QCS	Queen Charlotte Strait
QPCR	real-time quantitative polymerase chain reaction
RBP	retinol binding protein
rT_3	reverse triiodothyronine
RXR	retinoid X receptor
SMRT	silencing mediator of retinoid and thyroid hormone receptor
TBG	thyroxine-binding globlin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	toxic equivalency factor
TEQ	toxic equivalent
TG	thyroglobulin
TH	thyroid hormone
TMB	tetramethylbenzidine
T_2	3,5-diiodo-l-thyronine
T_3	triiodothyronine
T_4	thyroxine
TPO	thyroid peroxidase
TRH	thyrotropin-releasing hormone
TR	thyroid hormone receptor
TRα	thyroid hormone receptor alpha
TRβ	thyroid hormone receptor beta
TRE	thyroid hormone response element
TSH	thyroid stimulating hormone
TT_3	total triiodothyronine
TT_4	total thyroxine
TTR	transthyretin

UDP-GT	uridine diphosphate glucuronosyltransferase
WA	Washington

Chapter 1

Introduction

1. Persistent organic pollutants (POPs)

More than 100,000 anthropogenic organic chemicals are currently in use, and the long-term effects of many of these chemicals on the health of humans and wildlife are unknown. The persistent organic pollutants (POPs) are of particular concern because of their resistance to biological and chemical breakdown in the environment. Unlike many other pollutants, POPs tend to travel long distances with atmospheric and oceanographic transport processes and have become widely distributed pollutants. POPs have been detected all over the world, including remote areas such as the Arctic and the Antarctic, far away from POP sources (1). In the environment, POPs are either associated with sediments, or bioaccumulate in the fatty tissues of organisms, due to their lipophilicity. POPs are thereby able to biomagnify in food webs. The species at the tops of food chains, such as predatory birds and marine mammals, can thus be exposed to elevated levels of these compounds and may be at risk for toxic effects (2). In wildlife, a number of adverse health effects have been associated with POP exposure, including reproductive dysfunction, eggshell thinning, metabolic changes, deformities and birth defects, tumors and cancers, behavioral changes, endocrine disruption, and immunosuppression (3-6).

Because of these observed deleterious health effects, many POPs have been the target of intergovernmental agreements attempting to reduce or eliminate the release of POPs into the environment. Twelve major chlorinated POPs, known as the "Dirty Dozen", were the first to be banned. Many of these were used as pesticides (aldrin, dieldrin, chlordane, 1,1,1-trichloro-2,2 (4 chlorophenyl)- ethane (DDT), endrin, mirex, heptachlor, toxaphene), whereas others were industrial chemicals {polychlorinated biphenyls (PCBs)} or industrial byproducts {polychlorinated dibenzo-p-dioxins (PCDDs) and

polychlorinated dibenzofurans (PCDFs)}. Hexachlorobenzene (HCB) falls under all three categories. Although the use and production of these POPs has now been reduced or eliminated in many countries, their global distribution and persistence have resulted in a slow decrease in environmental concentrations, and species at the top of food web continue to be exposed to POPs.

1.1. PCBs, dioxins, and furans

PCBs, PCDDs, and PCDFs are structurally-related compounds consisting of two benzene rings and various numbers of chlorines. Depending on the position and number of chlorines, there are 209 theoretically possible congeners of PCBs, 75 PCDDs and 135 PCDFs (Figure 1.1). Whereas the PCDDs and PCDFs were never intentionally produced, PCBs were once widely applied as coolants and lubricants in electrical transformers, flame retardants, adhesives, sealants, paints, and in carbonless copy papers (5;7). Although the production and use of PCBs in most industrialized nations was terminated in the 1970s due to their identification as persistent chemicals in wildlife (8), over 1.2 million tons of PCBs were released worldwide (660,000 tons in North America alone) during their approximately 30 years of use (9). Current use of PCBs is restricted only to closed systems in most of developed countries. However, PCBs continue to be released into the natural environment through accidents, leakage, or improper disposal, and the amount of this current release is unknown (5).

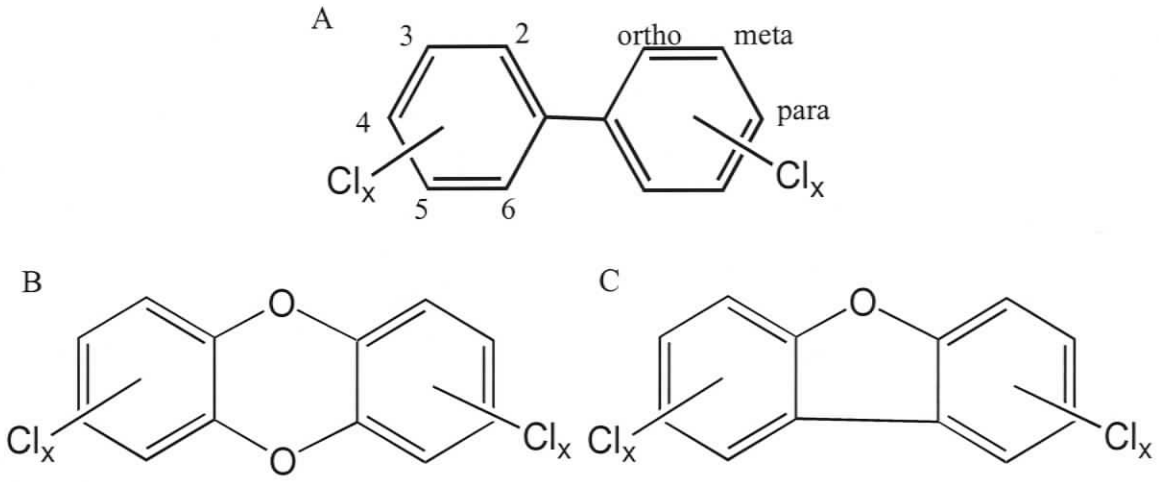

Figure 1.1. Chemical structures of PCBs and structurally related compounds.
(A) polychlorinated biphenyls (PCBs), (B) polychlorinated dibenzo-para-dioxins (PCDDs), and (C) polychlorinated dibenzo-furans (PCDFs) are structurally similar group of industrial POPs. PCB congeners with one or no substitution in the ortho position form planer configurations like PCDDs and PCDFs.

PCDDs and PCDFs are formed as by-products in many industrial and combustion processes. The production and use of organic chemicals containing chlorine, including the production of pesticides and wood preservatives, as well as processes historically used during pulp and paper bleaching, are the primary sources of PCDD/PCDF contamination in environment (7;10). PCDDs and PCDFs are also produced by natural events such as forest fires and volcanic eruptions. Since PCDDs and PCDFs are subject to degradation in natural environments, and such events are taking place gradually over time, these natural releases are less critical compared with anthropogenic releases.
Among all the congeners of PCDDs, PCDFs, and PCBs, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) is known to be the most toxic compound ever produced by humans. Its toxicity is believed to be mediated *via* the aryl hydrocarbon receptor (AhR).

Besides TCDD itself, six PCDD congeners, 10 PCDF congeners, and 12 PCB congeners have the ability to bind to the AhR with lesser affinities, but share the resulting toxic effects. AhR acts primarily as ligand-dependent transcription factor, and the binding of dioxins and dioxin-like compounds changes the expression of AhR dependent genes (e.g., detoxification phase I enzymes, cytochrome p-450 oxidase (CYP) 1A1, CYP 1A2, and CYP 1B1), causing a wide range of toxic effects including reproductive defects, developmental abnormality, endocrine disruption, immunotoxicity, cancer and liver damage (5;11).

Based on similarities in structure and toxicity, the toxic equivalency factor (TEF) approach was developed for application in hazard and risk assessments of dioxins and dioxin-like compounds in various environments. Dioxin itself, with the highest binding affinity to AhR, was given a TEF value of 1.0, and all other compounds were assessed as fraction of 1.0, based on their relative ability to bind the AhR (12). TEFs multiplied by the concentrations of individual congeners calculate a value that assesses the toxicity of complex mixtures of contaminants by expressing them as a toxic equivalency (TEQ) to TCDD.(12).

Although the TEF/TEQ approach is an important tool in the assessment of dioxin-like environmental contaminants, it is important to note that non-dioxin-like compounds in the PCB group of contaminants are also abundant in the environment, and may also contribute to adverse health effects. However, their toxic effects and the mechanisms by which they are mediated are less understood (3;13;14). In addition, some PCB metabolites, particularly hydroxyl PCBs (OH-PCBs), are known to exert toxicological effects, which are different from those exerted by parent compounds (15;16).

1.2. Organochlorine pesticides

Many of the organochlorine contaminants other than PCBs are pesticides that have been widely used for the control of disease-transmitting insects, termites, and soil insects affecting agricultural production (Figure 1.2). Like PCBs, these compounds tend to be lipophilic, bind easily to sediments, and are extremely persistent. For example, mirex has a half-life of up to ten years in soil, endrin up to 12 years, and chlordane over 20 years. Organochlorine (OC) pesticides may also be volatilized, transported in air currents, and deposited into colder regions far from their sources. Hexachlorocyclohexane (HCH), commonly detected in Arctic regions, represents an example of the latter group.

Many of the OC pesticides have been targeted together with the PCBs under the Stockholm Convention (United Nations Environmental Programme, http://www.chem.unep.ch/pops). However, because there are no effective replacement chemicals available, or the alternatives are financially unattractive to developing nations, parts of Asia and Africa continue to use OC pesticides. In these particular cases, the risks of OC exposure and the associated risks of adverse health effects are still small compared to the economical and industrial impacts of insects on crops and human health. For many of the OC pesticides, acute toxicity targets the nervous system (17). Chronic effects are varied but include carcinogenicity, reproductive effects, and endocrine disruption (17). Dichlorodiphenyltrichloroethane (DDT) is one of the earliest and the most widely used pesticides. Beginning in the 1940s, it was used for controlling insects spreading human disease (e.g., malaria and typhus) among both military troops and civilians.

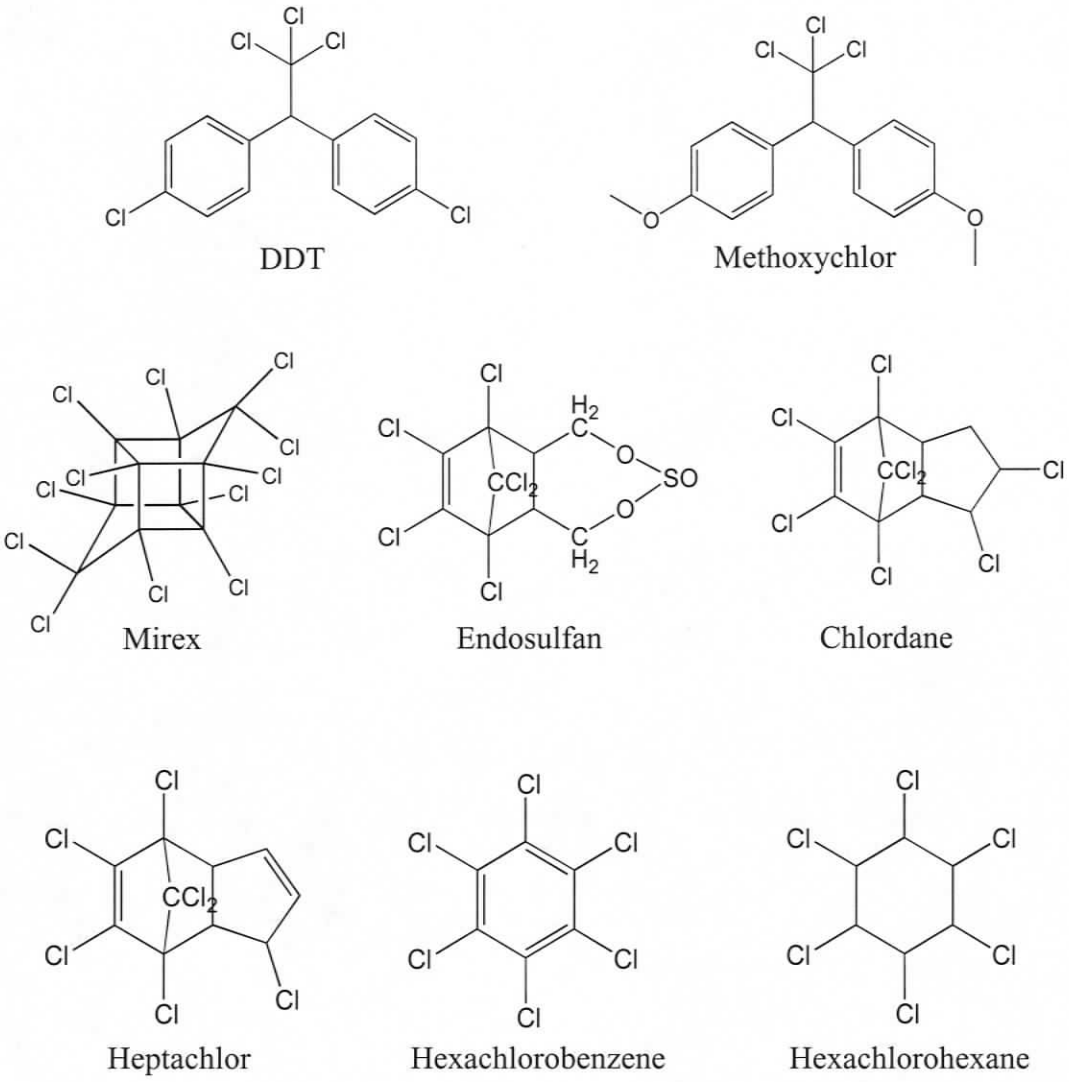

Figure 1.2. Chemical structures of major organochlorine pesticides.

A variety chemical structures is observed among the organochlorine pesticides. Four primary groups can be identified: (1) dichlorodiphenylethanes; DDT, methoxychlor, (2) chlordecone; mirex, (3) cyclodines; endosulfan, heptachlor, chlordane, and (4) chlorinated benzenes and cyclohexanes; hexachlorobenzene and hexachlorohexane.

The mechanism by which DDT kills insects involves the opening of sodium channels in neurons, causing the neuron to signal constantly, eventually leading to uncontrolled spasms and death. In wildlife, DDT exposure has been related to impaired egg-shell quality in fish-eating birds, and impairment of reproduction in fish. Concern as to the potential effects of DDT on human health led to the banning in the 1970s of this pesticide in agricultural applications in many developed countries (18;19).

1.3 Fate of POPs in the marine environment

POPs are widespread environmental contaminants, reaching even the most remote areas, because of their chemical properties. The relatively volatile and more water soluble compounds, such as lesser chlorinated PCBs and HCH, are transported readily as gases or aerosols by atmospheric processes. After traveling long distances, they can be deposited through precipitation and condensation in colder regions. Conversely, the less volatile and less water soluble POPs, such as the more highly chlorinated PCBs, tend to be absorbed onto particulate matter, and are either ingested by organisms or deposited into sediments.

POPs are resistant to biotic and abiotic degradation in the marine environment. In general, the more highly chlorinated the compounds, the more resistant it will be to degradation. The primary routes of POP degradation are biodegradation by micro-organisms (20) and degradation by sunlight through photolysis. Lastly, enzymatic

transformation and excretion through detoxification systems of higher organisms are also important factors influencing POP degradation.

In vertebrates, the main mechanism of POP metabolism involves two reaction phases. A Phase I reaction introduces polar functional groups (e.g. –OH, -NH_2, -SH, or –COOH) onto the lipophilic POPs, making the compounds more water soluble. This intermediate structure is also important for the attachment of a larger molecule during the Phase II reaction (e.g. glucuronidation, sulfation, acetylation, methylation, and conjugation), which increases hydrophilicity. The final molecules can be excreted from the body. The less chlorinated POPs are more easily metabolized and excreted as compared with the more highly chlorinated POPs. Resistance to metabolism also depends on the substitution pattern of the chlorines. Due to the interspecies variation in activities of the biotransformation enzymes, the capacity to detoxify POPs differs among species. The capacity has been observed at the lowest levels in invertebrates, at intermediate levels in fish, and at the highest levels in mammals and birds (21;22). Among mammals, marine mammals represent an exception as they have relatively low POP detoxification capacities thus resulting in greater POP body burdens (23).

The metabolism of POPs not only removes these compounds from the body, but also produces metabolites which may be more toxic and more persistent than the parent compounds. For example, an oxidative reaction induced by phase I enzymes creates OH-PCBs that have mutagenic and carcinogenic potential, and can disrupt certain hormone systems. Dechlorinated metabolites of DDT, such as Dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloromethane (DDD), retain their lipophilicity, and are

therefore persistent in the body and able to produce toxic effects. For example, DDE is known to induce a number of toxicological effects in wildlife, such as eggshell thinning (prevention of calcium fixation), and thyroid and adrenal gland disruption (24;25).

2. Marine mammals

Certain groups of mammals have evolved secondary adaptations over the last 50 million years, enabling them to switch from a primarily terrestrial existence to a marine-based life style (26). Currently, more than 120 living marine mammal species (defined as mammals that are primary ocean-dwelling or depend on the ocean for food) are recognized. They are represented within three orders; 1) Pinnipedia, including the seals, sea lions and walruses, 2) Cetacea, including the whales and dolphins, and 3) Sirenia, including the manatees and dugongs. Although marine mammals share many characteristics with terrestrial mammals, each group of marine mammal has also developed unique physiological and biological adaptations to survive in an aquatic environment.

2.1. Harbour seals

Harbour seals (*Phoca vitulina*) are the most widely distributed pinnipeds in the north Atlantic and north Pacific regions. Their population is estimated to be between 400,000 and 500,000 worldwide (Seal Conservation Society, http://www.pinnipeds.org). Harbour seals can be grouped into four primary subspecies: the eastern Atlantic (*P.v. vitulina*), the

western Atlantic (*P.v. concolor*), the eastern Pacific (*P.v. richardsi*), and the western Pacific harbour seals (*P.v. stejnegeri*). There are also harbour seals known to inhabit freshwater ecosystems, including the Ungava seals (*P.v.mellonae*) in lakes and rivers on the Ungava peninsula of northern Québec.

The eastern Pacific harbour seal population ranges from northern islands in Alaska to southern California and Mexico, and its number was estimated at approximately 285,000 individuals (Seal Conservation Society, http://www.pinnipeds.org). In British Columbia (BC), harbour seals are abundant, with a population of over 100,000, which is believed to be approaching its carrying capacity (27). Geographical variation in pigmentation patterns, different pupping seasons, and size differences represent evidence of possible subpopulations in eastern Pacific harbour seals. Recent evidence based on mitochondrial and microsatellite DNA analysis suggests that the eastern Pacific subspecies can be grouped as three distinctive populations. They are 1) Japan, Russia, Alaska, and northern BC, 2) southern BC and Puget Sound, Washington, and 3) the outer coast of Washington, Oregon and California (28).

Harbour seals are generally considered to be sedentary animals (29). They generally feed within 25 km of the shore, but some individuals occasionally travel long distances (more than 100 km) to find their prey. They are opportunistic feeders, preying on locally- and seasonally- abundant species, including, a wide range of fish species, crustaceans, and molluscs. Therefore, each population may have differing feeding ecologies (30-32). For instance, the diet of harbour seals inhabiting the Strait of Georgia (BC, Canada) is comprised primary of Pacific hake (*Merluccius productus*) and Pacific herring (*Clupea pallasi*) (70%) (33), whereas harbour seals in Puget Sound (WA, USA) feed on a wider

variety of species, with about 50% of the diet made up of Pacific tomcod (*Microgadus proximus*) and Pacific herring (34).

Generally, harbour seals live in small groups and haul out to rest during low tides, breeding and pupping, and moulting. Their haul out habitats vary, from rocky shores, reefs, and sand and gravel beaches, to artificial objects such as rafts and buoys (29). Females give birth to a single pup in the summer (35), which is particularly precocious. The well developed pup enters the water within hours of birth (36). Pups along the coast of BC are born with an average weight of 11.2 kg and spend approximately 32 days with the mother (37). During the nursing period, they consume their mother's high fat-content milk (as much as 45 % fat), and gain about 400 g daily (37). Although the energy transfer from the mother to the pup is quickly completed, the development of pups takes much more time, especially with respect to oxygen stores, swimming muscles, and neural pathways for diving and foraging. Young harbour seals are the most vulnerable to mortality. In the first year, pup mortality ranges from 20% to 60%. After that the first year, mortality rates decrease to between 5% and 20% (38), and an average life span of 25 to 30 years can be attained.

Harbour seals are one of the most intensely studied marine mammals in the field of biology, physiology, behaviour, immunology, endocrinology, and toxicology due to their abundance and relative ease of handling (small size and non-aggressive characteristics) compared to other pinnipeds and cetaceans. Their non-migratory nature and their wide distribution allow an evaluation of harbour seals populations associated with different POP exposures. For example, contaminant levels and their effects in harbour seals have been evaluated in the heavily industrialized area of Puget Sound (PS) in WA and the

more remote areas of Vancouver Island, BC for more than 10 years (39). The concentrations of POPs (mainly PCBs) are approximately 7 times higher in PS seals compared to those in seals inhabiting remote areas of BC. POP exposure has been related to adverse health effects in these animals, including the alteration of circulatory vitamin A levels (40). Thus, harbour seals inhabiting these areas may be at risk of toxicological effects.

2.2. Blubber physiology

Blubber represents one of the tissues easily obtained from free-ranging marine mammal species. This thick, vascularized layer of fat under the skin represents the animal's primary fat deposits, and is therefore the primary energy source. However, blubber has other critical functions, such as insulation and thermoregulation, buoyancy control, and sculpting of the external surface as a hydrodynamic streamer.

Blubber is a unique tissue, anatomically and biochemically different from the types of adipose tissues found in terrestrial mammals. Like other adipose tissues, blubber is mostly composed of fat cells, or adipocytes, which fill and empty with fat in their lipid vacuoles. Adipocytes are held in place by a mesh of structural fibers, including collagen, elastic, and reticular fibers. While most adipose tissues contain only small amounts of collagen, blubber is rich in collagen and elastic fibers, increasing its strength, flexibility and elasticity (41). Blubber has abundant blood vessels and contains specialized shunts, which allow blood to flow faster at greater volume than would be possible through capillaries alone. This circulatory system enables marine mammals to thermoregulate

relatively quickly and easily (41). By restricting blood flow to the blubber surface, marine mammals can conserve their body heat; whereasI increased blood flow will lower their body temperature.

Blubber is not only structurally unique, but its biochemical lipid composition is also different from other mammalian adipose tissues. In mammals, dietary intake of lipid is mostly in the form of triglycerol. It breaks down in the digestive system as fatty acids and glycerol, which are subsequently taken up by tissues. The fatty acids are either used for energy or stored as reformed triglycerol in adipocytes. Fatty acids in the marine food web are extremely complex, and include high levels of long-chain polyunsaturated fatty acids (PUFAs), which are neither modified nor synthesized upon digestion in marine mammals. Therefore most fatty acids found in marine mammal blubber are from the dietary intake of prey and contain unusually high levels of long chain PUFAs. Since unsaturated fatty acids have lower melting points than saturated fatty acids, this helps the blubber to remain fluid in order to function as an effective insulator.

Blubber is the most important tissue for energy storage in marine mammals. Stored lipids in blubber play critical roles during annual migrations, the breeding season and molting period, and during lactation, when food intake is low or absent in many marine mammal species. For instance, some of the large baleen whales, such as gray whales do not feed during their 1000 mile migrations from tropical waters where they give birth to their feeding areas in polar regions (42). In some of the phocid seals, such as the gray seal, females fast throughout the entire lactation period, in addition to producing a fat rich milk for weeks. During these occasions, marine mammals rely on their abundant blubber (up

to 50% of their body mass) for energy, and switch almost completely to a fat-based metabolism. The thickness of the blubber generally dictates how healthy the pup is at the end of the lactation period (43). Furthermore, the newborn pup depends on a sufficient amount of blubber deposition during lactation for thermoregulation and energy stores, needed for several weeks of fasting and survival until they learn how to forage on their own.

2.3 Partitioning and concentration of POPs in blubber

It is important to understand the partitioning and distribution of POPs among tissues in order to assess the toxic effects of POPs. Due to their lipophilic nature, POPs dissolve in lipid droplets in fat-containing tissues. In the case of marine mammals, the majority of the body burden of POPs is stored in blubber. However, lipid weight based concentrations of POPs are fairly comparable among different tissues, implying equilibrium among the lipid contents of the different body compartments.

Changes in lipid content and nutritional condition through diet and biological events, such as fasting and lactating, can dramatically change lipid concentration of POPs. For example, during a period of little or no dietary intake (e.g., during fasting), marine mammals utilize the lipids in the body (mainly blubber) to meet energy demands. During this time, POPs will increase in concentration in the blubber, or can be mobilized into the blood. This may increase the toxic risk of POPs.

When female marine mammals produce lipid-rich milk in the mammary glands, the concentration of POPs in the milk is comparable to concentrations in other tissues. However, when concentration of individual PCB congeners were compared between milk and blubber of the harbour seal, highly chlorinated PCBs were significantly less abundant in milk. The concentration of the lower chlorinated PCBs were comparable in blubber and milk, and the composition of PCB burdens in milk and pup blubber was also comparable (44). This indicates a lower mobility of lipophilic compounds through the aqueous environment of blood and mammary glands. Pups may have a low ability to metabolize these compounds, resulting in their accumulation in blubber.

The characteristics of the relatively less mobile, higher chlorinated POPs may explain the stratification in the concentration of POPs in marine mammal blubber. Inner blubber is more metabolically active in lipid use and deposition compared to outer layers. Since lactation, fasting, and extensive foraging periods can dramatically change dietary lipid consumption, the concentrations of POPs following these events change mainly in the inner layer of blubber. For example, during their fasting period, captive harp seals utilize their lipid in blubber and showed a significant time- dependent increase in blood concentration of highly lipophilic POPs. Whereas the concentrations in outer layers from the same animals stayed relatively constant suggesting that POPs detected in blood were mobilized from the inner blubber layer (45).

3. Thyroid hormones

Thyroid hormones (THs) are important in physiological processes in vertebrates, such as development, differentiation, and metabolism. THs play also essential roles in species-

specific processes such the metamorphosis of amphibians and fish and the moulting of snakes, birds, and pinnipeds (46). Because thyroid disorders are common in humans, medical investigations have provided insight in the importance of THs. One out of twenty people in Canada have thyroid problems during their life (Thyroid foundation of Canada, http://www.thyroid.ca), and the World Health Organization has estimated that 740 million people worldwide suffer from iodine deficiency, which leads to hypothyroidism (an insufficient amount of TH). For example, the critical role of THs in brain development (from the first weeks of gestation through the fist year of life in humans) has been demonstrated through the study of mothers with hypothyroidism, whose children suffer from mental retardation and growth defects (47). Therefore, developing organisms may be particularly vulnerable to any small changes in THs.

3.1. Thyroid hormone economics

The thyroid is a bilobed gland located on both sides of the vertebrate trachea, and is the site of TH synthesis and storage. THs are synthesized from tyrosine and iodine in the follicular cells of the gland. Iodine is acquired in the diet and is combined with tyrosine to form a large glycoprotein called thyroglobulin (TG). Through the action of thyroid peroxidase (TPO), iodine is covalently bound to the tyrosine residues of TG to produce THs, mainly in the form of 3,5,3',5'-tetraiodothyronine (T_4), and, to a lesser extent, the more active 3,5,3'-triiodothyronine (T_3) (Figure 1.3).

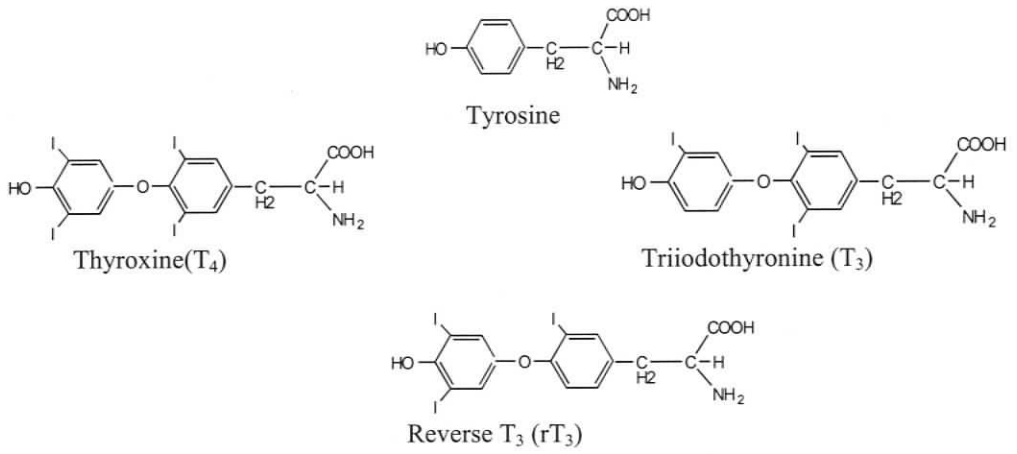

Figure 1.3. Chemical structures of tyrosine and thyroid hormones.
Thyroid hormones; thyroxine (T_4), triiodothyronine (T_3) and reverse triiodothyronine (rT_3) are formed from two of tyrosines and iodines.

Once a release signal reaches the thyroid gland, T_4 and T_3 diffuse into the blood. In circulation, most of the THs are bound to transport proteins, mainly thyroxine-binding globulin (TBG), and a small amount of transthyretin (TTR) and albumin, with only a small fraction circulating as free hormones (0.5% of T_4 and 0.3% of T_3 in humans). Although TBG is the least abundant transport proteins in the human circulatory system, more than 75% of THs bind to TBG due to its high affinity. Only certain mammals have TBG, and fish, amphibians, reptiles, and birds use TTR and albumin as their main TH carriers (48). Levels of circulating THs have been documented in seals, however, it is still unknown which is the main TH transport protein.

THs are regulated through various pathways in several organs and tissues. Metabolic processes play a role in regulating the amount and form of THs. The main metabolic mechanisms are deiodination and conjugation. Iodothyronine deiodinase (ID) (types I, II, and III) is involved in the bioactivation of THs. Type I and type II enzymes convert T_4

into T_3, whereas type III enzymes convert T_4 and T_3 into their inactive forms of reverse T_3 (rT_3) and 3,5-diiodo-l-thyronine (T_2), respectively (49). Conjugation of THs occurs primary in the liver and involves conjugation with either glucuronic acid (mainly T_4) or sulphate (mainly T_3) at the phenolic hydroxyl group. The resulting conjugates are excreted in the bile into the intestine. A portion of the conjugated material is hydrolyzed in the intestine, and the resulting free hormones are reabsorbed into the blood. The remaining conjugated material is excreted in the feces.

Changes in thyroid status or circulatory thyroid concentrations can be interpreted by the hypothalamus (Figure 1.4). The hypothalamus responds to such a signal by the secretion of thyrotropin-releasing hormone (TRH). TRH acts upon the anterior pituitary gland, in stimulating the synthesis and release of thyroid stimulating hormone (TSH). Pituitary TSH is transported in the blood stream to the thyroid gland. TSH can then bind to the TSH receptor, located at the plasma membrane of thyroid gland follicle cells. Binding of TSH will stimulate the synthesis and secretion of THs. Both the release of TRH by the hypothalamus and of TSH by the pituitary gland are controlled by the negative feedback of high circulatory concentrations of THs (mainly total T_4). When blood concentrations of THs have decreased, the negative feedback signals stop, and the release of hormones by the brain increases again (49).

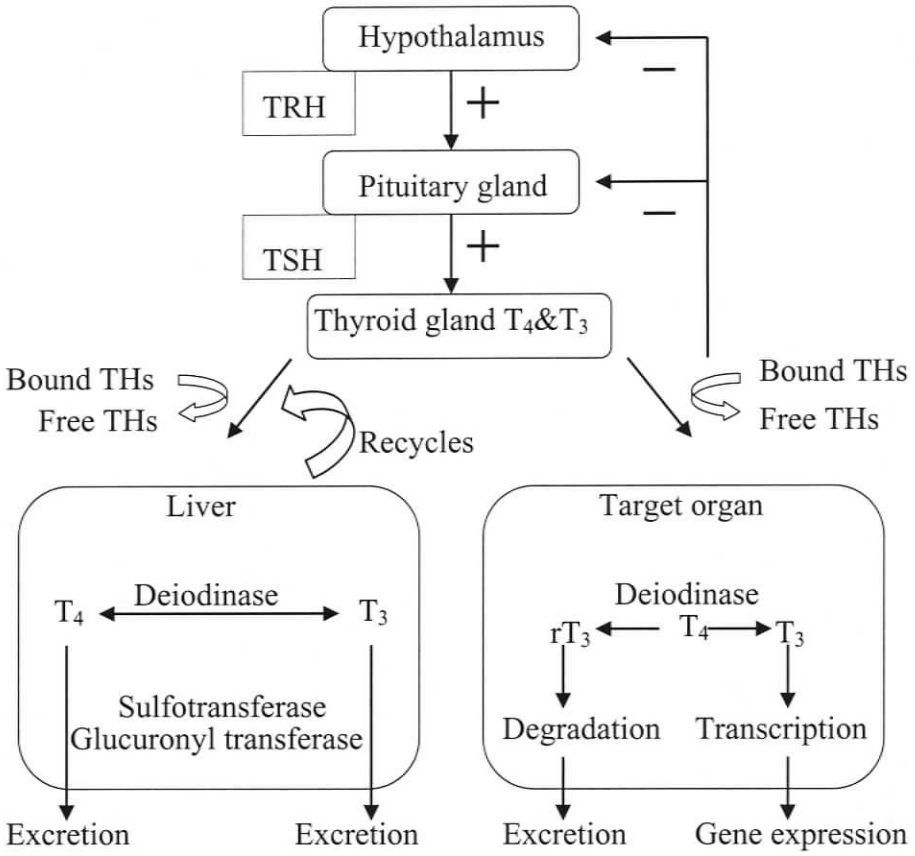

Figure 1.4. Regulation of thyroid function (hypotharamic-pituitary-thyroid axis and metabolic pathway).

The regulation of THs includes various pathways. The hypothalamic-pituitary-thyroid gland axis controls the levels of circulating THs through a classic negative feedback mechanism. The regulation of THs also involves activation and inactivation metabolism though deiodinase pathways, glucuronidation, and sulfatation.

THs function as effector signaling molecules that interact with nuclear thyroid hormone receptors (TRs) to regulate their transcription activation and repression activities. The lipophilic nature of THs allows them to come across cell membranes and access their target cells. T_4 is converted into T_3 by IDs in the cytoplasm. In the nucleus, T_3 binds to TRs with an affinity of approximately 10^{-10}M (50;51). TRs are members of

the nuclear receptor superfamily that also includes steroid, retinoid, and vitamin D receptors (52). Nuclear receptors share common structures with structural/function domains, namely, a DNA binding domain (DBD), a ligand binding domain (LBD), and a transactivation domain. TRs are encoded by two genes, THRA and THRB, and are designated thyroid hormone receptor alpha (TRα) and beta (TRβ), respectively. Although alternative splicing or alternative promoter usage from each gene yields isoforms of each receptor, the isoforms share highly conserved functional domains.

Once T_3 binds to the LBD in the TR, conformational change occurs in LBD, which determines whether coactivators interact with the TR. The DBD of TRs then acts as a high affinity binding component of the TR for specific DNA regions, called thyroid hormone response elements (TREs). TRs can bind to TREs as a monomer, homodimer or as heterodimers with other members of the nuclear receptor superfamily. Binding of such heterodimers (e.g. TR-retinoid X receptor (RXR)) to TRE increases the affinity of TR binding. In contrast to other receptors from the superfamily, TRs can also bind to TREs in absence of the ligand, T_3. However, the biological effects of TRE binding by the presence or absence of ligand are dramatically different.

In the absence of ligand, TR associates with co-repressor proteins such as nuclear receptor corepressor (NCoR) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Figure 1.5.a)(53). These co-repressor proteins can interact with Sin 3 and histone deacetylases (HDACs) (54). The resulting deacetylation of core histones increases the positive charge of histones, and thereby the electric interaction between DNA and histones increases. Consequently, the chromatin condensates and the access transcription factor binding at the promoter region is reduced or eliminated.

In the presence of ligand, dissociation of present corepressors and the association of co-activators from the TR-RXR complex can occur (Figure 1.5.b) Co-activators such as CBP/p300, c/CAP, and p160 have histone acetyltransferaes (HAT) activity. The lysine-rich amino terminal domains are acetylated by HATs, which lead to a reduction in the affinity of the histone core for DNA and the establishment of the transciptionally active chromatin conformation (opposite to the Sin 3 and HDACs in the co-repressor protein complex). Activated TRs can associate with thyroid hormone receptor-associated protein (TRAP)220, and subsequently with a number of different TRAP complex of auxiliary cofactors (Figure 1.5.c). TRAP 220 directly interacts with the transcription factor TATA box-binding proteins and recruits this crucial component of the basal transcription machinery to TH-responsive promoters to enhance gene expression.

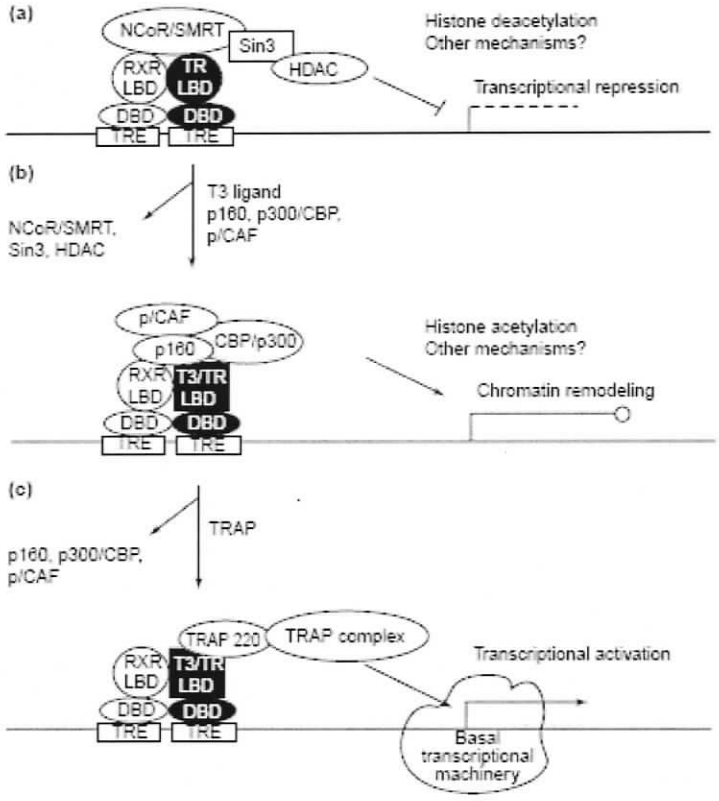

Figure 1.5. Model of thyroid hormone receptor interactions with corepressors and coactivators.

Unliganded TRs are complexed with corepressors, including NCoR and SMRT, leading to repression of transcription (a). Attachment of a ligand, such as T_3, leads to a conformational change in the TR and dissociation of the corepressor (b). Interaction of a ligand TR with coactivators, including CBP/p300, c/CAP, and p160 leads to transcription activation (c).

Modified from Wu and Koenig (55).

3.2. POP- related disruption of thyroid hormone physiology

3.2.1. Mechanisms of disruption

Due to the critical role of THs in the development of the fundamental vertebrate body and fetal development, transgenerational exposure to POPs which have chemical

structures similar to THs, such as PCBs, are of special concern. Such contaminants may pose a risk as antagonistic and agonistic effectors on thyroid systems in the young. Thyroid hormone concentrations have been found to be affected by the intake of PCB-like compounds in laboratory rodents, wildlife (including marine mammals), and humans (4). At least three mechanisms of action have been described: (a) disruption of thyroid gland function and regulation, (b) alterations in thyroid hormone metabolism, and (c) changes in thyroid hormone transport binding protein activity. Recently, evidence of interference of PCBs in thyroid hormone mechanism of action has been revealed.

Thyroid gland function

Morphological evidence of a direct effect of PCBs on thyroid glands was observed in many experimental studies with rats exposed to PCBs in their diet (56-60). Abnormalities within the follicular cells related to PCB exposure, such as an increase in the number and size of lysosomes and colloid droplets, and irregularly branched microvilli were described. Normally, colloid droplets containing TGs fuse with lysosomes. Subsequently, TGs are enzymatically digested by lysosomal enzymes into free thyroid hormones that can be released into blood. However, the abnormal lysosomes are unable to interact with colloid droplets in a normal manner (56). The resulting accumulation of colloid droplets and lysosomes leads to a displacement of synthetic organelles such as the endoplasmic reticulum and Golgi apparatus. These ultrastructural lesions in thyroid gland were associated with reduction in circulating thyroid hormone (mainly T_4) and led to moderate compensatory hyperplasia (an increase in the number of cells in a tissue or organ) and hypertrophy (increase in the size of cells and decreased colloid area) in the follicular cells(56).

Thyroid hormone metabolism

Dioxin and dioxin-like compounds induce hepatic biotransformation enzymes that are involved in chemical detoxification through the AhR pathway. The biotransformation enzymes are important for thyroid hormone dynamics. T_4 is glucuronylated by phase II enzyme, uridine diphosphate glucuronosyltransferase (UDP-GT). Increased biliary secretion and hepatic elimination of T_4 have been reported in rats following exposure to individual PCB congeners such as PCB 77, PCB169 and PCB mixture Aroclor 1254 (61;62). In most cases, an increase in T_4 glucuronidation was correlated with reduced plasma T_4 levels in the same animals. Sulfation of T_4 is also a target mechanism of PCBs. Sulfate conjugates of TH are either quickly degraded by deiodination or freed by desulfation. OH-PCBs were a potent inhibitor of sulfotransferase activity *in vitro* (63).

Other enzymes involved in thyroid hormone metabolism are IDs. Exposure of rats to PCBs and dioxin resulted in inhibition of hepatic ID-1, leading to a reduction in plasma T_4 levels (64). On the other hand, increased ID-2 activity was observed in brains of fetal and neonatal rats born from dams exposed to Aroclor 1254 (65). Since conversion of T_4 to T_3 is mainly controlled by deiodination in the brain, this observed increase in ID-2 activity may be a compensatory response by the brain to maintain T_3 levels when circulating T_4 levels are low.

Plasma transport

TTR and Retinol binding protein (RBP) form complexes in circulating blood (Figure 1.6). They contain binding sites for T_4 and retinol and transfer these hormones to their target cells. Some OH-PCBs (with the hydroxyl groups in meta or para positions with one or more adjacent chlorine substitutions) have a much greater binding affinity for TTR

than T_4 (66). Binding of OH-PCBs to TTR can also decrease the affinity for RBP and the accompanying retinol. Unbound T_4 and retinol are then easily filtered by the kidney and eventually excreted (67). Rats fed a PCB mixture of Aroclor 1254 showed reduction of plasma TT_4 and FT_4, corresponding to a reduction in the ex vivo binding of ^{125}I-T_4 to TTR (60). Moreover, maternal exposure to PCBs could result in transplacental accumulation of OH-PCBs in fetal mice (68). Reduction of T_4-TTR binding was observed and consequently, T_4 levels in fetal plasma and brain were reduced (68;69). Since THs are critical for development, reduction of THs due to altered TH transport through tranplacental exposure can cause serious health effects in the fetus.

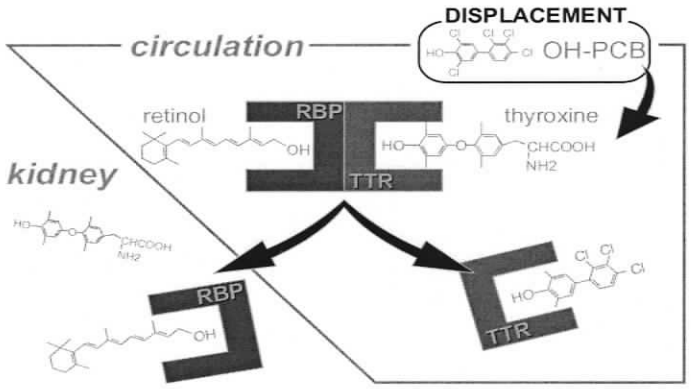

Figure 1.6. The disruption of circulatory thyroid hormone transport by OH-PCBs.
Some OH-PCBs have greater affinity than the natural TH, T_4, for the binding site on TTR. Displacement of T_4 causes the conformational change in TTR and breaks the TTR/RBP complex. Subsequently, free hormones are filtered by the kidney and excreted in bile and urine.

Alteration of the thyroid hormone signaling pathway

An insufficient amount of plasma THs is known to cause irreversible damage to brain development in laboratory animals and humans. Thus, neurotoxic effects of PCB

exposure to developing brain by relative hypothyroidism have been a concern (70;71). Interestingly, PCB exposure does not always produce consistent effects on rat development which are indications of TH insufficiency, such as timing of eye opening (71-73). PCBs and their structurally related compounds, therefore, may directly interfere with TH signaling pathway and produce perhaps unpredictable toxic effects.

Possible bindings of PCBs and their metabolites to TRs have been investigated. Surprisingly, parent PCBs and their metabolites, OH-PCBs, had little or no competitive binding affinity to human and rat TRs compared to natural THs by competitive binding assay (74;75). Therefore, PCBs and their related compounds may not compete directly with THs for TR binding sites. Also, there is evidence indicating PCB-related alteration of TH signaling pathways, but the nature of the responses varied between studies. Agonistic effects of PCBs were observed using human neural progenitor cell lines. 2,3',4,4',5- pentacholobiphenyl (PCB-118) treated cells showed T_3-like activity by increasing oligodendrocyte differentiation, and the PCB mediated effect was blocked by TR antagonist (76). Moreover, three congeners of OH-PCBs showed weak thyroid activities in two-hybrid assays using yeast cells containing human TRα (77). On the contrary, two mono-ortho-OH-PCBs suppressed T_3-mediated transactivation by TR in various cell lines, especially brain-derived cell line TE671 (78), but not in a glucocorticoid response element. This result indicates that the molecular toxic effect of PCB is TR specific and its effect may be especially significant in the brain. Its following study hypothesized that the suppression of transcription by PCBs probably occurred via the dissociation of the TR/RXR heterodimer complex from a TRE (79).

It is clear that PCBs interfere with the TH signaling pathway, perhaps with multiple and complex mechanisms. Therefore, the consequences may differ depending on the specific interactions. The actually effects of PCB may depend upon variations in TR isoforms, promoter structure, coactivators, and heterodimarization. Moreover, the effects also may be dependent upon PCB molecular structure as well as the presence of PCB metabolites. Since developing brains are especially vulnerable to PCB exposure, these complex effects need to be addressed.

3.2.2. Evidence of POP - related thyroid disruption in wildlife

Reports of alternation in thyroid gland morphology (hypertrophy and follicle cell hyperplasia) by PCBs exist for several wildlife species. Hypertrophy and follicle cell hyperplasia in predatory fish and herring gulls from the Great Lakes were likely due to PCB-induced hypothyroidism (80;81). In marine mammals, such as harbour seals (*Phoca vitulina*), harbour porpoises (*Phocoena phocoena*) and beluga whales (*Delphinapterus leucas*), abnormal thyroid gland morphology has also been related to PCB exposure. In a highly PCB-contaminated population of harbour seals inhabiting the North Sea, lesions of thyroid glands were found more often than in relatively less polluted seals from Iceland (82). Histological lesions (colloid depletion and interstitial fibrosis) were found in the thyroid glands of harbour seals, and similar lesions, were observed in the harbour porpoises, albeit to a lesser degree. Since the abnormal follicular cells in the contaminated harbour seals were similar to the follicular cells of rats exposed to high concentrations of PCBs (under laboratory conditions), altered seal thyroid glands are likely cause by exposure to PCBs. Beluga whales in the St. Lawrence Estuary, known to

be one of the most contaminated group of mammals in the world, showed no evidence of changes in thyroid gland morphology or function similar to those observed in seals. However, other lesions such as abscesses in the thyroid gland and small thyroid adenomas were noted in these animals (83).

Weight of evidence suggests that many POPs alter TH concentrations in experimental animals and wildlife species. However, consequences of the exposure are divergent, even in well-controlled laboratory animal studies (84). Exposed to TCDD, dioxin-like PCBs, and mixtures of PCBs such as Aroclor 1254, resulted in reduction in TT_4, with little or no changes in TT_3 in rats and rodents (4). *In utero* exposure to Aroclor 1254 also resulted in reduction of plasma T_4 levels in late gestation and new born rats (85). In contrast, PCB exposed mink showed increased plasma T_4 and T_3 concentrations (86).

Reductions of plasma TT_4, FT_4, TT_3, and retinol were observed in captive harbour seals which were fed highly PCB-contaminated fish (87). The negative correlation between circulatory THs and accumulated PCB concentrations has been reported in free-ranging pinnipeds, including Northern elephant seals, grey seals, harbour seals, large seals, ribbon seals and California sea lions (88-92). PCB exposure associated reductions in TT_4, TT_3, and retinol were observed in Northern elephant seals (91). In a study of California sea lions, the concentration of total PCBs and total PCB TEQ were negatively correlated with plasma TT_4, TT_3 and retinol (89). However, blubber concentration of PCBs did not correlate with TT_4, but did correlate with TT_3 and (free T_3) FT_3 in young, weaned grey seals (92). Similar results were obtained in a study of adult large seals in Japan (90). Plasma TT_3 levels were negatively correlated with PCB-170 and PCB-180 in a study of ribbon seals (90). Finally, grey seal pups from the UK showed no correlation

between total PCBs and THs, but there was a negative correlation between the ratio of T_3:T_4 and a single PCB congener, PCB-169 (88). In highly contaminated polar bears from Norway, PCBs had a larger affect on TT_3 concentration than on TT_4.(93).

The difference of thyroid toxicity among wildlife species may be due to the nature of complex mixture of POPs or species-specific vulnerability to POP exposure. It is also important that thyroid hormones dynamically change with environmental and biological conditions, such as changes in temperature, age, and status of nutrition. Thus, in order to assess the effects of POPs on the thyroid system, it is important to minimize these variable factors.

4. Biomarkers

Humans and wildlife have been exposed to and accumulated various POPs. Since the endocrine system is very sensitive to perturbation, even low levels of exposure to certain POPs may cause deleterious health effects, particularly during critical developmental stages (embryonic, fetal, and early postnatal stages)(94). Therefore, it is important to monitor the levels of these POPs in environments and to develop "biomarkers", which can identify exposure to and effects of contamination in wildlife.

Biomarkers are defined as "… biochemical, cellular, physiological, or behavioral changes that can be measured in a tissue, biological fluid, or whole organisms that provide evidence of exposure to, and/or toxic effect of, one or more contaminants" (95). Biomarkers are generally used as biomarkers of exposure and biomarkers of effects. Biomarkers of exposure include all the responses which indicate individuals, populations, or communities have been exposed to a single or multiple chemical compounds, whereas

biomarkers of effects include all the responses indicating that exposure to the chemical compounds resulted adverse biological effects for individuals, populations, or communities.

Several criteria must be met for a biomarker to be effective. First, biomarkers should be sensitive even to low concentrations of contaminant exposure. Therefore, prior to manifestation of serious health effects in populations and entire ecosystems, the contaminant stress can be identified (96). Secondly, biomarkers should be highly specific, relatively robust, and stable to changes in natural, physiological events, and environmental factors (e.g., temperature). In addition, biomarkers should be set up based on well-known biological and physiological functions. Thus, it is relatively easy to identify the difference in biomarker response between contaminant exposure and natural factors. Finally, biomarkers should be quantitative with different levels of contaminant exposure (dose-response). This relationship is central in determining hazardous levels of potential pollutants and predicting the toxicological risks in population levels (97).

Induction of CYPs has been widely used as an exposure of biomarker in many wildlife species including fish (98), birds (99), and marine mammals (100;101). The activity of CYPs seems sensitive, relatively inexpensive, and easily measured. The downside of this assay is that sampling of liver can be destructive and challenging, particularly for free ranging marine mammals. It also raises ethical difficulties. Alternative non-invasive sampling methods, such as the use of skin biopsies, are under investigation (102;103).

On the other hand, development of biomarkers of effects which can precisely indicate the effect of POPs in wildlife is still insufficient. Quantification of circulatory hormones

such as thyroid hormone and vitamin A has been used as a biomarker in wildlife, including marine mammals (97). Use of molecular biomarkers which measure contaminant-related changes in gene products (transcripts and proteins) is a powerful approach to assess contaminant effect. Genetic effects can occur with lower contaminant exposure prior to biological and morphological changes. Regardless of the sensitivities of genetic biomarkers, their precision may decrease in field –exposed organisms due to the complexities of contaminant exposure in the environment. Practical molecular biomarkers of effects have not been established for marine mammal species.

5. Research hypothesis and thesis outline

The main objective of the present thesis was to design, evaluate, and apply a useful biomarker for the assessment of potential POP-related health effects in free-ranging harbour seal pups. THs are highly conserved and essential hormones for normal development in all vertebrates. Due to similarities in chemical structure, some POPs, including PCBs, mimic natural TH activities. The mechanisms of thyroid toxicity related to POP exposure have been relatively well evaluated in laboratory animals, and the interaction of POPs through TR related molecular mechanisms has become increasingly clear. However, despite the importance of the molecular mechanisms as the basis of thyroid hormone action, nothing is known about the molecular structure of TRs and/or their regulation systems in marine mammals. Thus, the goal of this study was to identify harbour seal TRs and use these as molecular biomarkers for POP-related effects. This newly developed molecular biomarker and levels of circulating thyroid hormone levels

were applied to assess the toxicological risk of POP exposure in harbour seal pup populations inhabiting the coast of British Columbia and Washington State.

Chapter 2

Thyroid hormone dynamics in nursing harbour seal pups from British Columbia, Canada

This chapter is in preparation for submission to Canadian Journal of Zoology (short communication). Tabuchi M. Dangerfield N, Helbing C.C. and Ross P.S.

1. ABSTRACT

While thyroid hormones (TH) are critical for normal development and differentiation in all vertebrates, they are of special importance in marine mammals where they function in moulting and thermoregulation. THs can be influenced by natural factors (i.e. age, sex and health condition), environmental factors (i.e. temperature, prey availability), and anthropogenic factors (i.e. environmental contaminants). Since many marine mammals carry a high body burden of environmental contaminants, they may be of special concern for thyroid hormone disruption. However, little is known about the natural factors affecting thyroid hormone dynamics in marine mammals, making it difficult to distinguish contaminant-related effects from other influences. In this study, circulatory thyroid hormones were measured in free-ranging harbour seal (*Phoca vitulina*) pups inhabiting British Columbia, Canada, during their nursing period. Harbour seal pups were born with high levels of the most abundant thyroid hormone, thyroxine (T_4) (97.5 ± 13.6 nmol/L) and its free form (FT_4) (56.0 ± 4.6 pmol/L) both of which gradually decreased with age. The concentration of the "active" form of thyroid hormone, triiodothyronine (T_3), measured as both total T_3 and free T_3, varied at birth and remained relatively constant with age. Since TH metabolic processes (i.e. deiodination and degradation) are ill-defined, the clinical significance of THs to the pups is unclear. However, the elevated T_4 observed in young pups in this study may indicate a response to a high demand for active metabolic function in seal pups with a limited blubber layer to maintain their core body temperature in cold water. The constant levels of T_3 may imply the homeostatic action of TH physiology. Understanding the baseline of circulatory THs is an important first step in using THs as a biomarker for exposure to environmental toxics.

2. INTRODUCTION

THs are essential for normal physiological function in all vertebrates. They play especially critical roles in the prenatal and postnatal mammal in the regulation of metabolism, growth, cell differentiation, as well as in development and immune system function (104). The importance of THs in brain development has been intensively studied in laboratory animals and humans (105;106). Abnormal TH levels also cause pleiotropic effects in many other organ systems (107).

Phocid seals, including harbour seals, are considered to be precocious, meaning they are well developed at birth and nurse for only a few weeks (108;109). During the relatively short nursing period, harbour seal pups gain their body mass at a rapid rate by consuming fat-rich milk (37). During the first days of life, pups have little protection against heat loss, and prior to adequate insulative blubber layer development, they have a high metabolic rate caused by shivering and non-shivering thermogenesis (110;111). The high concentrations of THs observed in newborn seal pups are speculated to be due to their roles in these processes (112).

Laboratory studies have indicated that some persistent organic pollutants (POPs) and their metabolites can interfere with the TH system on multiple levels: hormone synthesis, circulatory transport, metabolism, and at the level of target tissues (4;113;114). Suspected POP-related alteration of circulating TH levels and abnormal thyroid glands have been observed in wildlife, including fish-eating birds and marine mammals inhabiting contaminated regions (84). Marine mammals are of concern, since they can accumulate high concentrations of POPs (115;116).

The measurement of TH concentrations has been touted as a possible biomarker of contaminant exposure in marine mammals. However, in order to use THs as an effective biomarker for POP exposure, the possible influence of natural factors such as age, health condition, and nutritional status must be eliminated, because these may compromise the utility of the biomarker.

Young phocid seals are exposed to POPs though lactation (44). Possible effects of POP exposure on their thyroid physiology could lead to negative effects on their development. In order to elucidate a possible disruption of TH, basic information on the "normal" thyroid hormone dynamics during this period is needed. To date, there is only one study that measured thyroid hormones in free-ranging harbour seal pups through the nursing period (112). The study measured total thyroxine (TT_4), fee thyroxine (FT_4), total triiodothyronine (TT_3), and free triiodothyronine (FT_3) in both nursing mothers and their pups of the western Atlantic subspecies (*P.v. concolor*) inhabiting Sable Island, Nova Scotia. Differences in pupping time, birth mass, nursing period, and growth rates have been observed among different harbour seal populations (35;117;118). For example, northeast Pacific harbour seal (*P.v. richardsi*) pups have half of the daily mass gain and a longer nursing period compared to Sable Island harbour seal (*P.v. concolor*) (37). These natural factors may have consequences for thyroid hormone physiology in pups. Thus, in this study we examined the thyroid dynamics of healthy free-ranging northeast Pacific harbour seal (*P.v. richardsi*) pups from British Columbia, Canada, to provide a much-needed baseline of normal thyroid physiology.

3. MATERIALS AND METHODS

3.1 Sampling collection

Young harbour seals (*Phoca vitulina*) were live-captured around Sidney Island, off the southern tip of Vancouver Island, British Columbia, Canada (48° 6'N, 123° 3'W) during the summer of 1998 (37;119). This field work was carried out for several developmental studies and so pups were marked at birth and recaptured throughout the nursing period. Thus, when pups were captured the first time, they were tagged on their head with color coded moulded epoxy resin balls in order to identify individuals from a distance as described elsewhere (37). Their haul-out sites were visited three days per week. The pups were recaptured whenever possible until they were weaned. At each capture, pups were aged, sexed, weighed, measured for length and axillary girth, assessed for general body condition, and blood samples were taken from the extradural vein using 18 gauge, 4cm needles (Becton Dickinson, U.S.A.) and 10mL Vacutainer tubes (Becton Dickinson, U.S.A.). Serum samples were separated by centrifuging at 400 g for 20 minutes. The details of capture and the blood sampling were described elsewhere (37;119). The serum samples were kept at -80°C until analyses of TH concentrations were performed.

3.2 Thyroid hormone assay

The concentrations of TT_4, FT_4, TT_3, and FT_3 were measured in pups which were captured twice or more (n=8) using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's recommended protocol (Adaltus, Montréal, PQ, Canada). Frozen (undiluted) serum samples were thawed on wet ice and four TH measurements of

each sample were obtained within six hours in order to avoid repeated freeze-thaw cycles, which can lower the quality of samples. Serum samples were incubated with horseradish peroxidase (HRP)-labeled hormones in anti-TH antibody coated polystyrene microtiter plates. HRP-labeled hormone and native hormones competitively bind to the antibodies on the wells. After washing off the unbound hormones, the amount of enzyme-labeled hormones was measured by adding substrate: a mixture of 3,3',5,5'-Tetramethylbenzidine (TMB) which changes color by reacting with the HRP. The color intensity of seal serum samples and TH standards was measured at 450 nm on a MRX microplate reader (Dynatech laboratories Inc. Chantilly, VA, USA). For each ELISA assay, reactions were prepared in triplicate and the sample data were subsequently averaged and compared to the standard curve in order to obtain representative TH concentration values.

Inter-assay variation was evaluated in two ways. First, by the regular inclusion of a reference pooled seal serum sample, whereby results were accepted for an assay only when standard results were ± 20% of expected values. Second, total hormone measurements (TT_3 and TT_4) were validated using the manufacturer's reference standard (Thyroid Cal-ver™ reagent; Casco Neal, Portland, ME, USA), and results were accepted for an assay only when concentrations were within ± 5% of expected values.

No purified harbour seal thyroid hormones are commercially available. With this in mind, we validated the thyroid hormone assays for harbour seals by conducting analyses of serial dilutions within a fixed sample volume, and using incremental spikes of seal serum with Thyroid Cal-ver™ reagent. Responses of serial dilutions of seal serum and standard additions of seal serum with the reference standard both produced linear results.

3.3 Statistical Analyses

All statistical analyses were performed using SPSS version 12 software (SPSS Inc. Chicago, IL). Eight pups were successfully recaptured more than twice. The recapture period was subdivided into four groups (day 1, 8-14, 15-21, and 22-28 days old). For each group, values were examined for normality with the Shapiro-Wilk test and for homogeneity of variance using Levine's test. Variance over time was analyzed using paired T-test.

For each individual, TH levels and weight were analyzed for correlation using the Pearson method for normally distributed data, or the non-parametric Kendall's tau_b method when data were not normally distributed.

4. RESULTS

All sampled animals were captured as newborns or one day-old pups. Thus, the age of the pups on recapture was precisely known. Pups were born with a mean weight of 11.16 kg (± 0.56 SEM) and gradually gained weight during their nursing period (Figure 2.1). Body weight was significantly increased at the age period of day 15 to 21 ($p < 0.05$, paired T-test) compared with the day one animals. Close to the end of the nursing period (according to the previous study; 32 d ± 1.5 d) (37), pup weight remained the same or was slightly reduced, although it was not a significant relationship (p=0.073, paired T-test).

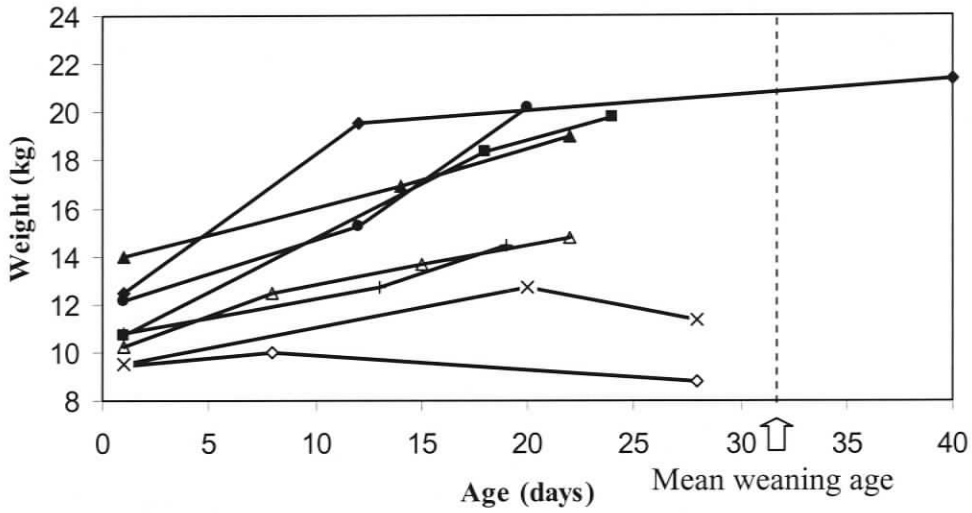

Figure 2.1. Change of body weight of harbour seal pups during their nursing period. Harbour seal pups gradually gain weight during their nursing period.
Each line represents a different seal pup.
Adapted from Cottrell *et al.*(37).

Circulating total thyroxine (TT_4) in day old harbour seal pups was high with a peak value of 97.5 ± 13.6 nmol/L, and gradually decreased as the pup aged (Figure 2. 2A). At day 22 to 28, the level of TT_4 was 33.6 ± 5.1 nmol/L, significantly lower than the levels in the day old pups ($p < 0.05$) (Table 2.1). Trends in free thyroxine (FT_4) concentration closely followed those of TT_4 (Figure 2. 2B). One-day old pups had significantly higher FT_4 (56.0 ± 4.6 pmol/L) compared with all other age groups. Total triiodothyronine (TT_3) concentrations in pups were highly variable over time for each individual animal (Figure 2. 2C). However, the variance became smaller once the animals got older. The same trend was observed in free T_3 (Figure 2. 2D). In day old pups, there was large variance,

followed by a steadier concentration range of 5 to 7 pmol/L at the end of the monitoring period.

A negative correlation was found between body weight and TT_4 (R= -0.45, $p< 0.05$) and FT_4 (R= -0.67, $p< 0.01$), but not between body weight and TT_3 (R= -0.16, $p= 0.49$) or FT3 (R= 0.11, p=0.65).

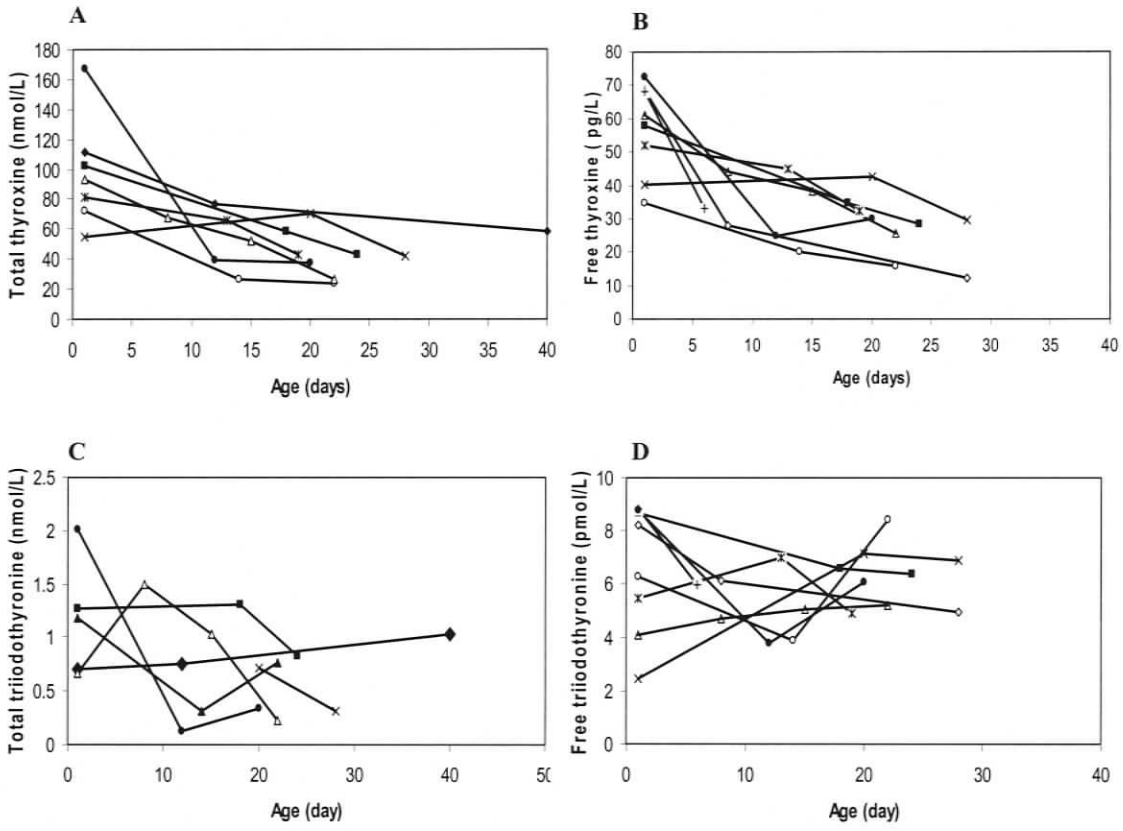

Figure 2.2. Serum concentrations of thyroid hormones in harbour seal pups through the nursing period.

A. total thyroxine, B. free thyroxine, C. total triiodothyronine, D. free triiodothyronine were measured. Each line represents a different seal pup.

Table 2.1.

Circulating thyroid hormone concentrations in harbour seal pups grouped by age class during the nursing period.

Age (days)	TT_4 (nmol/L)	FT_4 (pmol/L)	TT_3 (nmol/L)	FT_3 (pmol/L)
1	97.5 ± 13.6	56.0 ± 4.6	1.12 ± 0.20	6.53 ± 0.84
	(55.2-166.9)	(34.8-72.6)	(0.67-2.02)	(2.43-8.79)
8-14	55.2 ± 9.6	32.4 ± 5.1*	0.68 ± 0.24	5.08 ± 0.63
	(26.1-76.9).	(20.2- 45.1)	(0.12-1.50)	(3.79-6.97)
15-21	52.2 ± 5.9	35.6 ± 2.2*	0.75 ± 0.20	5.96 ± 0.44
	(37.2- 70.4)	(29.9-42.6)	0.33-1.31)	(4.88-7.16)
22-28	33.6 ± 5.1*	22.3 ± 3.4*	0.53 ± 0.15*	6.37 ± 0.62
	(23.3-43.0)	(12.2-29.4)	(0.23-0.83)	(4.96-8.40)

Mean ± standard error of mean (SEM) with the range in parenthesis are shown. Significant differences in THs compared with their concentrations in day 1 are denoted by an asterisk ($p<0.05$).

5. DISCUSSION

Thyroid hormones (THs) are important for growth and metabolism in all vertebrates. Additionally, THs play special roles in moulting, thermoregulation, and lipid metabolism in phocid seals (120-123). A weight of evidence strongly suggests that some environmental contaminants, including POPs, alter thyroid hormone systems in wildlife and humans (4). Young seal pups may be particularly vulnerable. However, identifying the possible effect of these environmental contaminants on the thyroid systems in free-ranging seal pups is challenging, because of the complex mixtures of POPs to which they are exposed, their multiple pathways of interaction with TH systems, and the difficulty in minimizing the influence of natural factors. Since TH concentrations change dramatically

during their nursing period, difficulty in using THs as a biomarker for POP exposure in seal pups has been reported (88).

In this study, we measured circulating thyroid hormones in free-ranging harbour seals on the southern coast of Vancouver Island in British Columbia (BC) in order to understand the thyroid dynamics during the nursing period. This is the first study to measure THs from free-ranging harbour seals on the Pacific coast of North America. Information from this study provides valuable reference material for future studies on the possible effect of environmental contaminants in highly polluted areas in the Pacific coast.

In this study, we found that circulating TT_4 concentrations were highest in newborn seals and subsequently dropped to relatively stable levels. This pattern, as well as the concentrations, is similar to results reported in previous studies on harbour seal pups from other locations and in studies of other phocid seals (Table 2.2). Phocid seals are born without the insulative blubber layers which are characteristic of older pups. Therefore, they require effective thermoregulatory mechanisms. High T_4 levels in newborn harp seals may serve a calorigenic function, such as the thermogenic mobilization of brown fat. Elevated THs in the southern elephant seal was thought to be important for the high metabolic rate necessary for thermogenesis (122). Newborn harbour seals may have similar metabolic needs (129). Studies on metabolic rates of grey pups showed that the basal metabolic rate (BMR) of newborns is twice as high in comparison with 14 day old pups (130). Thus, the high levels of TT_4 seen in this study may reflect an adaptation of the precocious harbour seal to ensure a high BMR for survival.

Table 2.2. Circulating thyroid hormone concentrations reported in phocid seal pups.

	Age	Physiological status	TT4 (nmol/L)	FT4 (pmol/L)	TT3 (nmol/L)	FT3 (pmol/L)	References
Harbour seal	1 d	Neonate	98 ± 14	57 ± 5	1.1 ± 0.2	6.6 ± 0.8	Present study
	8-14 d	Nursing	55 ± 10	33 ± 5	0.7 ± 0.2	5.1 ± 0.6	Present study
	15-21 d	Nursing	52 ± 6	34 ± 2	0.8 ± 0.2	6.0 ± 0.4	Present study
	22-28 d	Nursing	34 ± 5	22 ± 3	0.5 ± 0.2	6.4 ± 0.6	Present study
	1 d	Neonate	102 ± 26	28*	2.1*	2.8*	(112)
	15 d	Nursing	55*	15*	2.8*	3.3*	(112)
	25 d	Nursing	50 ± 17	9*	1.9*	1.8*	(112)
	Adult	Nursing	35*	5*	0.9*	0.5*	(113)
	Adult	Not pregnant	6.7 ± 5.8	2.5 ± 1.2	0.6 ± 0.1	0.4 ± 0.03	(124)
Harp seal	9 h	Neonate	112*		2.1*		(125)
	1 d	Neonate	62		1.2		(126)
	1 d	Neonate	245*		2.1*		(127)
	10 d	Weaned	18 ± 3		2 ± 0.3		(126)
	14 d	Weaned	26*		1.4*		(125)
	3 w	Weaned	82*		5.1*		(127)
Grey seal	9h	Neonate	85*		1.6*		(125)
	1-2 d	Neonate	128± 42				(128)
	1-2 w	Nursing	91 ± 15		4.3 ±1.2		(127)
	10 d	Nursing	32*		2.7*		(125)
	3-23 d	Nursing	49 ± 19	23 ± 14	1.5 ± 0.6		(128)
Hooded seal	1 d	Neonate	77*		2.5*		(125)
Southern Elephant seal	6 h	Neonate	54 ± 12		3*		(122)
	20 d	Weaned	21 ± 12		1.3*		(122)
North elephant seal	9 d	Nursing	52*	15*	1.2*		(123)
	18-22 d	Nursing	18*	5*	1.0*		(123)
	2 w[a]	Weaning	23*	6*	0.7*		(123)
	8 w[a]	Weaning	30*	7*	0.7*		(123)

± standard error of mean (SEM)
[a] Early (second week post weaning)
[b] Late (eighth week post weaning)
* approximate values estimated from figures

Adapted from Hauena et al. (112).

Our study showed that harbour seal pups in BC have lower concentrations of TT_3 compared to harbour seals from Sable Island, Nova Scotia (112). The TT_3 concentration in BC pups was closer to that of adult seals in Sable Island. While analytical differences between the two assays may partially explain this difference, it is also possible that the difference can be attributed to physiological differences between two subspecies.

Northeast Pacific harbour seal (*P.v. richardsi*) pups in this study grow more slowly and have a longer nursing period than those from Sable Island (*P.v. concolor*) (37). Interestingly, FT_4 and FT_3 concentrations were both high in BC seals. Since the role of thyroid hormone is largely dependent on the activation of their receptor by free thyroid hormones, the elevated free hormones in BC animals may reflect a physiological compensation for the low level of TT_3. Since BC seals grow more slowly, they might have a higher need for active thyroid hormones.

The difference in TH levels between the northeast Pacific and Sable Island subspecies is one of the examples of natural variance of TH systems. Therefore, it may not be appropriate to assess POP exposure effects between different seal populations without knowing their baseline TH levels. A strong correlation between body weight and TT_4 or FT_3 was observed in this study. Thus, age must be considered in study design in order to interpret TH levels in seals and their possible relationship with environmental contamination. These variations with age and between populations highlight the importance of identifying and minimizing the influence of natural factors on TH dynamics in order to establish THs as useful biomarkers.

Evidence of POP-related effects, including reduction in circulatory vitamin A and immune function, has been observed in harbour seal pups from Puget Sound, Washington, U.S.A (40). Since thyroid hormones share circulatory transport proteins with vitamin A and play a role in the function of the immune system, harbour seals in the Puget Sound area may also have POP exposure effects on the thyroid system. This study provides a baseline of TH physiology in northeast Pacific harbour seal pups and it provides valuable

information for further studies on environmental contaminant-related alteration of THs in marine mammals.

Chapter 3

Persistent organic pollutant (POP)-related alteration of thyroid hormones and thyroid hormone receptor gene expression in free-ranging harbour seals (*Phoca vitulina*)

The majority of this chapter has been published in

Tabuchi M, Veldhoen N, Dangerfield N, Jeffries S, Helbing C.C, and 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 114 (7): 1024-31.

1. ABSTRACT

Many persistent organic pollutants (POPs), due to their lipophilic properties and long half-lives, bioaccumulate within aquatic food webs and may posses inherent endocrine disruptive activity. Predatory marine mammals, such as harbour seals (*Phoca vitulina*), are particularly at risk to accumulation of high POP concentrations resulting in increased toxicological risk. The highly conserved thyroid hormones (THs) represent one vulnerable endocrine endpoint which is critical for metabolism, growth, and development in all vertebrates. The emerging development of gene expression biomarkers directed towards a variety of wildlife species shows great promise in the early detection of exposure effects of POPs. Thus, in this study, a sensitive gene expression biomarker was developed by using specific thyroid hormone receptor (TR) gene expression, with a combination of a minimally invasive skin/blubber biopsy-based tissue collection method. Circulatory THs, TR gene expression in blubber, and POP concentrations (PCBs, PCDDs, PCDFs, DDTs and other organochlorine pesticides) were measured to assess the POP related alteration of thyroid economy of free-ranging harbour seal pups from British Columbia, Canada and Washington State, USA. A contaminant-related increase in blubber TRα gene expression (ΣPOPs; $r = 0.698$; $p < 0.001$) was observed, as well as concomitant decrease in circulating total thyroxine concentrations (ΣPOPs; $r = - 0.749$; $p < 0.001$). Consistent with results observed in carefully controlled laboratory and captive feeding studies, findings in this study suggest that the thyroid hormone system in harbour seals is highly sensitive to disruption by environmental contaminants. Such a disruption may not only lead to adverse effects on growth and development, but could have important ramifications for lipid metabolism and energetics in marine mammals. The

application of specific gene expression biomarkers can be extended to other species at risk in order to assess the effects of chemical contaminants within the environment.

2. INTRODUCTION

A wide range of chemicals produced either directly or indirectly as a result of human activities have contributed to the contamination of aquatic food chains around the world. Marine mammals occupying high trophic levels in aquatic food webs are often contaminated with relatively high concentrations of persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins, (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated diphenylethers (PBDEs), and dichlorodiphenyltrichloroethane (DDT). This is due to food web-related biomagnification, the extent of which reflects the persistence of the chemical, coupled with the long lifespan and limited detoxification capacity of marine mammals (23;131). Many studies have shown that exposure to these complex POP mixture can lead to developmental abnormalities, reproductive impairment, endocrine disruption, and immunosuppression in harbour seals (87;132;133) and other marine mammals (134;135). Marine mammals may therefore serve as indicators of marine environmental contamination, something that is relevant to both human and ecological risk assessments (2).

While the concept of endocrine disruption in wildlife has garnered much international scientific attention (136), contaminant-related alteration of thyroid hormones (THs) has received less attention, yet may still adversely affect vertebrates. Laboratory-based studies have indicated that some POPs and their metabolites interfere with TH physiology

at multiple levels including hormone synthesis, circulatory transport of TH, and TH metabolism in the liver and brain (4;113). Reductions in circulating TH levels have been observed with increasing exposure to PCBs and related compounds in laboratory animals, aquatic birds, and both captive and free-ranging marine mammals, highlighting the sensitivity of this endocrine endpoint to disruption by environmental contaminants (84).

In addition to effects on circulating thyroid hormones, recent *in vitro* and laboratory animal evidence suggests that PCB and PCDD exposure can affect thyroid hormone receptor (TR) activity and TH-responsive gene expression (114). Thus, there is great concern for the current accumulation of environmental chemical contaminants that may possess the potential to disrupt thyroid hormone action and alter gene expression programs critical for the normal development of wildlife and humans. While circulating TH levels are often used as biomarkers of contaminant exposure in harbour seals and other wildlife (88;90;137-139), gene expression endpoints that exploit the cellular functions of TH could provide a more sensitive and mechanistically-based means to characterize the thyroid-toxic potential of complex contaminant mixtures in the real world. Such gene expression analysis might also form the basis of an early detection approach for POP exposure prior to the manifestation of higher-level health effects, such as developmental abnormalities and neurotoxicity, especially when results are consistent with laboratory-based observations.

Obtaining liver or blood samples from free-ranging marine mammals is generally fraught with logistical and ethical challenges. Skin/blubber biopsies have been used to generate useful information on contaminant concentrations and, more recently, on toxicologically-relevant endocrine endpoints (103;140;141). Gene expression analysis

using small biopsies has the potential to become a useful, sensitive, and minimally-invasive biomarker strategy in wildlife toxicology.

The objectives of this study were 1) to develop thyroid hormone receptor gene expression biomarkers using skin/blubber biopsies, 2) to confirm the utility of using circulating THs as biomarkers of POP exposure, and 3) to assess the feasibility of using TH-related gene expression biomarkers in free-ranging harbour seals.

3. MATERIALS AND METHODS

3.1 Sample collection

A total of 39 healthy, young harbour seals (*Phoca vitulina*) of comparable body weight and condition were live-captured from five locations in southern British Columbia (BC) and northern Washington State (WA), during the summer of 2003 (Figure 3.1). These locations included three Canadian sites in Queen Charlotte Strait (QCS) (northeast Vancouver Island; n=10) and the Strait of Georgia (City of Vancouver; n=8; and Hornby Island; n=7), and two US sites in Juan de Fuca Strait (Smith Island; n=7) and Puget Sound (Gertrude Island (GI); n=7).

Both the accumulation of POPs and biological endpoints in marine mammals are age dependent (chapter 2), so we restricted our sampling to pups ranging in age from 3.5 to 5 weeks. Seals hauled out on sandy beaches were captured using a rapidly deployed seine net (142), while those hauled out on rocky inlets were captured one at a time using a salmon-landing net (119). Seals were kept in hoop nets until sampling, then removed from the net and manually restrained for tissue and blood collection. Seals were typically

captured at low tides (peak haul out times), with time of capture ranging from 08.25 h to 15.40 h across all sites.

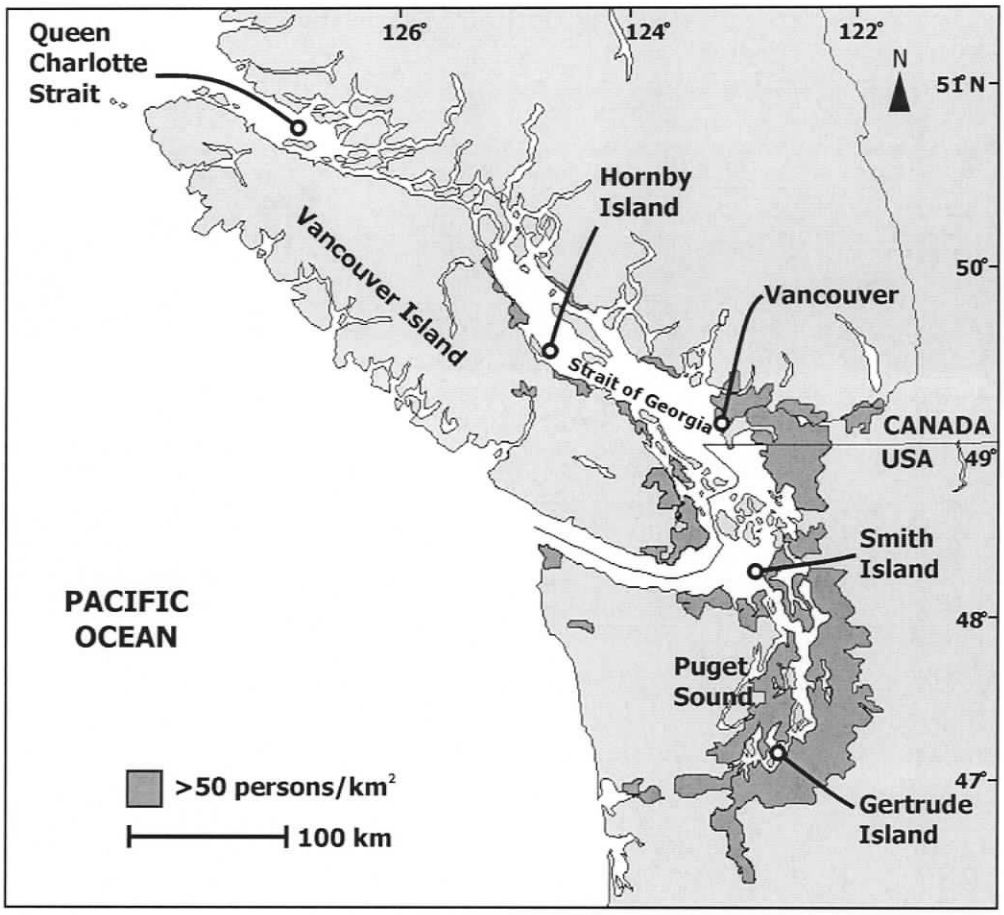

Figure 3.1. Sampling locations.

Harbour seal pups were live-captured from three sites (Queen Charlotte Strait, Hornby Island and Vancouver) in British Columbia and two sites (Smith Island in Juan de Fuca Strait and Gertrude Island in Puget Sound) in Washington State. Human population densities greater than 50 persons/km^2 are denoted in dark grey as a proxy for possible regional contaminant sources.

Blood samples were taken from the extradural vein using a Vacutainer™ blood collection system with an 18 gauge needle and serum collection tube (Becton-Dickenson,

Franklin Lakes, NJ). All collected blood samples were stored at 4° C in the field. Blood samples were centrifuged within five hours after collection at 400 × g for 20 min. Serum was aspirated and stored in 1 ml aliquots in cryovials either on dry ice (-80 °C) or in liquid nitrogen (-196°C) during transport, and in a (-80 °C) freezer until analysis of TH concentrations was performed.

Skin/blubber biopsy samples were taken from an area 20 cm lateral to the spinal column and anterior to the pelvis. The area was shaved first with an electric shaver (Sculptor™ with type-50 blades, Oster, Niles, IL) and cleaned using Betadine™ (Purdue Frederick, Pickering, ON, Canada) followed by 95 % isopropyl alcohol. Two biopsy samples were collected; one was 3.5 mm and the other 8 mm in diameter and approximately 2 cm to 3 cm in depth (Acuderm, Ft. Lauderdale, FL, USA). Following sample collection, the biopsy area on the animal was disinfected using Betadine™ and Aquaphor™ (Beiersdorf, Wilton, CT) iodine ointment. The 8 mm diameter biopsy samples were wrapped in hexane-rinsed aluminum foil and placed in 2 ml cryovials and stored immediately in liquid nitrogen in the field. The 3.5 mm diameter biopsy samples were placed into 1.0 ml of the RNA stabilization solution, RNAlater™ (Ambion, Houston, TX, USA) in RNase-free 1.5 ml cryovials and stored on wet ice in the field. Blubber samples frozen in liquid nitrogen were subsequently transferred to -80 °C in the laboratory and biopsy samples in RNAlater were stored at -20 °C.

Animals were subsequently weighed, sexed, measured for length and axillary girth, assessed for general body condition and then released. Captive time was approximately 15 to 20 min for captures using the landing net and less than 60 minutes for captures using the seine net method. All procedures were carried out under the auspices of the

respective animal care committees and scientific research permits for researchers in British Columbia (Fisheries and Oceans Canada Animal Care Committee using guidelines from the Canadian Council on Animal Care; Scientific Research Permit) and Washington State (United States Marine Mammal Protection Act Permit No. 835).

Prior to this one particular field study, various tissue samples taken from a euthanized animal were used for evaluating the difference of TR expression in each tissue. The samples were taken from a pup previously captured in 2000. The animal was a healthy 26.5 kg male, however, he had a broken jaw with savior infection probably inflicted by another seal. Thus animal was euthanized and autopsized by a veterinarian. Adrenal gland, heart, muscle, blubber, skin, spleen, liver, kidney, lung, and intestine were sampled and stored in -20 °C until analysis of TR expression was performed.

3.2 Tissue homogenization

Since a possible stratification within blubber biopsies could influence the results, we evaluated the steady-state levels of the normalizer gene, ribosomal protein L8, in skin, as well as upper and lower blubber sections collected from all animals (Figure 3.2.A). For this, each 3.5 mm diameter tissue biopsy was separated into skin (approximately 2 mm) and blubber sections using a razor blade prior to homogenization. Blubber samples were further divided into lower (close to the muscle) and upper (close to the skin) sections of 4 mm in depth.

All blubber samples were homogenized in TRIzol™ reagent (Invitrogen Canada Inc, Toronto, ON, Canada) using a Retsch MM301 mixer mill as described elsewhere (143) and with the following modifications. 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 any given sample, an additional 3 min to 6 min of mixing was performed if unhomogenized tissue remained following the initial 6 min homogenization period. Due to the presence of a substantial amount of connective tissue, the mixer mill procedure was incapable of efficient homogenization of the skin samples. These samples were homogenized using a PowerGen 125 tissue homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Skin samples were minced with a razor blade and placed into a 2.0 ml microcentrifuge tube (Mic Rew™ Simport Plastics Ltd., CA, USA) containing 400 μl TRIzol™. The shearing head was placed directly into each sample tube and gradually ramped from 8,000 rpm to approximately 30,000 rpm for a total of 3 min with 1 min cooling period for every 15 seconds of homogenization. In order to minimize heat production in the skin samples, tubes were kept on wet ice during the entire homogenization procedure.

Frozen adrenal gland, heart, muscle, blubber, skin, spleen, liver, kidney, lung, and intestine sample was cut into 5mm cubes or less and directly put into TRIzol™ reagent and homogenized using the same procedure for skin samples.

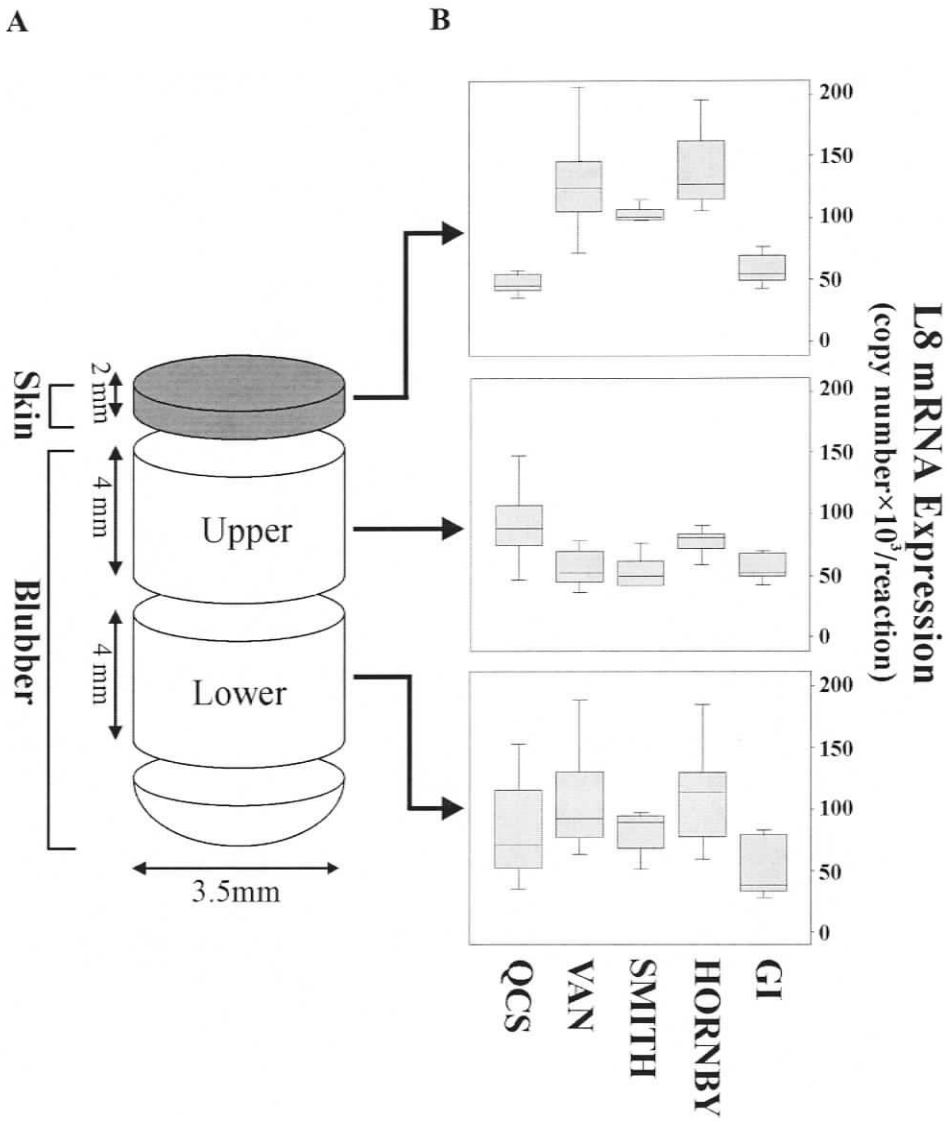

Figure 3.2. Ribosomal protein L8 (L8) expression in different sections of harbour seal biopsy samples.

Steady-state *L8* mRNA levels were measured using total RNA isolated from skin, as well as upper and lower sections of blubber. A) Diagram of the specific tissue regions examined. B) *L8* mRNA expression levels for each tissue section indicated. Animals from five geographic locations were compared including; Queen Charlotte (QCS) (n=5), Vancouver (VAN) (n=8), Smith Island (SMITH) (n=7), Hornby Island (HORNBY) (n=7) and Gertrude Island (GI) (n=6). Each box plot shows the median, first and third quartiles, and maximum and minimum population values.

3.3 Isolation of total RNA and preparation of cDNA

Total RNA was isolated from the tissue homogenates in TRIzol™ reagent as described by the manufacturer. Following phase separation, 1 μl of glycogen (Roche Diagnostics, Laval, QC, Canada) was added to each retained aqueous phase and RNA precipitated with the addition of isopropanol and an 1 hr incubation at -20 °C. Total RNA was resuspended in diethyl pyrocarbonate-treated distilled, deionized H_2O (20 μl for blubber and 40 μl for skin samples) and stored at -70 °C. For cross tissue samples total RNA was resuspended into 20 μl for lung, spleen, blubber and skin and 40 μl for adrenal gland, intestine, kidney, heart, liver and muscle samples.

Before eukaryotic DNA can be cloned into prokaryotic cells which are unable to remove introns, eukaryotic mRNA need to be isolated and used to make DNA without introns. Therefore, total complementary DNA (cDNA) was produced from total RNA using Superscript II RNase H^- reverse transcriptase as described by the manufacturer (Invitrogen Canada Inc). One microgram of total RNA from each sample was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Baie D'urfe, PQ, Canada) at 65 °C for 10 min and placed on wet ice. The assembled 20 μl cDNA synthesis reactions were incubated at 42 °C for 2 h and diluted 20-fold prior to QPCR analysis.

3.4 Cloning of thyroid hormone receptor cDNA sequences

Target cDNA sequences representative of the gene transcripts TRα and TRβ as well as our control gene, ribosomal protein L8 (L8) were amplified using primers designed using Primer Premier v4.1 software (Premier Biosoft International, Palo Alto, CA, USA)

and synthesized by AlphaDNA (Montreal, PQ, Canada) (Table 3.1). Each 25 μl DNA amplification reaction included 2 μl of 20-fold total cDNA template, 20 pmol of each primer, 200 μM equimolar dNTPs (dATP, dCTP, dGTP, and dTTP), and 2.5 units of Taq DNA polymerase (Invitrogen, Canada Inc). DNA amplification was performed on a Gene Amp®PCR System 9700 (PerkinElmer Biosystems, Foster City, CA, USA) using the following thermocycle conditions: denaturation at 95 °C (5 min); 35 cycles of 94 °C (1 min), 53 °C (1 min), and 72 °C (2 min); and an elongation step at 72 °C (7 min). DNA products were separated on a 1.5 % agarose gel and visualized with ethidium bromide staining on a ChemiImager 4000 (Alpha Innotech Corp., San Leandro, CA, USA). DNA bands representing TRα (631 base pairs; bp), TRβ (801 bp), and L8 (602 bp and 126 bp) were excised and isolated by three repeated 5 min freeze/thaw cycles followed by a 10 min centrifugation at 12000 x g (144). Isolated cDNA products (4 μl) were cloned into pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Canada). Plasmid DNA was purified from selected transformants using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, ON, Canada) and the presence of insert sequence confirmed by restriction analysis using *EcoRI* (Amersham Biosciences). The identity of each cloned cDNA was determined by DNA sequencing followed by BLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST). The sequences were deposited in Genbank (Table 3.1).

Table 3.1. DNA primers used in the cloning and QPCR analysis of harbour seal target genes.

Method	Gene	GenBank Accession #	Amplicon size (bp)	Primer sequences
Cloning	*ribosomal protein L8*[a]	DQ212693	126	5'-GGTGTGGCTATGAATCCTGT-3' 5'-ACGACGAGCAGCAATAAGAC-3'
	ribosomal protein L8	DQ212694	602	5'-CCGCCATGGGCCGTGTGATC-3' 5'-CGTACTCGTGGCCAGCAGTT-3'
	TRα	DQ212695	631	5'-TGCTGCATTATCGACAAGATCAC-3' 5'-GTGACTTGCCCAGTTCAAAGATGG-3'
	TRβ	DQ212696	801	5'-TATTCCTGTAAATATGAAGG-3' 5'-GTAATTGATATAGTGTTCAAA-3'
QPCR	*TRα*	–	231	5'-CGACGGAAGGAGGAAATG-3' 5'-GATCTTGGTAAACTCGCTGAA-3'
	TRβ	–	425	5'-AGAGGCTGGCAAAGAGGA-3' 5'-ACTTTCTGGGTCATAGCG-3'

[a]These primers were also used for QPCR analysis.

3.5 Real-time quantitative polymerase chain reaction (QPCR) assay

Primers specific for seal TRα, TRβ, and L8 were designed for the reverse transcription-QPCR assay (Table 3.1). Quantitative DNA amplification reactions (15 μl) were performed on a MX4000 system (Stratagene, La Jolla, CA, USA) as described previously (145). Briefly, each 15 μL reaction included 2μL of 20-fold total cDNA template, 10 pmol of each primer, 200μM dNTPs, 1.0 unit of Taq polymerase (Invitrogen, Canada Inc.), 10 mM Tris HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.01 % Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR), and 83.3 nM ROX reference dye (Stratagene, La Jolla, CA). The thermocycle conditions were denaturation at 95 °C (9 min); 40 cycles of 95 °C (15 sec), 55 °C (30 sec), and 72 °C (45 sec). Each sample was prepared in quadruplicate and the derived copy number

values averaged. The copy number for each gene transcript was determined from standard curves generated from the cloned plasmids in the previous section. TRα and TRβ expression values were normalized to those of the expression of the L8 internal control. The expression of this gene has been found to be invariant in many tissue types during developmentally-associated changes in endogenous TH concentrations in reptiles (146) and amphibians (147).

3.6 Measurement of thyroid hormone in blood

The concentrations of total thyroxine (TT_4), free thyroxine (FT_4), total triiodothyronine (TT_3), and free triiodothyronine (FT_3) were measured in animals from all five locations (n=39) using related EIAgen ELISA kits and by following the manufacturer's recommended protocol (Adaltus, Montréal, PQ, Canada) as described in chapter 2. Inter- and intra-assay variation was evaluated with the same regulation used in chapter 2.

3.7 Measurement of POP concentrations in blubber

Each frozen (-80 °C) 8 mm tissue biopsy was cut vertically and the upper skin layer (approximately 2mm) removed. A portion of each blubber sample (100 mg to 300 mg wet weight) was used for measuring POPs in two methods. Cost considerations for these analyses resulted in the use of 24 animals from QCS, Smith Island, and Gertrude Island. Firstly, all congeners of PCBs, as well as specific congeners of PCDDs and PCDFs were analyzed at the Fisheries and Oceans Canada Regional Contaminant Laboratory (Institute of Ocean Sciences, Sidney, BC, Canada). Briefly, the blubber sample was ground with

anhydrous sodium sulfate and spiked with a mixture of $^{13}C_{12}$-labeled PCBs, PCDDs, and PCDFs (Cambridge Isotope Laboratories, Andover, MA, USA). Using dichloromethane/hexane (1:1 ratio), the sample was extracted and the extract was evaporated to dryness and weighed. Total lipid concentration was determined related to the original sample weight. The residue was resuspended in dichloromethane/hexane (1:1), and analyzed by using high resolution gas chromatography and high resolution mass spectrometry analysis. Details of the chromatography and mass spectrometry conditions, the criteria used for chemical identification and quantification, and the quality assurance and quality control practices have been previously described (148).

Secondly, 12 organochlorine pesticides (OCs) were analyzed at Trent University (Peterborough, Ontario, Canada). The OCs were DDT (and its metabolites DDD, DDE), chlordane and components, HCH isomers, endosulfan and its metabolites, endrin and its metabolite, heptachlor and heptachlor epoxide, deldrin, aldrin, mirex, hexachlorobenzene (HCB), methoxychlor, and octachlorostyrene. Briefly, the blubber sample was ground with sodium sulfate and extracted with dichloromethane: hexane (1:1 ratio). Using gel permeation chromatography (GPC), lipids were removed from the sample. Percentage of lipid was determined by gravimetric analysis. The lipid portion of GPC fraction was further fractionated by using silica-gel column chromatography and the concentrations of OCs were quantified using gas chromatography in combination with electron capture detection. Quality control and assurance were in agreement with established laboratory procedures, and consisted of procedural blanks and a National Institute of Standards and Testing reference material.

In order to make the data comparable to other studies, the concentration of all POPs was expressed on a lipid weight (lw) basis. Although 154 PCB peaks were quantified (out of 209 theoretical congeners), many congeners were not detectable in all of the samples. Total PCB concentration was therefore calculated using the following rules. If a congener was detected in more than 70% of the sample population, the minimum detection limit substitutions were made. Where congeners were detected in less than 70% of samples, the minimum detection limit was set at zero. Total PCDDs and total PCDF concentrations were also calculated using the same rules as total PCBs. The pattern of PCBs was also examined. Individual PCB congeners were normalized by the most persistent PCB congener in seal, PCB-153. Then, the pattern difference of each congener between the sampling locations was calculated by:

Log (normalized congener X of location Y / normalized congener X of location Z).

Total toxic equivalents (TEQs) to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were calculated for all dioxin-like PCBs (12 congeners), PCDD (7 congeners), and PCDF (10 congeners) using the most recently reported international mammalian toxic equivalent factors (TEFs) for mammals (12). Total TEQ was calculated from its individual toxic equivalency factors (TEFi) by:

$$TEQ = \Sigma(PCDDi)\ TEFi + \Sigma(PCDFi) \cdot TEFi + \Sigma(PCBi) \cdot TEFi.$$

OC pesticide concentrations were calculated with no detection limit substitutions. The sum of related compounds (parent compound and metabolites) was calculated by concentration addition for DDT, chlordane, heptachlor, HCH, endosulfan and endrin.

Finally, the sum of all contaminants measured from seals (PCBs, PCDDs, PCDFs, and all OC pesticides) was represented as total POP concentration.

3.8 Statistical analyses

All statistical analyses were performed using SPSS version 12 software (SPSS Inc. Chicago, IL). Results were evaluated a) by site and b) among all individuals. For the former, harbour seal pups from five sites were compared for circulating thyroid hormone concentrations and steady state mRNA expression levels in skin and blubber biopsy samples. Seals from the remote Queen Charlotte Strait (QCS), previously (and in this study) shown to be relatively uncontaminated (39) were used as a reference group. For each group, values were examined for normality with the Shapiro-Wilk test and for homogeneity of variances using Levine's test. Groups that were normal with equal variance were evaluated using one-way analysis of variance to assess inter-group differences followed by Tukey's honest significant difference test. If the data were not normally distributed, a Kruskal-Wallis test was used followed by a Mann-Whitney U test for pairwise comparison of groups. Significance was defined as $p<0.05$. Extreme outliers were removed, having been defined as more than three times the inter-quartile range.

For the among-individual assessment of the entire study group, correlation analysis was carried out using the Pearson method for normally distributed data or the non parametric Kendall's tau_b method when data were not normally distributed. Given our concern that body weight (~age) of the harbour seal pups might influence either contaminant concentrations or thyroid endpoints, we conducted regressions between body weight and contaminant concentrations, thyroid hormone concentrations, and TR levels.

If body weight exhibited a significant relationship with contaminant or thyroid measurements, we conducted multiple regression analysis to identify the relative contribution of each variable.

4. RESULTS

4.1 Sample collection

Of the 39 harbour seals sampled, availability of adequate tissue quality and cost considerations for contaminant analyses resulted in the analysis of 39 serum samples for TH measurement, 35 biopsies (3.5 mm) for gene expression analysis, and 24 blubber biopsies (8 mm) for PCBs, PCDDs, PCDFs, and OC pesticides analysis.

The mean body weight was 20.6 kg ± SEM 0.52 kg (range 14.1 to 27.0 kg). Our ANOVA results revealed a significant difference among sites. A subsequent Tukey's HSD test indicated that only Smith Island seals differed, being slightly heavier than QCS seals (p=0.038).

4.2 Thyroid hormone receptor gene sequences in the harbour seal

Partial cDNA sequences were isolated from biopsied harbour seal blubber that represented expressed mRNA of TRα, TRβ genes, and our control gene, ribosomal protein L8. Both TR sequences obtained are predicted to encompass approximately half of the estimated open reading frame region within the mRNA transcripts and include sequence located between the encoded DNA binding and ligand binding domains (Figure 3.3.A and B). A comparison of harbour seal TR sequences with six other species using the ClustalW alignment program (http://www.ebi.ac.uk/clustalw/) indicated that the

mRNA sequence for TRα and TRβ are highly conserved among these vertebrates (Figure 3.3.A and B, and Table 3.2). This is particularly evident within mammals, where the putative protein sequence of harbour seal TRα and TRβ display >99% amino acid identity.

Table 3.2. Comparison of harbour seal thyroid receptor α and β cDNA and putative protein sequences of other species.

	Phoca vitulina TRα			*Phoca vitulina* TRβ		
	Genbank accession #	Nucleotide[a] (584bp)	Amino acid[a] (194aa)	Genbank accession #	Nucleotide[a] (760bp)	Amino acid[a] (253aa)
Homo sapiens	BC035137	95	100	NM00461	94	100
Ovis aries	Z68308	96	100	Z68307	89	99
Mus musculus	BC046795	92	100	NM009380	90	99
Gallus gallus	NM205313	84	93	NM205447	86	95
Danio rerio	U54796	77	85	NM131340	75	89
Xenopus laevis	L76285	79	92	M35361	79	93

[a] Values represent identity for the comparable nucleotide and putative amino acid sequences.

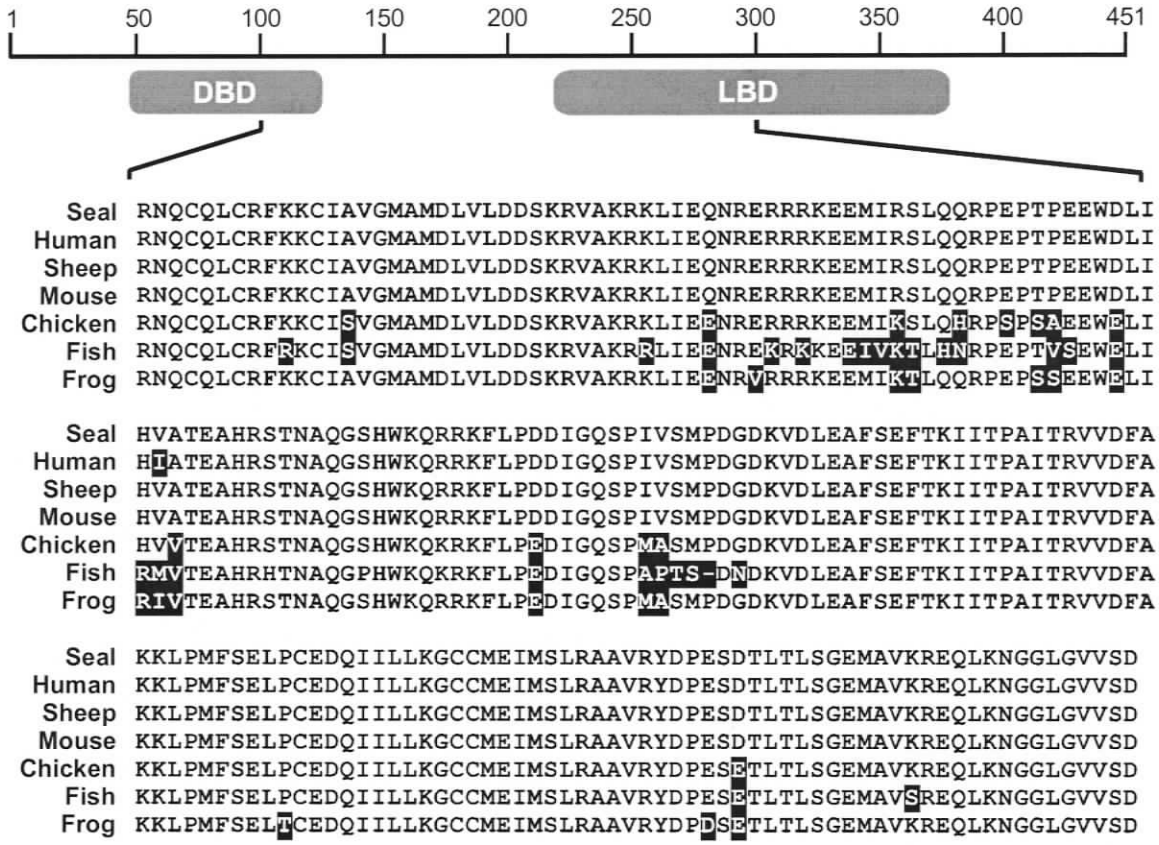

Figure 3.3.A. Comparison of amino acid sequence for thyroid hormone receptor α fragment between harbour seal and other species.

The upper scale represent complete human TR amino acid sequence. The position of DNA binding domain (DBD) and ligand binding domain (LBD) are indicated by the gray box. Harbour seal partial TRα (194aa) sequences was compared with TRα of human (*Homo sapiens*), sheep (*Ovis aries*), house mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*) and African clawed frog (*Xenopus laevis*). Dark background indicates the difference in amino acid compared with harbour seal TRα sequence.

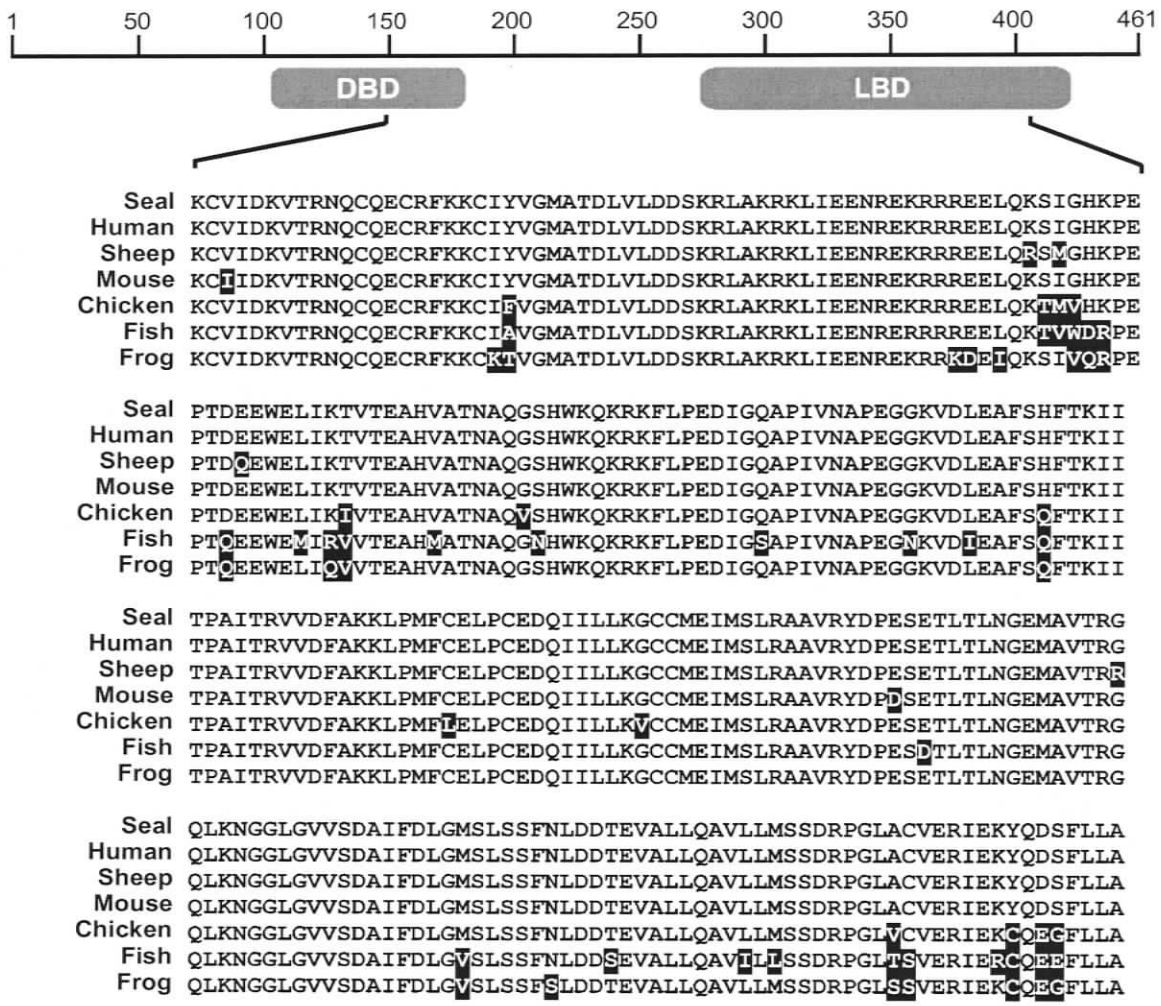

Figure 3.3.B. Comparison of amino acid sequence for thyroid hormone receptor fragment between harbour seal and other species.

The upper scalerepresent complete human TR amino acid sequence. The position of DNA binding domain (DBD) and ligand binding domain (LBD) are indicated by the gray box. Harbour seal partial TRβ (253aa) sequence was compared with TRβ of human (*Homo sapiens*), sheep (*Ovis aries*), house mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*) and African clawed frog (*Xenopus laevis*). Dark background indicates the difference in amino acid compared with harbour seal TRβ sequence.

4.3 Measurement of Thyroid hormone receptor expression

4.3.1 Thyroid hormone receptor expression in cross tissues

Based on the cDNA sequence obtained, oligonucleotide primers were developed for QPCR analysis of specific gene expression biomarkers (Table 1). Significant variation in L8 mRNA expression ($p<0.05$, Tukey's HSD or Mann-Whitney U) among the different tissue sample was found. L8 copy number (per reaction) was extremely high in spleen (193,000), while others were between 17,000 and 68,000. Even after eliminating the spleen sample, L8 expression varied significantly among 9 tissue samples ($p<0.05$, Tukey's HSD or Mann-Whitney U) (Figure 3.4). This demonstrates the difficulty in the evaluation of gene expression across tissues. In this study, we also sequenced β-actin gene (777bp) from harbour seals in order to use as an internal control. However, a recent study showed that β-actin expression levels were significantly decreased after exposure to the PCB mixture 1254 in *Xenopus laevis* tadpoles (149). Therefore, we did not further investigate the use of β–actin as a normalizer. Even though L8 expressions were significantly different among the different tissues, in later experiments, L8 was not significantly different in the lower part of the blubber from different populations of seals. Therefore, the experiments were continued without investigating another normalizer gene for this particular experiment.

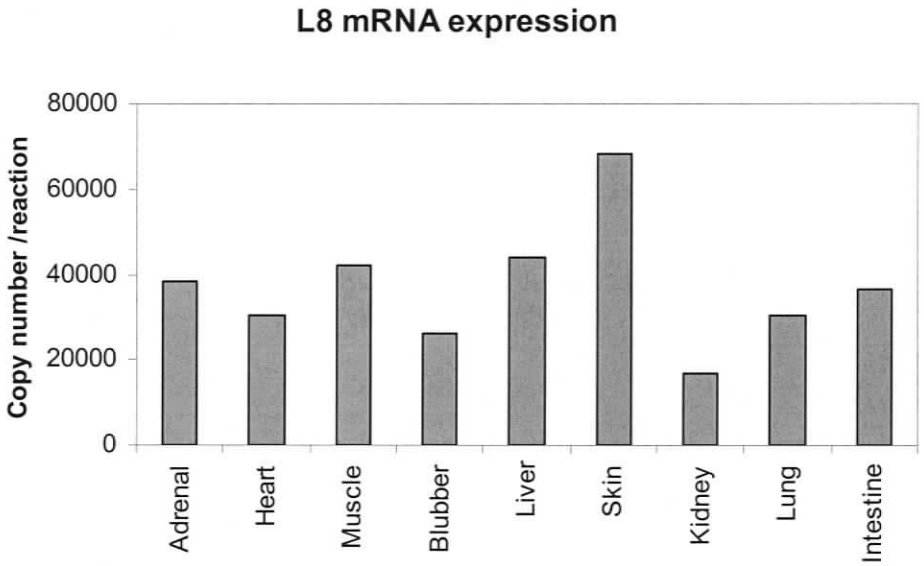

Figure 3.4. Ribosomal protein L8 (L8) expression in different tissues of harbour seal. Steady-state L8 mRNA levels were measured using total RNA isolated from 10 different tissues. Spleen had extremely high L8 expression (193,000/reaction). Thus, it was eliminated from this graph, in order to compare the relative L8 expression of other 9 tissues. These data were generated from a single animal.

TR mRNA expression was still measured and compared among different tissue samples without normalizing the data with L8 because each cDNA sample had an equalized amount of total RNA input. Both TRα and TRβ mRNAs were expressed in all sampled tissues (Figure 3.5). However their relative abundance of TRα and TRβ mRNA expression varied across tissues. TRα mRNA was expressed highest in the heart, whereas TRβ mRNA was high in the lung and the adrenal gland.

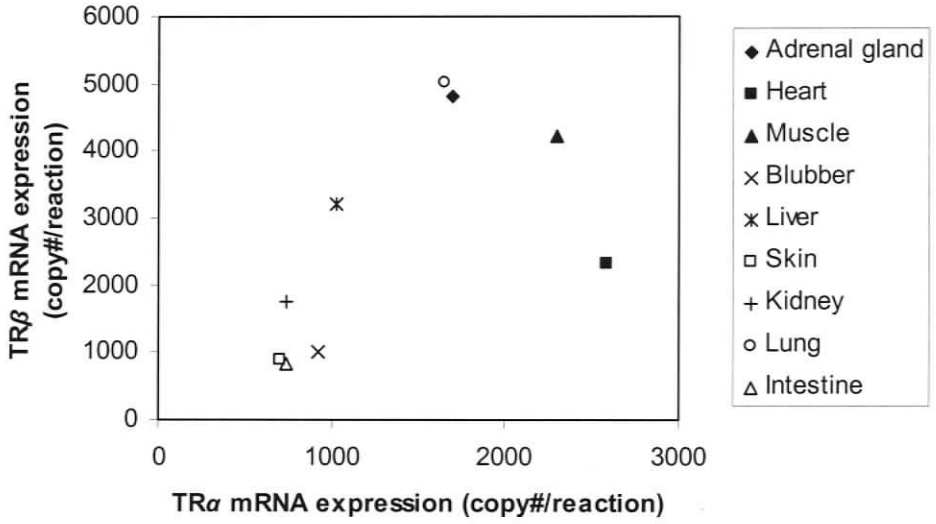

Figure 3.5. Thyroid hormone receptor α and β mRNA expression across tissues of harbour seal pup.

4.3.2 Measurement of thyroid hormone receptor expression in skin/blubber biopsies

Significant variation in L8 mRNA expression ($p<0.05$, Tukey's HSD or Mann-Whitney U) within the vertical plane of the biopsy tissue sample was observed for all sample sections, with the exception of QCS and Gertrude Island (Figure 3.2.B). Then each section (skin, upper blubber and lower blubber) was compared separately across the animals from different locations. Both the skin and the upper blubber (adjacent to skin) sections showed a significant difference in L8 mRNA gene expression among sites (skin $p<0.001$, Kruskal-Wallis; upper blubber $p=0.028$, Kruskal-Wallis). However, L8 steady state transcript levels in the lower blubber region did not differ among sites ($p=0.05$, ANOVA; $p>0.05$, Tukey). Therefore, we chose the amount of L8 transcript in the lower blubber as a suitable normalizer gene for the comparison of gene expression levels

between the different seal populations. All subsequent QPCR analyses of *TR* transcript copy numbers are presented for lower sections blubber only.

TRα mRNA abundance was found to be significantly higher than that of TRβ (p=0.004, Tukey) in all the individuals examined (Table 3.3). In addition, the relationship between TRα and TRβ mRNA expression patterns was positively correlated (R=0.651, p<0.05). In comparisons of animals from different geographic locations, Gertrude Island samples displayed a significant elevation in both TRα (p<0.001, Tukey) and TRβ (p=0.011,Tukey) transcript levels compared with animals from the QCS reference site.

Table 3.3. Steady state levels of thyroid hormone receptor α and β transcripts measured in the lower blubber biopsy section from harbour seal pups in coastal British Columbia and Washington State.

	QCS	Vancouver	Hornby Island	Smith Island	Gertrude Island
N	10	7	7	5	6
TRα	1309 ± 125 (699-1863)	1341 ± 193 (590-1927)	2459 ± 531 (988-4561)	802 ± 148 (464-1185)	3633 ± 446* (2539-5163)
TRβ	973 ± 246 (437-2984)	799 ± 88 (519-1254)	920 ± 194 (362-1732)	511 ± 26 (458- 603)	1387 ± 142* (936-1863)

Mean copy numbers per reaction ± standard error of mean (SEM) with the range in parentheses are shown. Data obtained from Queen Charlotte Strait (QCS) animals were used as the reference site for statistical analyses. Significant differences in gene expression are denoted by an asterisk (p<0.05).

4.4 Measurement of thyroid hormones in blood

The concentrations of different TH forms were measured in serum collected from the seal pups by site (Table 3.4). Among the seals sampled from different locations, Gertrude

Island animals had significantly lower TT_4 and FT_4 compared with reference site QCS animals ($p<0.001$, Tukey and $p<0.001$, Mann-Whitney). A strong positive correlation was found between measured TT_4 and FT_4 levels ($R= 0.844$, $p<0.001$) among all individuals, whereas no correlation existed between TT_3 and FT_3 serum concentrations ($R= 0.260$, $p=0.121$). A negative correlation between circulating TT_4 and TRα mRNA levels ($R=-0.456$, $p<0.05$) and circulating FT_4 and TRα expression ($R=-0.481$, $p<0.05$) were detected. No correlation was observed between any serum TH measurement and TRβ transcript levels.

Table 3.4. Circulating thyroid hormone concentrations in harbour seal pups from five sites in coastal British Columbia and Washington State.

	QCS	Vancouver	Hornby Island	Smith Island	Gertrude Island
N	10	8	7	7	7
TT_4 (nmol/L)	71.8 ± 9.0 (41.1-120.5)	74.4 ± 6.6 (47.2-98.5)	59.4 ± 4.9 (43.3-81.5)	56.5 ± 9.8 (12.6-86.7)	26.3 ± 4.3* (15.1-44.8)
FT_4 (pmol/L)	37.8 ± 2.4 (28.3– 49.5)	39.2 ± 2.6 (25.1-47.4)	32.7 ± 0.8 (29.2-35.5)	32.9 ± 3.0 (24.0-46.6)	22.3 ± 2.1* (16.9-32.7)
TT_3 (nmol/L)	0.72 ± 0.14 (0.22-1.57)	0.80 ± 0.08 (0.55-1.18)	0.28 ± 0.08 (0.08-0.64)	0.64 ±0.07 (0.28-0.84)	0.70 ± 0.10 (0.40-1.10)
FT_3 (pmol/L)	7.61 ± 0.67 (4.49-10.15)	7.15 ± 0.51 (5.41-9.42)	7.91 ± 1.58 (3.69-12.13)	6.81 ± 0.58 (4.91-8.90)	8.99 ± 0.99 (5.17-13.33)

Mean ± standard error of mean (SEM) with the range in parentheses are shown. Data obtained from Queen Charlotte Strait (QCS) animals were used as the reference site for statistical analyses. Significant differences in TH concentrations are denoted by an asterisk ($p<0.05$).

4.5 POP concentrations in blubber

4.5.1 PCB concentrations and their patterns

Out of a total of 154 PCB congener peaks quantified a maximum of 135 peaks were detected in QCS seals, 142 peaks in Smith Island seals, and 153 peaks in Gertrude Island seals. The concentration of total PCBs varied among the sampling locations (Table 3.5). Seal pups located in the Gertrude Island showed an approximate 10 times higher total PCB concentration (6239 ± 1008 μg/kg, lw) compared to animals from the reference QCS (682 ± 144 μg/kg, lw, $p<0.001$, Mann Whitney) and 2 times higher than animals from Smith Island (1355 ± 190 μg/kg, lw, $p<0.05$, Mann Whitney U) compared to QCS animals.

Table 3.5 Concentrations of persistent organic pollutants in blubber biopsies from harbour seal pups in coastal British Columbia and Washington State.

		QCS	Smith	Gertrude Island
ΣPOPs	(μg/kg, lw)	1630 ± 365	2310 ± 501	7386 ± 1192*
ΣPCBs	(μg/kg, lw)	682 ± 144	1355 ± 190*	6239 ± 1008*
ΣPCDDs	(ng/kg, lw)	125 ± 17	103 ± 25	152 ± 23
ΣPCDFs	(ng/kg, lw)	47 ± 19	48 ± 27	39 ± 29
ΣOC pesticides	(μg/kg, lw)	947 ± 225	1148 ± 265	1147 ± 196
ΣTEQ	(ng/kg, lw)	15.4 ± 3.4	24.9 ± 11.6	61.7 ± 13.3*
PCB-TEQ	(ng/kg, lw)	5.7 ± 0.9	9.4 ± 1.1	56.0 ± 13.6*
PCDD-TEQ	(ng/kg, lw)	6.8 ± 2.6	9.0 ± 6.2	3.1 ± 0.8
PCDF-TEQ	(ng/kg, lw)	2.8 ± 0.6	4.4 ± 3.6	2.4 ± 1.6

Mean concentrations ± standard error of mean (SEM). Data obtained from Queen Charlotte Strait (QCS) animals were used as the reference site for statistical analyses. Significant differences in POP concentrations are denoted by an asterisk ($p<0.05$).

Although the concentration of total PCBs significantly differed among the populations, the main congeners contributing to the total PCBs were indistinguishable. In all three populations, half of the total PCBs concentrations were contributed by the di-*ortho* PCBs, in the order of PCB-153, 138, 99, 101 (Figure 3.6).

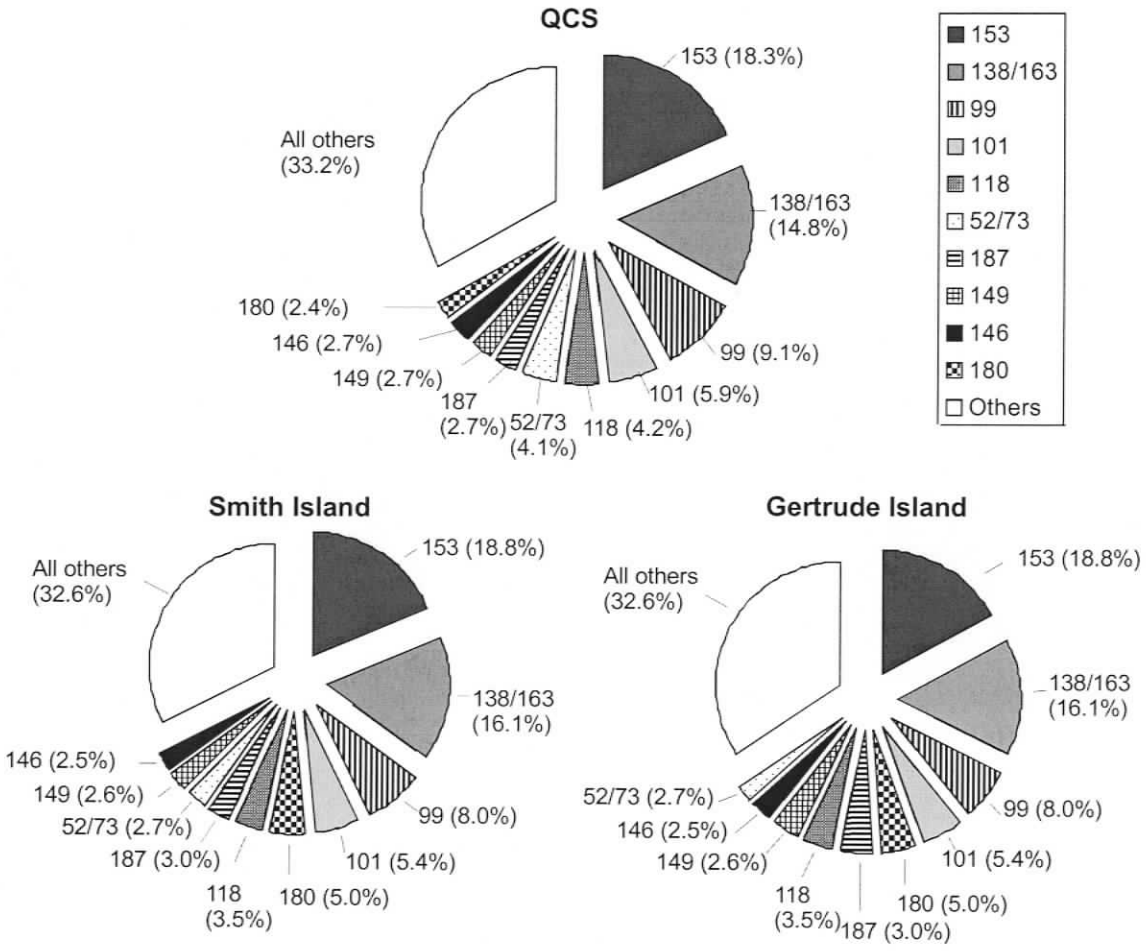

Figure 3.6. Individual PCB congener contribution to total PCBs in harbour seal pups from Queen Charlotte Strait (QCS), Smith Island, and Gertrude Island.

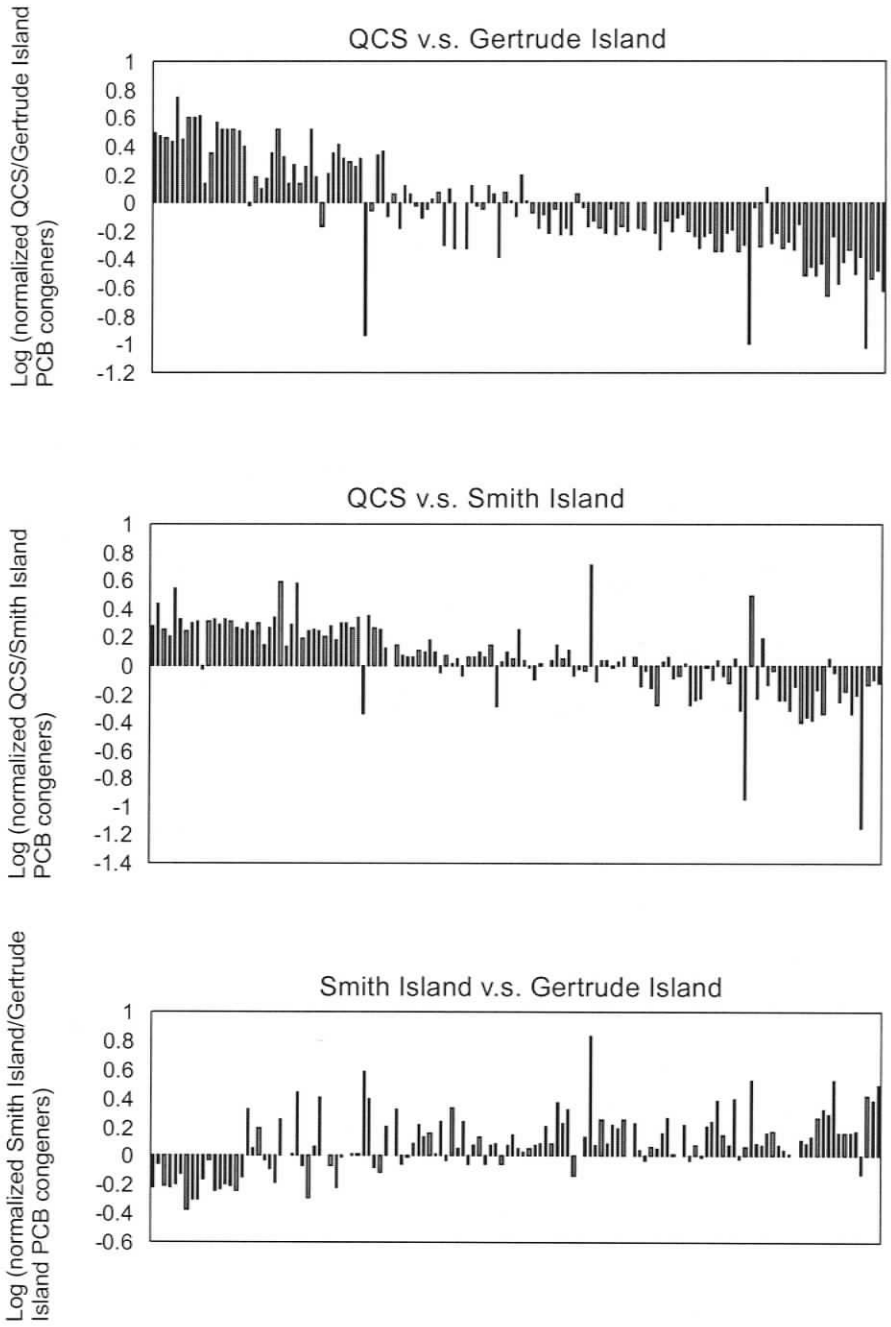

Figure 3.7. Comparison of individual PCB congener patterns in harbour seal blubber from Queen Charlotte Strait (QCS), Smith Island and Gertrude Island.

By comparing the ratio of each congener to the most persistent PCB-153, pattern and levels are standardized, independent to the concentrations. (Note: this figure also appears in L. Mos's doctoral thesis)

PCB pattern analysis revealed that there were distinctive differences among the sampling locations (Figure 3.7). QCS animals had a greater abundance of less chlorinated PCBs (lower molecular weight) and lower abundance of higher chlorinated PCBs (heavier molecular weight) compared to Gertrude Island animals. Similar trends were found between QCS and Smith Island animals, but to a lesser extent.

4.5.2. Toxic Equivalent Quotients (TEQ)

Three out of 24 seals were identified as extreme outliers in the TEQ calculations and were removed from further analysis. Calculated total TEQ values for PCBs, PCDDs and PCDFs were significantly higher in seal pups in Gertrude Island (61.7 ± 13.3 ng/kg) compared to animals from QCS (15.4 ± 3.4 ng/kg, $p<0.05$, Mann Whitney), but did not differ significantly from Smith Island (24.9 ± 11.6 ng/kg, $p= 0.540$, Mann Whitney) (Table 3.5).

PCBs were the dominant contributor to total TEQ (PCBs represented an average of 43.4% in QCS seals; 61.1% for Smith Island seals; and 91.1% for Gertrude Island seals) (Table 3.5). Among all three populations, PCB-118 (mono-*ortho*) was the main congener contributing to the total PCB TEQ (more than 60%) followed by the mono-*ortho* PCBs, 105, 156, and 157. These four congeners contributed more than 95% of total PCB TEQ (Figure 3.8). Consequently, there was a strong positive correlation between the logarithm of total PCBs and total TEQs ($R= 0.803$, $p<0.001$, Figure 3.9). On the other hand, the relative contribution of PCDD and DCDF to total TEQ was much higher at QCS and Smith Island (PCDF represented an average of 35.1% in QCS seals; 28.2% for Smith

Island seals; and 5.9% for Gertrude Island, and PCDF represent 21.4%, 10.7%, and 4.0% respectively.

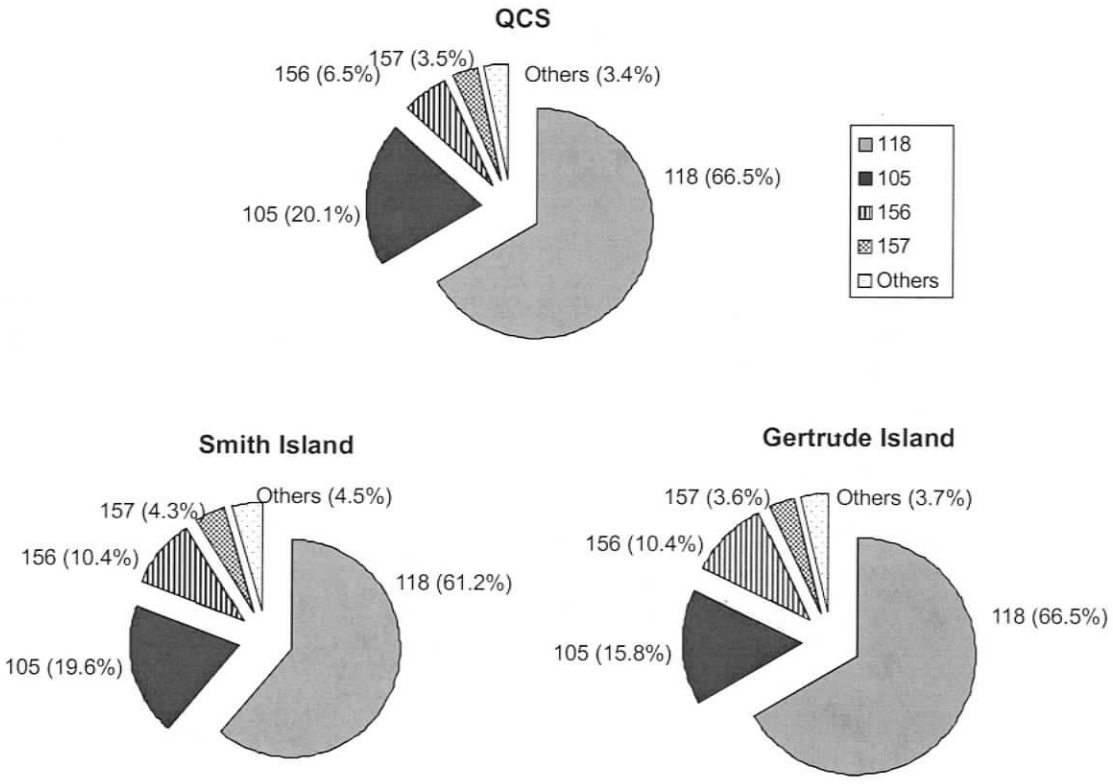

Figure 3.8. PCB congener contributions to PCB total TEQ in harbour seal pups from Queen Charlotte Strait (QCS), Smith Island, and Gertrude Island.

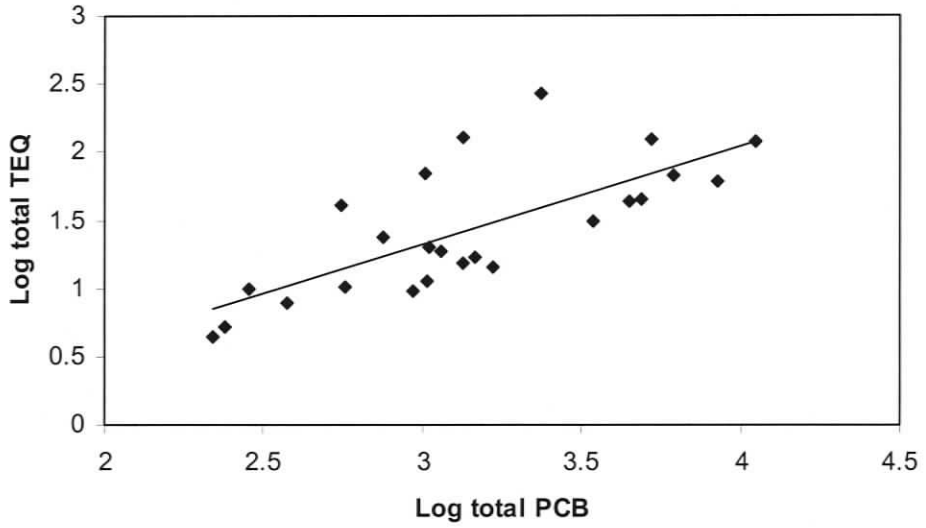

Figure 3.9. Correlation between logarithm of total PCBs and total TEQs.
Total PCBs and total TEQs were highly correlated (R=0.803, p<0.001) and both parameters were used as a measurement/biomarker of contaminant exposure in this study.

4.5.3. Organochlorine pesticide concentrations

Twelve OC pesticides were measured in QCS, Smith Island and Gertrude Island (Table 3.6). DDT and its metabolites were detected in all animals (n=24) and were also the most abundant (Table 3.6). Aldrin and methoxychlor were detected in only a few samples with less than 1 μg/kg (lw). Therefore, no statistics were applied. Chlordane, heptachlor and hexachlorocyclohexane (HCH) were detected in all animals in relatively high concentrations (Table 3.6). Other pesticides were detected in low concentrations. Main OC pesticides found in the seals were DDT and its metabolites. Total DDT concentration was compared among the populations, however there was no statistical difference (p=0.783, ANOVA). Gertrude Island animals had a significantly higher concentration of total chlordane (154.3 ± 34.5 μg/kg lw) compared to QCS animals (75.8 ± 8.8 μg/kg lw, p<0.05, ANOVA) (Table 3.6). White mirex was found in relatively low

concentrations among all populations, Gertrude Island animals showed significantly higher concentrations of mirex (4.7 ± 0.4 μg/kg lw) compared to animals from QCS (1.5 ± 0.4 μg/kg lw, $p<0.05$, ANOVA).

Table 3.6. Concentrations of organochlorine pesticides in blubber biopsies from harbour seal pups in coastal British Columbia and Washington State.

Organochlorine pesticides	QCS	Smith	Gertrude Island
Σ DDT	719 ± 190	923 ± 261	840 ± 148
Σ Chlordane	75.8 ± 8.8	73.0 ± 6.7	154.3 ± 34.5*
Σ Heptachlor	76.3 ± 22.4	58.0 ± 7.5	66.4 ± 14.1
Σ HCH	64.0 ± 8.7	48.5 ± 7.4	47.4 ± 7.8
Σ Endrin	2.1 ± 0.7	4.3 ± 1.5	3.4 ± 1.9
Σ Endosulfan	1.6 ± 0.6	5.5 ± 0.8	1.0 ± 0.6
Dieldrin	8.4 ± 1.1	8.1 ± 0.5	12.3 ± 2.5
Hexachlorobenzene	5.9 ± 0.5	5.7 ± 1.1	5.5 ± 0.3
Mirex	1.5 ± 0.4	2.7 ± 0.8	4.7 ± 0.4*
Methoxychlor	ND	ND	ND
Octachlorostyrene	0.8 ± 0.1	0.7 ± 0.3	4.1 ± 0.5*
Aldrin	ND	ND	ND

Mean concentrations ± standard error of mean (SEM) (μg/kg) (lw). Data obtained from Queen Charlotte Strait (QCS) animals were used as the reference site for statistical analyses. Significant differences in organochlorine pesticide concentrations are denoted by asterisk ($p<0.05$). ND = not detected.

The sum of all 12 OC pesticide concentrations was presented as total OC pesticides. Unlike total PCBs or total TEQ, Gertrude Island seal pups were not significantly different in total OC concentrations (1147 ± 196 μg/kg, lw) compared to QCS seals (947 ± 225 μg/kg, lw, $p=0.230$, Mann Whitney) or Smith Island seals (1148 ± 265 μg/kg, lw, $p=0.205$, $p=0.193$, Mann Whitney) (Table 3.5).

4.5.4 Correlation of POP concentrations

All contaminants measured from seals and their calculated TEQ values were analyzed for correlations. Many POPs correlated with each other (Table 3.7). total DDT and total heptachlor showed strongest correlation among OC pesticides (R=0.652 $p<$ 0.01). Whereas, there is no significant correlation among dioxin like compounds (ΣPCBs, ΣPCDD, and ΣPCDF). Total PCBs were strongly correlated ($p<.001$) with the three highest concentrations of OC pesticides (ΣDDT R=0.423, ΣChlordane; R=0.529, and Σheptachlor; R=0.399), Mirex (R=0.697) and octachlorobenzene (R=0.414). On the other hand, total PCDD was only significantly correlated with total Chlordane (R=0.304, p=0.037) and total PCDF was correlated with hexachlorobenzene (R=0.290, p=0.047). In the correlation metric, the strongest correlation was found between total PCBs and total POPs (R=0.897, $p<0.001$).

Table 3.7
Correlations among contaminants in harbour seals from QCS, Smith Island, and Gertrude Island (n=24).

	ΣPCB	PCB TEQ	ΣPCDD	PCDD TEQ	ΣPCDF	PCDF TEQ	ΣTEQ	ΣDDT	Σ chlordane	Σhepta-chlor	ΣBHC	Σendrin	Σendo-sulfan	dieldrin	hexa-chloro-benzene	mirex	methoxy-chlor	octa-chloro-styrene	aldrin	ΣPOPs
ΣPCB	1.000	**.881****	.162	.083	-.107	-.225	**.613****	**.423****	**.526****	**.399****	.012	-.004	.030	.194	.059	**.697****	.150	**.414****	-.153	**.897****
	.	.000	.279	.579	.476	.132	.000	.005	.000	.008	.937	.978	.848	.196	.692	.000	.388	.006	.376	.000
PCB TEQ	**.881****	1.000	.154	.075	-.115	-.265	**.715****	**.431****	**.423****	**.407****	-.012	-.021	.047	.138	-.028	**.689****	.087	**.374***	-.139	**.810****
	.000	.	.303	.616	.444	.077	.000	.004	.005	.007	.937	.891	.764	.355	.853	.000	.617	.013	.421	.000
ΣPCDD	.162	.154	1.000	**.348***	**.384****	.188	**.312***	.174	**.304***	.072	.022	-.028	.093	.123	.181	**.294***	.089	.055	-.052	.254
	.279	.303	.	.017	.009	.197	.037	.234	.037	.620	.882	.857	.540	.399	.215	.044	.602	.710	.758	.083
PCDD TEQ	.083	.075	**.348***	1.000	**.399****	**.290***	**.360***	.275	.145	.261	.210	-.178	.085	.094	.225	.004	-.133	.128	.117	.152
	.579	.616	.017	.	.006	.047	.016	.059	.321	.074	.150	.245	.574	.519	.124	.980	.434	.385	.489	.298
ΣPCDF	-.107	-.115	**.384****	**.399****	1.000	**.558****	.075	.123	-.036	.094	.130	-.178	.241	-.087	**.290***	-.004	-.089	-.230	.156	-.043
	.476	.444	.009	.006	.	.000	.616	.399	.804	.519	.372	.245	.113	.552	.047	.980	.602	.118	.356	.766
PCDF TEQ	-.225	-.265	.188	**.290***	**.558****	1.000	-.043	-.087	-.145	-.072	.109	-.178	.023	-.210	.167	-.156	-.074	-.142	-.039	-.181
	.132	.077	.197	.047	.000	.	.771	.552	.321	.620	.457	.245	.878	.150	.254	.286	.664	.333	.817	.215
ΣTEQ	**.613****	**.715****	**.312***	**.360***	.075	-.043	1.000	**.368***	**.312***	**.344***	.020	-.166	-.030	.138	.020	**.539****	.008	**.414****	-.097	**.636****
	.000	.000	.037	.016	.616	.771	.	.014	.037	.022	.895	.287	.848	.355	.895	.000	.964	.006	.573	.000
ΣDDT	**.423****	**.431****	.174	.275	.123	-.087	**.368***	1.000	**.290***	**.652****	**.355***	.083	.163	.268	**.312***	**.381****	-.148	.120	.026	**.529****
	.005	.004	.234	.059	.399	.552	.014	.	.047	.000	.015	.588	.283	.066	.033	.009	.384	.413	.878	.000
Σchlordane	**.526****	**.423****	**.304***	.145	-.036	-.145	**.312***	**.290***	1.000	**.319***	.254	.036	.054	.239	.065	**.454****	-.059	.128	-.104	**.514****
	.000	.005	.037	.321	.804	.321	.037	.047	.	.029	.083	.816	.721	.102	.655	.002	.728	.385	.538	.000
Σheptachlor	**.399****	**.407****	.072	.261	.094	-.072	**.344***	**.652****	**.319***	1.000	**.428****	-.075	.140	.283	.283	**.410****	-.222	.040	.195	**.486****
	.008	.007	.620	.074	.519	.620	.022	.000	.029	.	.003	.624	.358	.053	.053	.005	.192	.785	.248	.001
ΣBHC	.012	-.012	.022	.210	.130	.109	.020	**.355***	.254	**.428****	1.000	-.186	.070	**.362***	**.406****	.069	**-.385***	-.018	.182	.058
	.937	.937	.882	.150	.372	.457	.895	.015	.083	.003	.	.225	.646	.013	.005	.637	.024	.901	.281	.691
Σendrin	-.004	-.021	-.028	-.178	-.178	-.178	-.166	.083	.036	-.075	-.186	1.000	.132	.147	-.147	-.056	.292	-.135	.050	.004
	.978	.891	.857	.245	.245	.245	.287	.588	.816	.624	.225	.	.410	.339	.339	.718	.104	.380	.780	.979
Σendo-sulfan	.030	.047	.093	.085	.241	.023	-.030	.163	.054	.140	.070	.132	1.000	.008	.078	.019	-.064	**-.402****	.223	-.062
	.848	.764	.540	.574	.113	.878	.848	.283	.721	.358	.646	.410	.	.959	.610	.898	.721	.008	.206	.683
dieldrin	.194	.138	.123	.094	-.087	-.210	.138	.268	.239	.283	**.362***	.147	.008	1.000	**.290***	.272	.207	.244	.273	.275
	.196	.355	.399	.519	.552	.150	.355	.066	.102	.053	.013	.339	.959	.	.047	.063	.223	.096	.106	.059
hexachloro-benzene	.059	-.028	.181	.225	**.290***	.167	.020	.312*	.065	.283	**.406****	-.147	.078	**.290***	1.000	.054	-.178	.149	.195	.130
	.692	.853	.215	.124	.047	.254	.895	.033	.655	.053	.005	.339	.610	.047	.	.710	.297	.309	.248	.372
mirex	**.697****	**.689****	**.294***	.004	-.004	-.156	**.539****	**.381****	**.454****	**.410****	.069	-.056	.019	.272	.054	1.000	.074	.248	.039	**.693****
	.000	.000	.044	.980	.980	.286	.000	.009	.002	.005	.637	.718	.898	.063	.710	.	.664	.091	.817	.000
methoxy-chlor	.150	.087	.089	-.133	-.089	-.074	.008	-.148	-.059	-.222	**-.385***	.292	-.064	.207	-.178	.074	1.000	.164	-.159	.119
	.388	.617	.602	.434	.602	.664	.964	.384	.728	.192	.024	.104	.721	.223	.297	.664	.	.338	.421	.487
Octachloro-styrene	**.414****	**.374***	.055	.128	-.230	-.142	**.414****	.120	.128	.040	-.018	-.135	**-.402****	.244	.149	.248	.164	1.000	-.183	**.419****
	.006	.013	.710	.385	.118	.333	.006	.413	.385	.785	.901	.380	.008	.096	.309	.091	.338	.	.281	.004
aldrin	-.153	-.139	-.052	.117	.156	-.039	-.097	.026	-.104	.195	.182	.050	.223	.273	.195	.039	-.159	-.183	1.000	-.078
	.376	.421	.758	.489	.356	.817	.573	.878	.538	.248	.281	.780	.206	.106	.248	.817	.421	.281	.	.644
ΣPOPs	**.897****	**.810****	.254	.152	-.043	-.181	**.636****	**.529****	**.514****	**.486****	.058	.004	-.062	.275	.130	**.693****	.119	**.419****	-.078	1.000
	.000	.000	.083	.298	.766	.215	.000	.000	.000	.001	.691	.979	.683	.059	.372	.000	.487	.004	.644	.

The correlation coefficients are shown with associated *p*-values.
Statistical significance is denoted by ** ($p<0.01$) and * ($p<0.01$) and also shaded grey.

4.5.5 Correlation of thyroid hormone and thyroid hormone receptor endpoints with POP exposure

Total PCBs, total PCDDs, total PCDFs, total OC pesticides, and their sum, total POPs, and total TEQ were tested for correlation with THs and TR expression in blubber (Table 3.8). Total POPs, total PCBs and total TEQ were strongly correlated with thyroid parameters with similar manners. They were negatively correlated with circulating TT_4 and FT_4. The strongest correlation was between total POPs and TT_4 (R= 0.749, p<0.001), Total PCBs and total TT_4 elicited the second strongest correlation (R= 0.744, p<0.001, Figure 3.10). In contrast, they were positively correlated with the level of TRα mRNA. Total PCBs were the most strongly correlated with TRα (R= 0.717, p<0.001, Figure 3.11). Weaker than that of TRα, but a significant positive correlation was also observed between total POPs or total TEQ and level of TRβ mRNA (ΣPOPs; R= 0.326, p=0.044, ΣTEQ; R= 0.345, p=0.039).

While we limited our studies to seals of a similar body weight (~age), the potential confounding influence of age on both PCB concentration and thyroid endpoints remained a concern. Subsequent regression analyses revealed that body weight did not correlate with TT_3, TRα, TRβ, total PCBs, or TEQ (results not shown). However, there were negative correlations between body weight and TT_4, FT_3 and FT_4. No correlation existed between FT_3 and PCBs, so we did not further evaluate this relationship. Multiple regression analysis showed that while both total PCB concentrations and body weight correlated with TT_4 and FT_4, PCBs were the primary exploratory variable in the observed thyroid changes, whereas body weight was not significant for TT_4 (PCBs: partial R = 0.71, p<0.001; body weight: partial R =0.27, p=0.08) or FT_4 (PCBs: partial R = 0.72,

p<0.001; body weight: partial R =0.33, p=0.14). There were no significant correlations between any of the endocrine endpoints and time of day for each capture (results not shown), suggesting that circadian rhythm did not unduly influence our results. Likewise, there was no correlation between time held (restraint) prior to release and any of the endocrine endpoints, suggesting that stress was not a factor (results not shown).

Table 3.8. Correlations between contaminant concentrations in seal blubber and circulating thyroid hormone concentrations and thyroid receptor gene expression from the lower blubber biopsy section for seals from QCS, Smith Island, and Gertrude Island.

	Serum Thyroid Hormone				Blubber	
	TT_4	FT_4	TT_3	FT_3	TRα	TRβ
ΣPOPs	-.749** (<.001)	-.682** (<.001)	-.135 (.540)	.002 (.994)	.698** (.001)	.326* (.044)
ΣPCBs	-.744** (<.001)	-.723** (<.001)	-.113 (.617)	.095 (.673)	.717** (.001)	.263 (.115)
ΣPCDDs	-.146 (.328)	-.020 (.895)	-.091 (.544)	-.154 (.303)	.284 (.080)	.105 (.516)
ΣPCDFs	.190 (.386)	.238 (.274)	-.046 (.836)	-.329 (.125)	-.331 (.154)	-.158 (.330)
ΣOC pesticides	-.448* (.032)	-.329 (.125)	-.131 (.552)	-.337 (.116)	.242 (.305)	.137 (.399)
ΣTEQ	-.585** (.004)	-0.528* (.011)	-.159 (.481)	.124 (.584)	.685** (.001)	.345* (.039)
PCB-TEQ	-.541** (<.001)	-.522** (<.001)	-.004 (.978)	.013 (.933)	.450** (.007)	.263 (.115)
PCDD-TEQ	-.111 (.614)	.015 (.818)	-.173 (.431)	-.237 (.276)	.057 (.813)	.172 (.469)
PCDF-TEQ	.178 (.235)	.290 (.654)	-.115 (.444)	.012 (.937)	-.084 (.604)	.032 (.846)

The correlation coefficients are shown with associated p values in parenthesis. Statistical significance is denoted by * (p<0.05) and ** (p<0.01).

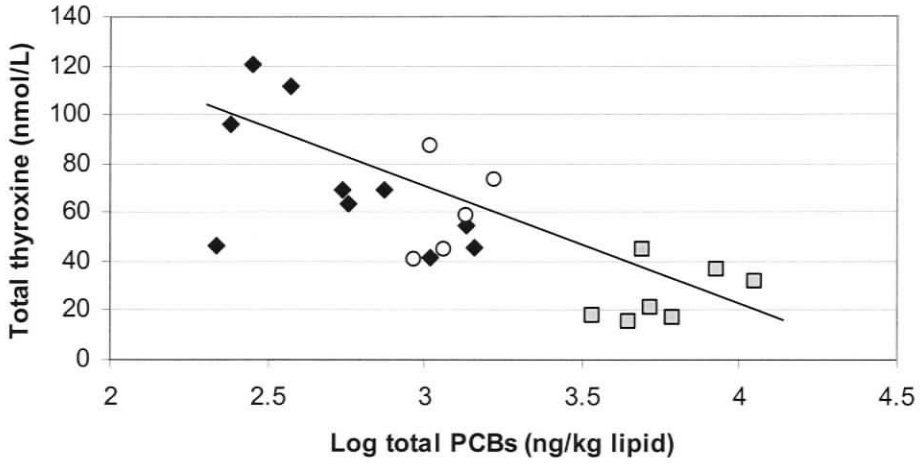

Figure 3.10. Correlation analysis of circulating total thyroxine levels with total PCBs measured in blubber of harbour seal pups from the British Columbia and Washington State. (Black-QCS, while-Smith Island, and Gray-Gertrude Island)

A significant negative correlation is noted. R= -0.744; p<0.01.

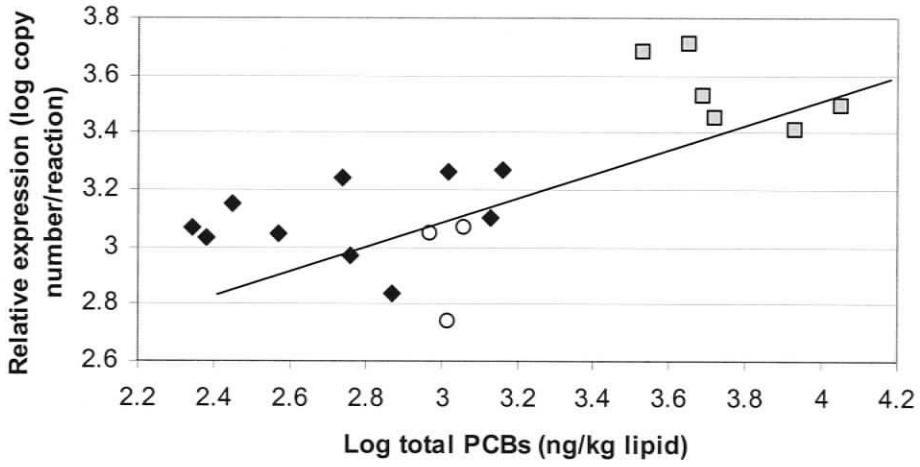

Figure 3.11 Correlation analysis of relative thyroid hormone receptor α mRNA expression in lower blubber with total PCBs from harbour seal pups from the British Columbia and Washington State. (Black-QCS, while-Smith Island, and Gray-Gertrude Island)

A significant positive correlation is noted. R= 0.717; p<0.01.

5. DISCUSSION

The extensive use of many persistent manmade chemicals has resulted in the contamination of the marine environment. Due to their long lifespan, apical position in the aquatic food web, and relative inability to metabolize POPs, marine mammals are prone to high contamination with POPs. Laboratory-based studies, captive feeding studies of seals, and studies of free-ranging marine mammals highlights the endocrine-disrupting nature of complex mixtures of POPs and many of their constituents (2). However, the mechanisms of action are often ill-defined in field studies since the presence of multiple POPs makes it difficult to sort out the roles of each individual chemical. On the other hand, it is important to study the effects in as wide a context as possible with the consideration of additive, synergistic, and antagonistic toxic effects of mixtures.

Hundreds of individual POP congeners were measured in this study. Due to their similarity in physical and chemical properties, such as lipophilicity and persistence, many POPs were correlated with each other. One of the dominant POPs, PCBs, are not only strongly correlated with total POPs, but also are correlated with the top three OC pesticides: DDT, chlordane and heptachlor. Thus, total PCB can be a useful indicator of 'contaminant' status on the harbour seals inhabiting the coast of BC and WA. Predominance of PCBs in blubber have been observed in other harbour seal populations and pinnipeds (150-152). Correlative evidence between PCB exposure and morbidity in marine mammals have been reported (88;90;137;138). By analyzing the correlation between total PCBs and biological or physiological parameters, the evidence for potential contaminant effect on harbour seals inhabiting in BC and WA might be provided.

Total POPs, mainly contributed by PCBs (more than 80%), and total TEQ were significantly higher in Gertrude Island (Puget Sound) seals compared to those sampled in the adjacent coastal waters of northern WA (Smith Island), and southern and central BC (Queen Charlotte Strait). The high ratio of PCBs detected in Gertrude Island seals seem to reflect that which remains from historical use of these industrial chemicals in Puget Sound areas in combination with its semiclosed geological nature (153). In fact, elevated levels of POPs, particularly PCBs, in sediments and fish (seal prey) have been documented in Puget Sound (154;155). Since harbour seals are non-migratory and reside within 20 km of their feeding areas, this result was certainly expected.

Moreover, the results were also consistent with previous contaminant studies in seals from the same study area in 1996 and 1997 (39). Surprisingly, in contrast to the predicted persistence of PCBs, total PCB concentration and total TEQ value were about one-third in seals captured in Puget Sound in 2003 compared to seals captured 6-7 years before. Although individual congeners contributing to total PCBs or total TEQ were very similar among the sampling locations, seals in Puget Sound contained higher chlorinated PCBs, which are also less volatile. Thus, they are more likely to attach to particles and be quickly deposited into sediments or into organisms, with less atmospheric transportation. It may suggest that the local PCB input in Puget Sound has been declining in the last several years, with a resultant reduction of PCBs in seals inhabiting in Puget Sound.

Elevated POP exposure has been associated with altered circulating vitamin A and immune function in harbour seals sampled from the same study areas (40;156). Finding a strong negative relationship between circulating TT_4 and total PCB concentrations or total TEQ in this study, contributes to the notion that PCBs represent a significant health

concern in Gertrude Island seals. These findings are consistent with previous observations of contaminant-related reductions in thyroid hormone concentrations in captive seals fed contaminated fish (87) and in free-ranging pinnipeds (88-90;139). Histopathological lesions, including fibrosis and colloid depletion, in thyroid glands of seals inhabiting PCB-contaminated areas (82) may explain reduced TH levels in our contaminated seals. Laboratory animal studies provide more information on possible mechanisms of action, where altered hormone synthesis in the thyroid gland, disrupted circulatory transport, and altered metabolic enzyme activity have been observed (4). Findings in this study suggest that the more contaminated seals from Gertrude Island may be considered hypothyroid (112), highlighting concerns about the health of high trophic level wildlife in this region.

Thyroid hormones play a critical role in regulating a wide range of physiological processes such as growth, development, and metabolism largely through binding to the nuclear receptors TRα and TRβ, and modulating their activity on TH-responsive gene promoters (55). In this study, TR mRNA was detected in a variety of tissue samples in harbour seal pups. This is evidence of broad TR activities in many important organs. In addition, the variation in relative abundance between TRα and TRβ indicates different functions of each tissue mediated by specific TRs. For example, TRα1 mRNA was predominantly expressed in heart (157) and its special importance for normal cardiac function was observed in significant reduction of heart rate in mice lacking TRα1 (158). Therefore, relatively high levels of TRα mRNA expression observed in the seal heart sample may indicate the common function of TRα in mammals. However, it is important to note that our results were from only one animal, and the levels of mRNA expression

may not correlate with receptor protein concentrations. Further research is necessary in order to characterize the abundance and functions of TR proteins in individual tissues in harbour seals.

Despite the physiological importance of TR action, laboratory studies have showen that PCBs are capable of direct effects on TR activity and TH-responsive gene expression (78;79). The observed differential relationship between TRα and TRβ transcripts in seal blubber samples relative to PCB levels may indicate a particular vulnerability of the TRα gene. This may be related to the apparent hypothyroidism observed in the more contaminated animals. Interestingly, increased TRα expression has been observed in the brains of hypothyroid compared to euthyroid rats (159). Positively TH-regulated genes were up-regulated in postnatal and fetal rat brains after *in utero* exposure to the PCB mixture Aroclor 1254 despite a reduction in the dam's circulating TH levels (72;73). These results suggest that PCBs may interfere directly or indirectly with TH signaling leading to changes in TH-responsive gene expression.

Altered circulating thyroid hormone levels in PCB-exposed marine mammals provide evidence of an effect on this endocrine endpoint. However, obtaining blood samples is not always feasible, and skin/blubber biopsies essentially represent the only obtainable samples for many marine mammals, including cetaceans. In addition, circulating TH levels can be influenced by a number of natural factors, and may not present a rigorous assessment of thyroid status. In this study, therefore, we investigated developing a gene expression biomarker approach using TR expression levels in blubber/skin biopsies in order to evaluate the utility of such an approach for harbour seals and other marine mammals. Using this biopsy-based sampling technique, it became possible to quantify

the expression of thyroid hormone receptor genes in blubber. Although TR expression was the only focus in this study, other emerging gene expression biomarkers could be examined in the same way. Expression of the aryl hydrocarbon receptor (AhR) or cytochrome p450 oxidase (CYP) as gene expression biomarkers in liver have already been suggested for marine mammals (160).

The positive correlation between blubber TRα and PCB concentrations in harbour seals suggests that contaminants either directly or indirectly affect this TH endpoint and may alter TH-regulated gene expression. The high degree of sequence conservation between harbour seals and other vertebrates accentuates the likely functional similarity of TRs between animal groups. While the results would suggest that PCBs affect systemic thyroid homeostasis in harbour seals, our detection of contaminant-related alteration of TR gene expression in blubber raises a toxicological concern of particular note for marine mammals. TH is known to play an important role in the maintenance and function of adipose tissue (161). T_3 treatment can induce adipocyte cell proliferation, fat cell cluster formation, lipid accumulation, and increased malic enzyme and glycerophosphate dehydrogenase activities in young rats as well as in preadipocyte cell lines (162;163). TRs within murine adipocytes predominantly comprise TRα, with little detectable TRβ isoform (164), consistent with our findings in blubber. Recently, several TH-regulated genes were identified in human and mouse adipose tissue that encode for protein products involved in lipid metabolism (165).

Blubber is a specialized adipose tissue layer under the skin of marine mammals, and is vital for energy storage, heat insulation, thermogenesis, and buoyancy control. Blubber is typically viewed as a storage depot associated with lipid reserves, within which lipids,

lipid classes and fatty acid profiles have been characterized in physiologic and energetic studies of marine mammals (31;166). However, blubber also represents an important storage site for micronutrients, holding as much as 66% of the body burden of vitamin A in harbour seals (141). The finding of TRα in blubber in this study indicates the possible function of TRα as a mediator of TH-dependent metabolism and homeostasis. Perhaps, contaminants might therefore present a risk to the structural and functional integrity of blubber, as metabolism within adipocytes may be altered. The influence of TH-related processes on body weight in laboratory animals and in humans (167;168) underscores the potential effects of a disruption of TR on such critical processes as energy storage and thermoregulation in marine mammals.

The biomarker-based thyroid assessment in this study may be applied to studies of other species for which blood samples are not available due to logistical constraints (e.g. cetaceans). The contaminant-associated decrease in circulating TH levels and concomitant up-regulation of TRα expression in blubber of harbour seals may indicate an increased risk of TH-dependent health effects, such as developmental abnormalities and neurotoxicity. In addition, altered TRα gene expression in blubber may have profound consequences for metabolic turnover and energetics in contaminated marine mammal populations.

Chapter 4

Conclusion and future directions

Thyroid hormones (THs) are critical for development and metabolism in all vertebrates. Because of their adaptation to living in an often cold aquatic environment, marine mammals also rely on THs for unique events, such as thermoregulation, molting, and fasting. However, laboratory studies have shown that some persistent organic pollutants (POPs) can disrupt thyroid economies in multiple pathways through multiple mechanisms. A reduction in circulating TH concentrations has been observed as a consequence of POP exposure in laboratory animals, captive seals, and wildlife species, including free-ranging harbour seals (4). Therefore THs have been touted as possible biomarkers of POP exposures in wildlife. More recent research on POP mechanisms of toxicity has focused on cell signaling pathways. Since some POP exposure-related changes in transcript and/or protein levels likely take place prior to physiological or pathological effects, the sensitivity and specificity of molecular markers support their application as novel biomarkers for POP effects.

Therefore, in this study we designed, verified, and utilized transcripts of thyroid hormone receptors (TRs) as novel biomarkers of POP effects using a minimally-invasive biopsy technique. In combination with the measurement of circulating thyroid hormones, this study evaluated thyroid physiology in free-ranging harbour seals from multiple sites in British Columbia and Washington State. This represents the first study in which TRs were partially sequenced in a marine mammal and their expression levels quantified in peripheral tissues. We observed a negative correlation between total/free T_4 in circulation and total POP concentrations measured in blubber. In contrast, there was a positive correlation between blubber TRα expression and POP concentrations.

PCBs were the dominant contaminants found in all seals in our study and they were correlated with the concentration of total POPs and the major OC pesticides: DDT, chlordane and heptachlor. Laboratory studies have shown that PCBs are able to interact directly, as well as indirectly, on thyroid hormone signaling pathways (71-73;76-79). Therefore, a positive correlation between blubber TRα and PCB concentration, concomitant with a negative correlation with circulating thyroxine observed in this study, suggest that a complex POP mixture, consisting largely of PCBs, is at the origin of our observed TH/TR disruption.

Biomarker-based studies of wildlife populations can be confounded by natural factors (169). We therefore examined THs in developing/nursing seals in order to better understand the possible effect of growth and development on this endocrine endpoint. In our developmental study, we observed constant but rapid weight gain in nursing harbour seal pups due to the consumption of fat rich milk and the consequent deposition of fat into blubber layers, described more fully elsewhere (37). The highest circulating concentrations of THs were observed at birth with gradual decline toward more constant levels. There was a negative correlation between total thyroxine (TT_4) or free thyroxine (FT_4) and body weight. In addition, concentrations of POPs change during the course of the nursing period, as pups ingest fat-soluble chemicals delivered through mother's milk (170). These results formed the rationale to restrict the capture and sampling of seals for our biomarker study to those aged between 3.5 to 5 weeks, when TH levels were found to be relatively constant. We also statistically evaluated the relationship between body weight and thyroid parameters and/or POP concentrations, and demonstrated that body weight (age) did not explain TH and TR variance.

This study raises a number of concerns. Seals inhabiting the highly contaminated Gertrude Island area in southern Puget Sound had significantly lower total and free TH levels, perhaps indicating hypothyroidism. The functions of THs are numerous and involve almost all cells within an organism, regulating protein, lipid and carbohydrate metabolism, and proper differentiation, development and growth. Therefore, POP-related reduction in THs may alter these functions. Particularly, the developmental neurotoxicity of PCBs, including abnormal cognitive and motor responses, delayed somatic and reflexive development, and permanent hearing loss have been attributed to hypothyroidism conditions (4). Therefore, among the sites visited, Gertrude Island pups are especially susceptible to such effects associated with thyroid toxicity. While the measurement of circulating TH levels is important in documenting the effects of POPs, the impact would likely be in the TH responsive target tissues since THs act through the interaction with TR at their target tissue. In target tissues, much of the T_4 is converted to active triiodothyronine (T_3) and so it may more appropriate to assess the POP exposure through target tissues. Moreover, THs may show different physiological effects dependent upon their molecular conditions, such as differences in TR isoforms and transcription factors. In addition, POPs may disrupt thyroid hormone signaling pathways without changing circulating TH levels. Since the physiological significance of TR in each target tissue is being clarified through TH (TR mutation)-resistant and genetically modified TR mice and cDNA microarray during recent years, the assessment of a POP effect in TH target tissue has only become recently possible.

Even though the majority of POPs are stored in the blubber of marine mammals, toxicological risk within this compartment has been largely overlooked because blubber

is generally viewed as a storage tissue with low metabolic activities. This has led to a possibly erroneous view that blubber POPs are inert. Concerns have therefore focused on the mobilization of blubber and redistribution of POPs from blubber through circulation into "target tissues" where POPs can cause endocrine disruption with interacting receptors. However, in this study, POP exposure-related alteration of TR gene expression was observed in the blubber of young harbour seals. Therefore, POPs may disrupt endocrine processes as a direct consequence of storage in the blubber, forcing us to view blubber as metabolically active "target tissue" rather than simply an inert storage compartment.

Blubber is unique adipose tissue found only in marine mammals, where its functions include energy storage, heat insulation, thermogenesis, and buoyancy control. TH status influences adipose tissue development, differentiation and metabolism (163). Particularly, THs stimulate the utilization of lipid substrates to increase mobilization of triglycerides stored in adipose tissue (171). Moreover, recent cDNA microarray experiments revealed the expression of genes encoding proteins involved in lipid metabolism, regulated by T3 in mature adipocytes (165). Since THs are highly conserved hormones among mammals, THs likely also have similar roles in blubber structure and function, and lipid metabolism in marine mammals.

An altered TH system may present a critical problem for young marine mammals in particular. Harbour seal pups are born with a thin layer of blubber, requiring more energy for thermoregulation. Our developmental study showing that elevated TH levels in young harbour seals consistent with the high demand of THs for energy metabolism. Our observation of a POP-related reduction in circulating THs and elevated levels of TRα in

blubber of highly contaminated Gertrude Island animals indicates the possible disruption of lipid metabolism. Interestingly, a recent study showed that transgenic mice lacking the TRα gene product had significantly lower body temperature than that of the wild type (172). Thus, the elevated levels of TRα found in highly contaminated seals may have reflected a compensatory mechanism for low levels of TH, and helped to accommodate for the demand for thermoregulatory demands through lipid metabolism. Alternatively, since blubber is the major storage site for POPs, elevated TRα observed in Gertrude Island seal blubber may be the result of a direct interaction of POPs and the TH signaling pathway.

However, to understand the toxic mechanism of altered thyroid hormone physiology in this study and its impacts on the health of marine mammals, further studies on mechanistic (cause and effect) toxicological studies, the nature of marine mammal blubber, and its relation to endocrine factors are clearly required. In this study, levels of TR mRNA were measured, but this may or may not correlate to TR protein expression, the latter of which is directly related to the functionality of the receptors. Future studies could help to confirm TR protein levels in the same blubber samples, in which TR mRNA and contaminants were measured.

Since it is difficult to obtain sufficient samples from live marine mammals, it is important to maximize the use of available samples. Since TH has been measured in fish and rodent tissue (173;174), we could adapt such methods for application to our seal samples. POPs (mainly PCBs) seem to have both direct (interference on TH signaling pathway) and indirect (based on hypothyroid or hyperthyroid condition) effects on thyroid hormone mechanisms of action. Therefore, measuring levels of THs in blubber

tissues could provide mechanistic explanations of elevated TR levels in Gertrude Island seals.

The assessment of POP effects using thyroid biomarkers and non invasive sampling methods may be useful in the study of other wildlife species, including cetaceans. Some cetaceans have much higher POP levels due to their longevity, trophic level, and inability to metabolize certain POPs (175). Cetaceans also contain larger amounts of blubber (up to 50% of body mass in large cetaceans). Thus, measuring TRα expression and perhaps some genes which are regulated by TH in blubber may provide important information on the heath effects of environmental contaminants. This study highlights a contaminant-related disruption of thyroid economy in free-ranging harbour seals, which may have serious consequences for energetics, metabolism, and ultimately, survival.

Toxicological studies of free-ranging marine mammals provide an overview of the effects of complex mixtures of environmental contaminants, but suffer from a lack of mechanistic insight. Future research could elaborate on mechanistic linkages, including carefully-controlled single chemical exposures in laboratory rodents and *in vitro* exposure using tissue culture would add to the information gleaned here from studies of free-ranging harbour seals.

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