Halogenated (Phenolic) Contaminants and Complex Mixtures in Model Gull Species and Competitive Binding Interactions With Major Thyroid Hormone Transport Proteins

By

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Abstract

Herring gulls *(Larus argentatus)* are suitable avian bio-monitors of ecosystems. No studies exist examining the interaction between organohalogen contaminants (OHCs) and thyroid hormones (THs) in birds from the Great Lakes. Polychlorinated biphenyl (PCB), polybrominated diphenyl ether (PBDE) flame retardant congeners are environmentally relevant OHCs, and as well as some of their hydroxylated (OH) analogues and recently perfluorinated compounds (PFCs) have been reported to have effects on THs-dependent processes.

TH transport proteins albumin (ALB) and transthyretin (TTR) from livers and brain of herring and glaucous *(Larus hyperboreus)* gulls were isolated, cloned, sequenced, purified and expressed, and used for competitive binding assays (CBAs) *in vitro* and compared with human ALB and TTR.

This is the first report on circulating £OH-PCB, £OH-PBDE, £PBDE and £MeO-PBDEs in herring gulls plasma and livers from the Laurentian Great Lakes. Results obtained shows that human ALB and TTR had higher preference for T_4 relative to T3 whereas it was reversed for gull recALB and recTTR. Complex neutral chemical fractions isolated from plasma had no effects, but phenolic fraction disrupted ($p>0.001$) the binding of human TTR with T_4, human ALB with T_4, and the gull TTR with T_3.

CBA with recALB and recTTR showed that relative to 2,2',4,4'-tetrabromoDE (BDE47) and 2,2',3,4',5,5',6-heptaCB (CB187) and the MeO-substituted (4-MeO-CB187 and 6-MeO-BDE47) analogues, 4-OH-CB187, 6-OH-BDE47 and 4'-OH-BDE49 had greater affinity than T3 or T_4. Also, Perfluorooctanesulfonamide (PFOSAJ had effects of human TTR binding of T_4; perfluoroundecanoic acid (PFUdA) had a disruptive effect on human and gull TTR binding to T_4, but no effect with T3. This is also the first report of PFCs in livers of herring gulls from the Great Lakes.

The combination of the more TH-like brominated diphenyl ether backbone (relative to the chlorinated biphenyl backbone), and having an OH-group, results in a high competitive ligand on gull Albumine and TTR relative to T3 and T_4. While in PFCs the carboxylated polar head appears to have higher affinity for Albumin than T_4 and T_3.

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Ottawa, November 2009.
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List of Publications and Conference Abstracts associated with Thesis.

Ucan-Marin. F, Arukwe A, W.A. Gebbink, Letcher RJ Perfluoroalkyl Compounds (PFCs) and PFC-containing complex mixtures isolated from herring gull liver (Lake Ontario): Comparative *in vitro* competitive binding with thyroid hormones on gull and human albumin and transthyretin transport proteins. *SETAC* North America 30th Annual Meeting, 19-23 November 2009, New Orleans, USA.

Ucan-Marin. F, Arukwe A, Mortensen A, Gabrielsen GW, Fox, G, Letcher RJ. Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species *(Larus argentatus* and *Larus hyperboreus). Toxicological Sciences* 107(2), 440-450 (2009).

Ucan-Marin. F, Arukwe A, Mortensen A, Gabrielsen GW, Fox, G, Letcher RJ. Recombinant albumin and transthyretin transport proteins from two gull species and human: Chlorinated and brominated contaminant binding and thyroid hormones. *(Environmental Science and Technology,* Article ASAP Publication Date (Web): December 3, 2009).

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Ecotoxicology, October 22, 2007. Second Mexican Conference of Graduate Students and Researchers in Canada, CEIMEXCAN Quebec City QC.

Ucan-Marin F, Arukwe A, Fox GA, Gabrielsen GW, Letcher, RJ., (2007). Cloning and Purification of thyroid transport protein transthyretin (pre-albumin) from *Larus argentatus* and *Larus hyperboreus* top avian predator models; and their competition binding potency on selected Organophenolic compounds. SETAC Europe 17th Annual Meeting, Porto, Portugal, 20-24 May 2007.

Ucan-Marin, F., Arukwe, A., Fox, G.A., Gabrielsen, G.W., and Letcher, R.J., (2006). Thyroid hormone transport in Great Lakes herring gull and Svalbard glaucous gull: Competitive transthyretin (TTR) binding of selected organohalogens. 26th International Symposium on Halogenated Persistent Organic Pollutants 2006, Oslo, Norway, August 21-25, 2006

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List of Abbreviations

AhR	Aryl hydrocarbon receptor
APPI	Atmospheric pressure, photo-ionization
BAT	Brown adipose tissue
CHL	Chlordane compounds
CNS	Central nervous system
CPs	Chlorinated paraffins
DDT	Dichlorodiphenyltrichloroethane
DDE	Dichlorodiphenyldichloroethylene
DNA	Deoxyribonucleic acid
DHEA	Dehydroepiandrosterone
ECNI	Electron capture negative ionization
EC-NWSB	Environment Canada's National Wildlife Specimen Bank
EDC	Endocrine disrupting compounds
EI	Electron impact
EROD	Ethoxyresorufin-O-deethylase
ESI	Electrospray ionization
FOSAs	Perfluorooctane sulfonamides
FTOHs	Fluorotelomer alcohols
FTUCAs	Fluorotelomer unsaturated carboxylic acids
GH	Growth hormone
GLHGMP	Great Lakes Herring Gull Monitoring Program
HBB	Hexabromobenzene
HBCD	(a)-hexabromocyclododecane
H-P-PG	Hypothalamic-pituitary-peripheral gland
HPT	Hypothalamic-pituitary-thyroid axis
HCH	Hexachlorocyclohexanes
HCB	Hexachlorobenzene
HH	Hamilton Harbour
His-Trap-FF	Histidine-tagged Protein Purification
GC-MS	Gas chromatography-mass spectrometry
MeO	Methoxylated
MeS02-CB	Methylsulfonyl PCB
$MeS0_2$-DDE	Methylsulfonyl DDE
mRNA	Messenger ribonucleic acid
MRM	Multiple reaction monitoring
NCBI	National Center for Biotechnology Information
NTNU	Norwegian University of Science and Technology
LN2	Liquid nitrogen
PBDEs	Polybrominated diphenyl ethers
PCA	Principal Component analysis
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-p-dioxins
PCDF	Polychlorinated dibenzofurans

PCR	Polymerase chain reaction
PFCs	Perfluorinated Compounds
PFCAs	Perfluoroalkylcarboxylates
PFOS	Perfluorooctane Sulfonate
PFSAs	Perfluoroalkylsulfonates
P450	Cytochrome P450
POP	Persistent organic pollutants
PBT	Pentabromotoluene
recALB	recombinant albumin
recTTR	recombinant transthyretin
RBP	Retinol-binding protein
rT_3	3,3'5'-triodo-L-thyronine
T_4	thyroxine
T_3	3,3',5-triiodothyronine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalency factor
TEQ	Dioxin toxic equivalent
THs	thyroid hormones
THBPs	Thyroid hormone binding proteins
TRH	Thyrotropin-releasing hormone
TRa	Thyroid receptor alpha
TRP	Thyroid receptor beta
TSH	Thyroid stimulating hormone
TT_4	Total Thyroxine
TT_3	Total Triiodothyronine
OH	Hydroxylated
OC	Organochlorines
OHCs	Organohalogen contaminants
SB	Scotch bonnet Island
UDPGT	Uridine diphosphoglucuronosyl transferase activity
4-OH-HpCS	4-OH-heptachlorostyrene

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CHAPTER ONE

General Introduction

1.1 Endocrine system in Vertebrates

The endocrine system is a complex network of glands and hormones that regulates many of the body's functions; including growth, development and maturation, as well as the way various organs operate. The endocrine glands - including the pituitary, thyroid, adrenal, thymus, pancreas, ovaries, and testes - release carefully-measured amounts of hormones into the bloodstream that act as natural chemical messengers, traveling to different parts of the body in order to control and adjust many life functions (NRDC 2009).

Vertebrates (Phylum Chordata: *Vertebrata)* are separable into at least seven discrete classes that represent evolutionary groupings of related animals with common features. The class Agnatha, or the jawless fish, is the most primitive group. Class Chondrichthyes and class Osteichthyes are jawed fishes that had their origins, millions of years ago, with the Agnatha. The Chondrichthyes are the cartilagous fishes, such as sharks and rays, while the Osteichthyes are the bony fishes. Familiar bony fishes such as goldfish, trout, and bass are members of the most advanced subgroup of bony fishes, the teleosts, which developed lungs and first invaded land. From the teleosts evolved the class Amphibia, which includes

frogs and toads (EBO 2009). The amphibians gave rise to the class Reptilia, which became more adapted to land and diverged along several evolutionary lines. Among the groups descending from the primitive reptiles were turtles, dinosaurs, crocodilians (alligators, crocodiles), snakes, and lizards. Birds (class Aves) and mammals (class Mammalia) later evolved from separate groups of reptiles. Amphibians, reptiles, birds, and mammals, collectively, are referred to as the tetrapod (four-footed) vertebrates. The endocrine system of the tetrapod vertebrates is the product of millions of years of evolution, and consequently the endocrine glands and associated hormones of the human endocrine system have their counterparts with more primitive vertebrates. By examining these animals, it is possible to document the emergence of the hypothalamic-pituitary-target organ axis, as well as many other endocrine glands, during the evolution of fishes that preceded the origin of terrestrial vertebrates (EBO 2009).

The hypothalamic-pituitary-peripheral gland (H-P-PG) endocrine (see Glossary) system (Figure 1) couples the vertebrate central nervous system (CNS) to peripheral organs (Norris 1997; Weichert 1953). A wide variety of ligands from the hypothalamus in the CNS regulate the release of hormones from the nearby pituitary gland. These hormones regulate the production of other hormones by the peripheral endocrine glands. Feedback loops complete the network of communication between the tissues of the system. The H-P-PG system regulates growth, metabolism, reproduction and response to stress.

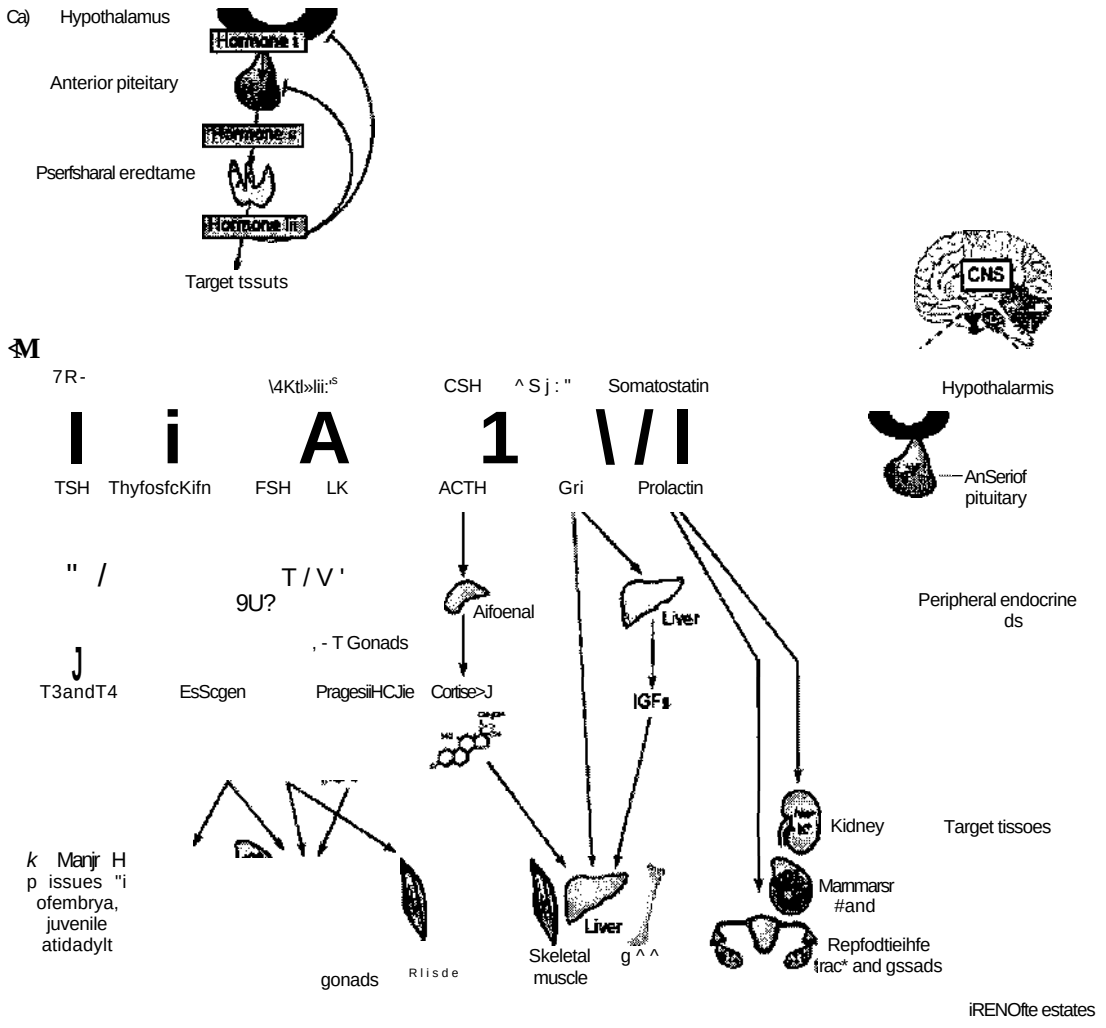

Figure 1. (a) Hypothalamic-pituitary-peripheral gland (H-P-PG) system. The vertebrate H-P-PG system generally shows three levels of ligand-receptor systems. Ligands that are produced by the hypothalamus (i) in the central nervous system (CNS) regulate the release of hormones from the nearby pituitary gland (ii). Pituitary hormones regulate the production of other hormones by peripheral endocrine glands (iii). Hormones from the peripheral endocrine glands can in turn influence functions of the hypothalamus and pituitary, forming positive or negative (shown in the figure) feedback loops, (b) Examples of vertebrate H-P-PG system connection to target tissues. The H-P-PG system coordinates physiological functions of diverse tissues in response to internal and external environments. Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone; FSH, follitropin; GH, growth hormone; GRF, growth hormone releasing factor; GnRH, gonadotropin releasing hormone; IGF, insulin-like growth factor; LH, lutropin; TRH, thyrotropin-releasing hormone; TSH, thyrotropin; T3, triiodothyronine; T4, tetraiodothyronine (thyroxine) (Source: Campbell et al, 2004).

Each of these physiological functions is regulated by a subset of H-P-PG ligands and receptors. This system appears highly conserved across vertebrates (i.e. from fish to mammals). Although the general scheme of linking neural and endocrine systems is also seen in invertebrates, there appears to be little conservation of the vertebrate H-P-PG tissues, ligands and receptors in invertebrates (Adams et al 2000).

1.2 Avian Thyroid System

1.2.1 Thyroid Function

The vertebrate thyroid gland secretes two hormones, thyroxine (T4), and triiodothyronine (T_3) that are important for development and metabolism. T_4 is the most abundant hormone, whereas T_3 is the most active with respect to its function in biological systems (i.e., main hormone that binds to nuclear receptor). The thyroid gland is under the control of the hypothalamic-pituitary-thyroid (HPT) axis. Thyrotropin-releasing hormone (TRH), from the hypothalamus, stimulates the release of thyroid stimulating hormone (TSH), from the anterior pituitary, which in turn causes the thyroid gland to produce and release hormone. TH feedback to the anterior pituitary regulates the control of TSH production, as well as TRH release, which is also modulated by feedback from TSH (McNabb 1992). TSH stimulation of the thyroid gland increases when circulating concentrations of thyroid hormones (THs) are low. Although small amounts of T3 are produced by the thyroid gland, most of the T3 in the body is produced by deiodination of T_4. Deiodination of T_4 occurs when iodine atoms are removed from either the

phenolic (outer) or tyrosyl (inner) ring. In 5' -deiodination (5'D), iodine is removed from the outer ring of T_4 yielding T3 (the most active hormone), and in 5-deiodination (5D), an iodine atom is removed from the inner ring, yielding rT_{3i} which is inactive (Figure 2 and 3). These products can then be deiodinated further to yield diiodothyronines (T_2) (other inactive form). There are three types of deiodination (D) pathways: type 1, type 2, and type 3. Type 1 (D1) deiodination provides a circulating source of T_3 to peripheral tissues. This type of deiodination is found mainly in the thyroid, liver, and kidney. Hyperthyroidism causes D1 to increase and hypothyroidism causes it to decrease, (i.e. the enzyme activity is substrate driven). Type 2 deiodination (D2) occurs in the brain, pituitary, and brown adipose tissue (BAT). In the brain and pituitary, T3 produced by D2I remains largely within the tissue where it is produced, maintaining the intracellular T_3 supply at euthyroid levels despite hypothyroid conditions in the rest of the body. In mammals, when T3 is limited, BAT type 2 5'D releases T3 to peripheral tissues, providing a circulating source of T3. In a hyperthyroid state, D2 decreases its activity. Type 3 deiodination (D3) involves removal of an iodine atom from the inner ring of iodothyronines (Figure 2). It is a major route for T3 degradation and inactivation of T4, producing rT3 (Leonard and Koehrle, 1996), consequently the role of transporters and deiodinases in the regulation of intracellular TH levels and, thus, in TH metabolism and action (Friesema et al, 2005) (Figure 3).

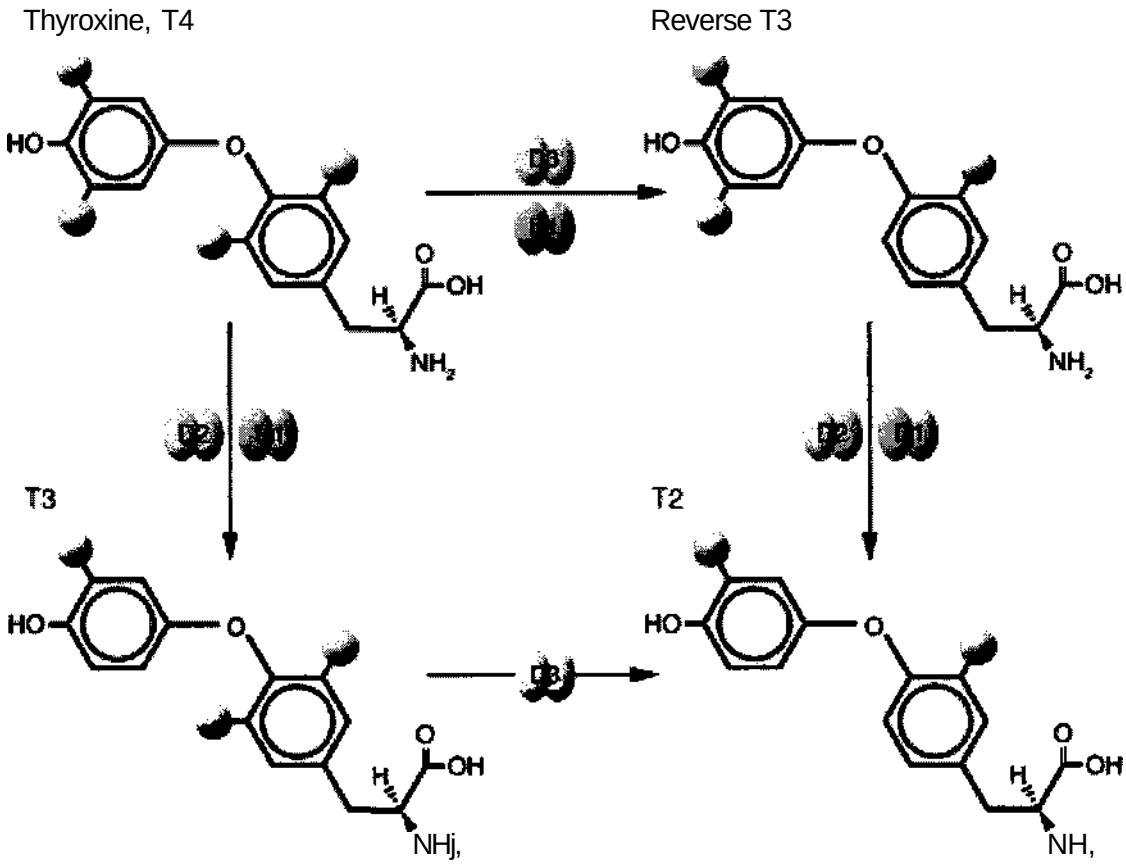

Figure 2. Basic deiodinase reactions. The reactions catalyzed by the deiodinases (D) remove iodine moieties (blue spheres) from the phenolic (outer rings) or tyrosil (inner rings) rings of the iodothyronines. These pathways can activate T4 by transforming it into T3 (via D1 or D2) or prevent it from being activated by converting it to the metabolically inactive form, reverse T3 (via D1 or D3). T2 is an inactive product common to both pathways that is rapidly metabolized by further deiodination (Bianco and Kim, 2006).

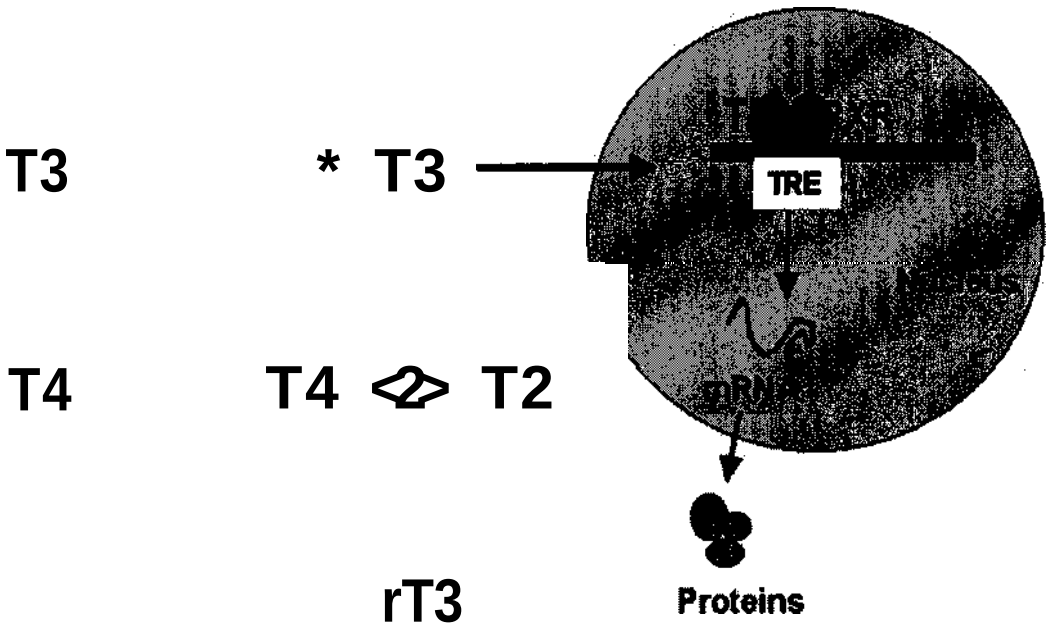

Figure 3. Role of transporters and deiodinases in the regulation of intracellular thyroid hormone levels and, thus, in thyroid hormone metabolism and action (RXR, retinoid-X receptor; TR, thyroid hormone receptor; TRE, thyroid hormone response element; D, deiodinase) (Friesema et al, 2005).

Binding proteins transport THs in the blood and ensure sustained TH supply to tissues despite fluctuations in thyroid gland function. They are present in different concentrations, with different numbers of hormone binding sites depending on the species. TTR, the major binding protein in mammals, binds to T4 with greater affinity than T_3, and the amount of hormone bound greatly exceeds the amount of free hormone in circulation. TH transport proteins play important roles in the availability and distribution of THs in the extracellular and intracellular compartments, and thus in regulating TH metabolism and kinetics (McNabb 1992) (Figure 3).

THs in birds regulate metabolic heat production (thermoregulation), growth, body weight, development of central nervous system, cell differentiation and maturation, hatching, molt, and reproduction (McNabb 2000; Merryman and Buckles 1998a, 1998b). Iodine, an essential element for TH synthesis, is stored in excess as iodide from dietary uptake (McNabb 1992). Avian THs are carried in the plasma bound to transport proteins, that is, albumin or TTR. Bird T_4 has been associated mostly with albumin, which has low-affinity binding sites with little specificity for T_4 or T_3 compared with TTR in mammals (Astier 1980; Davidson et al 1978; Merryman and Buckles 1998a). Factors that influence thyroid functions include dietary iodine (I-) availability, activity, ambient temperature, photoperiod, body condition, seasonality, and age (McNabb 2000).

1.2.2 Avian Thyroid Development

Avian developmental patterns range from chicks that are independent at the time of hatching (precocial) to those that are completely dependent on parental care after hatching (altricial). Precocial birds, like domestic chickens and Japanese quail, have their eyes open, are covered with down feathers, are capable of locomotion and self-feeding at hatching and show metabolic responses to cooling starting from time of hatching. In contrast, altricial birds as pigeons, sparrows and starlings, have closed eyes, lack down, are incapable of locomotion and are completely dependent on parental care for some period after hatch (Starck and Ricklefs 1998).

Altricial birds have relatively immature thyroid function at hatch. Their thyroid function increases post hatch and stabilizes at about the time when they develop thermoregulation (McNabb and Cheng 1985). TH concentrations are low at hatching and do not increase until several weeks after hatching, these different patterns of avian thyroid development have implications for the time during which chemical pollutants are likely to alter thyroid function and cause other downstream effects on morphology and physiological development (McNabb and King 1993). The thyroid glands are controlled by the HPT axis for precocial birds by mid incubation/gestation and after hatching for altricial birds (Thommes 1987; Freeman and McNabb 1991).

There is a lack of information about herring gulls and glaucous gull regarding their thyroidogenic system; and more studies are necessary to improve the knowledge of the mechanistic interactions between the TH transport proteins and THs in birds.

1.2.3 Deiodination of Thyroid Hormones

At hatch, the chicks of various species of birds differ markedly in the degree of maturation of many aspects of their behavior, physiology, and anatomy. Variation among taxa in these developmental characteristics has led to the separation of avian species into precocial and altricial developmental types. The two main groups of hormones involved in growth in birds are growth hormone (GH)

together with the associated insulin-like growth factors, and T4 and T_3. Normal growth of post hatching birds requires GH, T4 and T_3 (Decuypere et al 2005).

TTR and albumin are the most important transport proteins of THs; however there are few studies that provide evidence of the binding properties and the specific targets (T4/T3) for transport proteins. TTR belongs to a group of proteins, which includes TBG and albumin, which binds to and transports THs in the blood. TTR is also indirectly implicated in the carriage of vitamin A through the mediation of retinol-binding protein (RBP) (Decuypere et al 2005). Hormonal control of avian growth has almost exclusively been studied in poultry species. More work on wildlife birds are necessary, to confirm our current views of hormonal control of developmental differences and the role of hormone protein carriers. The present thesis examines the hormonal control of growth in several species of the genus *Larus,* all of which are not domesticated birds.

The generally accepted model of THs action assumes that T3 is the primary hormone and that the principal function of T_4 is to serve as a precursor of T3 in the deiodination of T4 by iodothyronine deiodinases (Oppenheimer 1983). The hypothesis holds that T_3 is bound to nuclear receptors (T3-R) with greater affinity than T4 and that an interaction of the hormone with the receptor initiates a cascade of nuclear events that results in the augmentation or inhibition of expression of those genes to which the T3-T3R complex binds. One of the benefits derived from the peripheral conversion of T4 to T3 is that the slower

fractional turnover of T_4 compared with that of T_3 helps to stabilize the level of circulating T_3. The level of serum T_4 plays an additional role in THs homeostasis. In states characterized by low concentrations of serum T4, such as that resulting from iodine deficiency, type II iodothyronine 5'-deiodinase activity rises in the brain and liver, resulting in enhanced conversion of T4 to T3, and, thus partially compensating for the diminished serum (Leonard and Koehrle 1996). The interrelation of T_4 and T3 in mediating the effects of THs has assumed particular importance in the case of brain development. The brain is especially rich in type D2 iodothyronine 5'-deiodinase (Leonard and Koehrle 1996). The demonstration that augmented type II 5'-deiodinase activity in brains of hypothyroid animals serves to preserve the level of intracellular T_3 strongly, suggests that the interaction of T3 with specific nuclear receptors is a critical step in mediating thyroid hormone action in the brain (Leonard and Koehrle 1996).

1.3 Birds as Bioindicators of POPs

1.3.1 Introduction

Considerable attention has been paid in recent years in understanding the transport, fate and distribution of the persistent organic pollutants (POPs) as they are found in wildlife all over the world including those from supposed pristine areas (e.g. Auman et all997; Muir et al 1999; Guruge et al 2001; Wyk et al 2001; Kunisue et al 2002; Tanabe 2002; Verboven et al 2009). An elaborate review by Elliott and Harris (2001/2002), examining the consensus among scientists, that

dichloro-diphenyldichloroethane (DDT) and other contaminants such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), Polychlorinated dibenzofurans (PCDFs) and other organochlorines (OCs) pesticides have contributed to the reduced nest success and population decline in bald eagle populations, did help to focus precisely on the susceptibility of birds from a toxicological point of view. As it is evident to researchers in this field, these chemicals are ubiquitous and present virtually in all the wildlife tissues. As a result of this, a dramatic increase in public concern about the state of the POPs contaminations has occurred in recent years, in relation to growing evidence that POPs cause severe toxicological impacts on animals.

A solution to the problems posed by POPs on a global level must start from monitoring these substances in a wide range of ecosystems. In order to monitor POPs, their environmental consequences and to assess ecosystem health it is necessary to use effective bioindicator species, which accumulate POPs from corresponding areas. An elevated number of published information on pesticide residues in birds is available indicating serious reproductive impairment in some birds, many of which had become endangered species (e.g. black-backed gull, *Larus fuscus)* (Ove Bustnes et al 2006). Biologists studying declining populations of predaceous birds had found relationships between dichlorodiphenyldichloroethylene (DDE) in bird tissues and egg-shell diameter decrease, but no studies had determined the exact cellular and genetic

mechanism of how DDE might be responsible for reproductive failure and population declines (Peakall and Kiff 1988).

Similar to marine mammals, fish-eating birds have been adversely affected by POPs contamination. The reduced capacity to detoxify (Tanabe 2002) and higher exposure via dietary intake enable some seabirds to accumulate high levels of PCBs (Walker et al 1984). Among the avian species (e.g. bald eagles, *Haliaeetus leucocephalus),* fish eating birds show serious teratogenic and reproductive dysfunctions because of high PCB accumulation (Tillitt et al 1992; Yamashita et al 1993) and most of the effects were generally associated with the dioxin-like planar PCBs (Guruge at al 2000).

Ratcliffe (1967) discovered that shells of raptor eggs from England weighed 18.9% less than they did before the dichlorodiphenyltrichloroethane (DDT) era began, which explained why the shells broke during incubation. Later, Hickey and Anderson (1968) found a direct inverse correlation between the DDE residues in eggs and the shell thickness in peregrines, bald eagles and osprey. However, Parslow and Jefferies (1977) demonstrated that for any particular bird species the amount of eggshell thinning is closely and linearly correlated with the DDE level in the egg and is not influenced by the levels of other pollutants such as dieldrin or PCBs (Furness et al, 1993). The authors also stated that once such a relationship is established one could use the very easily measured eggshell thickness of regularly sampled eggs to monitor the level of DDT pollution in a

region. Some argued that DDE was not responsible for eggshell thinning and population decline in birds (Edwards 1972; Beatty 1973), but the positive response of birds to the ban on DDT in USA proved these criticisms wrong (Peakall, 1990). Beyer et al (1996) reviewed the problem assessments supported by residue analyses in birds, in their book *Environmental Contaminants in Wildlife - Interpreting Tissue Concentrations.* Contaminants that are identified include organochlorine insecticides, polychlorinated biphenyls, heavy metals, dioxins, etc. The conclusions reached in these studies provided a basic perspective on the occurrence and effects of pollutant contamination on wildlife.

Bans and restrictions imposed by several countries have reduced domestic contamination and the situations have improved in some domestic bird populations by reducing the exposure of resident wild birds and their prey. However, the problem existed in a different way, at least in the case of birds. The migratory species of both prey and predator birds often are exposed elsewhere during their travels (Ramesh et al 1992; Tanabe et al 1998; Muir et al 1999; Tanabe 2002; Kunisue et al 2003). Residue analysis again helped to define the nature and locations of migrant exposure. For example, Henney et al (1982) found that the peregrine falcons accumulate DDE in their bodies during their annual migration to Latin America. Migratory birds of the Arctic are the best-documented examples of such a contamination. Muir et al (1999) has stated that the birds of the Arctic, which breed in the north and over-winter in more temperate and industrialized latitudes, contain higher levels of OCs than the birds

that over-winter in the north. Levels of contamination are taken up by feeding on the overwintering grounds and transported north each spring when the birds migrate back to their breeding grounds.

The seabird eggs have also been shown to be an efficient, conservative tool for monitoring OC levels in the marine environment (Gilbertson et al 1987; Oxynos et al 1993; Braune et al 2001). The authors compared the levels of various OCs like DDE, PCB, HCB, oxychlordane etc. in the eggs of different birds occurring at the Arctic Circle and found a declining trend in the concentrations of these chemicals from the mid-1970s to late 1980s. In fact, as stated earlier, the toxic effects by POPs on birds have been first detected in bird eggs (Moore and Ratcliffe 1962). Later Peakal (1974) extracted remnant lipids from museum eggs and showed that DDE was present in the peregrine eggs collected in 1948.

The contaminant residue studies in birds and bird eggs can help to define problems such as mortality, behavioral aberrations, eggshell thinning, reproductive failure and population decline in birds and also the resultant exposure of humans to these contaminants, because many of such birds make important food items to humans. This may raise toxicological concerns for both wildlife and mankind, based on historical and ongoing trends in the use of organochlorines. In this aspect the necessity of continual monitoring and surveillance of these substances in birds is recognized.

Unlike in the case of developed countries (e.g. Pain et al 1999; Guruge et al 2000; Guruge et al 2001; Sakamoto et al 2002; Helgason et al 2008; Verboven et al 2009; Elliot et al 2009) data on the pollutant loads from the developing countries of Asia, South America and Africa are very much limited (Goldstein et al 1996; Lacher et al 1997; Senthilkumar et al 1998; Tanabe et al 1998; Wyk et al 2001; Minh et al 2002; Kunisue et al 2003; Bouwman et al 2008) and hence collection of data on the resident and migratory birds from the developing nations has become mandatory. At the same time the possibility of the usage of bird species as bioindicators of POPs pollution in respective regions may be justified for several reasons such as their high mobility, easy susceptibility to pollutants, excretion via several routes, etc.

1.3.2 Factors affecting POPs accumulation in birds

The life of birds is regulated by opportunities for finding food and avoiding predators. They have to face drastically different conditions of life between summer and winter. Without much exception, all birds have at least a small range of migratory territory (Alerstam 1993). Avian species are useful bioindicators for monitoring OC contamination of the environment, because they are often at relatively high position in the food chain. The resident birds, which principally have localized feeding and breeding grounds throughout the year may reflect the background pollution of the inhabiting area through the levels of contamination in their bodies (Kunisue et al 2003).

Henny and Blus (1986) reported that the individuals of black-crowned herons reproducing at Ruby Lake, Nevada contained different levels of pesticide residues depending upon the different wintering grounds they have visited. Springer et al (1984) also found that the contaminant profiles (relative amounts of different compounds) among populations of peregrine falcons helped to evaluate the origin of residues in falcons and did not reflect the levels in the ambient environment from where they were collected.

Minh et al (2002) and Kunisue et al (2003) in their work on the persistent organochlorine residues in resident and migratory birds from Asia found considerable variations in the levels of DDTs, PCBs, HCH (hexachlorocyclohexanes) isomers (HCHs), chlordane compounds (CHLs) and HCB (hexachlorobenzene) between the resident and migrant species collected from the same location. Kunisue et al (2003) predicted the predominant contaminants in India, Japan, the Philippines, Russia (Lake Baikal) and Vietnam; using residue levels data from resident birds. At the same time, the migrant birds from different countries had different patterns of OC residues, reflecting that each species has inherent migratory routes and thus has exposure to different contaminants. Dauwe et al (2003) also cautioned while recommending Great tit *(Parus minor)* nestlings as biomonitors of OC pollution that the contamination on natal and breeding sites of the females are different which may cause differences in body burden among breeding females. Recently, Elliott et al (2009) reported that bald eagles *(Haliaeetus leucocephalus)* at pelagic marine sites in the pacific

North American coast showed high-trophic level and marine input, while eagles at freshwater sites analyzed showed low-trophic levels; and eagles at the estuarine and inshore marine sites had intermediate values. The authors concluded that it was a relationship between trophic level and marine input that may reflect longer food chains in pelagic, compared to terrestrial ecosystems. Where the sum of PCBs and DDE concentrations generally increased with trophic level and marine input (with the exception of the freshwater sites), while PBDEs, hydroxylated-PBDEs and hydroxylated-PCBs increased with marine input, independently of their trophic level.

1.3.3 Tissue Specific Accumulation in birds

Several organs and tissues of birds and whole eggs are generally used for evaluating the contaminant burdens in birds and their environment. Kunisue et al (2003) used the breast muscle, liver and whole body homogenates of different birds collected from India, the Philippines, and Russia for analyzing the POPs like PCBs, DDTs, CHLs, HCB and also HCHs and have normalized the values for lipid weight basis for interpretation of the data. Kallenborn et al (1998) analyzed the nine egg samples and three liver samples of *Cinclus cinclus* L. and found that the sum concentrations of the DDTs and PCBs in liver and egg samples cover more than 90% of the total pollutant burden. Moreover, the sum concentrations of the contaminants in eggs show higher average values than in liver samples. They also found different patterns of pollutants in egg and liver samples which were attributed to the differences in matrix composition,

especially lipid content.

Olafsdottir et al (1998) while evaluating the seasonal fluctuations of organochlorine levels in the common eider *(Somateria mollissima)* in Iceland found varying levels with season in breast muscle and liver, which they have attributed to the shrinking fat in winter for energy requirements. Wyk et al (2001) selected liver, heart, kidney, pectoral muscle, whole blood and clotted blood from three species of vultures from different localities of South Africa purposefully for measuring 14 OCs including HCH, Chlordane, dieldrin, endosulfan and heptachlor epoxide, due to the fact that they represent major systems in the body. The authors have selected heart and blood because they perform the circulatory functions in the body, muscle represents the dominant body mass, liver is the chief site of metabolism, and kidney the site of excretion for these contaminants. These authors have observed statistically different concentration ranges among different tissues, whole blood, liver, kidney, heart and muscle for all the five toxicants. But these authors have reported all their values on a wet weight basis and have not reported the fat percentage of their samples. Normalizing the values on a fat weight basis might have abated these differences largely. In an elaborate work on Indian birds, Tanabe et al (1998) found male-female differences of organochlorines on a whole body basis and related this to females excreting OCs via their eggs.

Connell et al (2003) evaluated the DDTs, PCBs, HCHs and CHLs in the eggs of two Ardeid species, the little egret *(Egretta gazetta)* and the black-crowned night heron *(Nycticorax nycticorax)* from Hong Kong to determine the exposure associated risk parameter. The eggs can be easily collected without much detriment to the population. However, whole body burdens of the contaminants may give an accurate picture of the contamination levels, at least in the case of small birds. Commonly, for the larger birds, the breast muscle has been traditionally the most suitable sample, rather than the organs like liver and kidney, which have the function of metabolizing and detoxifying the contaminants. However, Griffin et al (2001) observed the changes in the plasma lipoprotein metabolism in chicks as response to exposure of PCBs; and Traag et al (2006) analyzed and compared the residues of polychlorinated dibenzo-p-dioxins (PCDD/Fs) and PCBs in eggs, fat and livers of laying hens following a consumption of contaminated feed. The authors concluded that there was a confident relationship of using livers as suitable organ bioindicator of bioaccumulation of POPs.

1.3.4 Using Birds as Bioindicators

Marine birds are useful as bioindicators of environmental pollution in estuarine and marine environments because they are often at the top of the food chain, ubiquitous, and many are abundant and common, making collecting possible. Seabirds have the advantage of being large, wide-ranging, conspicuous, abundant, long-lived, and easily observed for people. Many avian species

belongs to the top of the food chain, where they bioaccumulate contaminants trhough aging (Burger and Gochfeld 2004).

Enormous numbers of publications are available explaining the spatial and temporal distributions of POPs in birds, but most of them explain the differences in POPs due to migration patterns (Klemens et al, 2000; Minh et al, 2002; Kunisue et al, 2002: Letcher et al, 2009). Birds are, no doubt, good bioindicators of POPs. Birds have been used in almost every part of the world as bioindicators of pollution in the place where they reside as well as in their migratory routes.

In the Great Lakes Ecosystem in Canada, colonial fish-eating birds have been used as convenient sentinel biological systems for detection and monitoring of the effects of chronic exposure to complex mixtures of persistent toxic environmental contaminants (Fox 1993). Studies of impairments to health using such biomarkers as induction of mixed function oxidases, alterations in heme biosynthesis, retinol homeostasis, thyroid function and DNA integrity and various manifestations of reproductive and developmental toxicity in these birds suggests the severity varies with time and location and generally decreased between the early 1970s and late 1980s. However, these studies confirm the continued presence of sufficient amounts of PCBs and related persistent halogenated aromatic hydrocarbons in forage fish to cause physiological impairments in these birds over much of the Great Lakes basin (Fox 1993).

Tanabe et al (2003) evaluated the levels of PCDDs, PCDFs and coplanar PCBs in five albatross species from the North Pacific and Southern Ocean for assessing the north-south differences in residue levels. They found that the Black-footed and Laysan albatrosses from the North-Pacific contained higher levels of PCDDs/Fs and coplanar PCBs than the albatrosses from the Southern Ocean, indicating that emission sources of these contaminants were predominant in the northern hemisphere. Residue levels in albatrosses from the remote North Pacific Ocean far from the point source of pollution were comparable or higher than terrestrial and coastal birds from contaminated areas of developed nations, suggesting specific exposure and accumulation of OCDDs/Fs and coplanar PCBs in albatrosses. The authors found that the relative proportions of PCDFs and coplanar PCBs were higher than those observed in birds inhabiting terrestrial and coastal areas, suggesting that these toxic chemicals may have higher transportability than PCDDs, by air and water. Earlier to this, Auman et al (1997) noticed significant differences among sampling periods, in the total concentrations of PCBs, DDT and DDE in the plasma between Layson albatross and black-footed albatross collected from remote areas of the North Pacific. They have attributed this to the differences in the mobilization of fat reserves due to extended periods of egg incubation without feeding and foraging over great distances to obtain food for the chicks. Recent works on the spatial and temporal variations in the POPs in birds from the Asian countries are comparatively less when compared with the northern hemisphere countries.

Recently, a extensive literature review was published by Letcher et al (2009), the review analyze recent studies on biological effects in relation to OHC exposure, and attempts to assess known tissue/body compartment concentration data in the context of possible threshold levels of effects to evaluate the risks. This review concentrates mainly on post-2002 studies, where new OHC effects data in Arctic wildlife and fish are published, where observable effects data for populations of several top trophic level species, including seabirds (e.g., glaucous gull *(Larus hyperboreus)),* polar bears *(Ursus maritimus),* polar (Arctic) fox *{Vulpes lagopus),* and Arctic charr *(Salvelinus alpinus),* as well as semi-captive studies on sled dogs *(Canis familiaris).*

OHC contaminant exposures in Arctic wildlife and fish effects are largely based on correlations between biomarker endpoints (e.g., biochemical processes related to the immune and endocrine system, pathological changes in tissues and reproduction and development) and tissue residue levels of OHCs (e.g., PCBs, DDTs, CHLs, PBDEs and in a few cases perfluorinated carboxylic acids (PFCAs) and perfluorinated sulfonates (PFSAs)). Some exceptions include semi-field studies on comparative contaminant effects of control and exposed cohorts of captive Greenland sled dogs, and performance studies mimicking environmentally relevant PCB concentrations in Arctic charr.

However, the authors suggest that the true (if any real) effects of POPs in Arctic wildlife have to be put into the context of other environmental, ecological and physiological stressors (both anthropogenic and natural) that render an overall complex picture. For instance, seasonal changes in food intake and corresponding cycles of fattening and emaciation seen in Arctic animals can modify contaminant tissue distribution and toxicokinetics (contaminant deposition, metabolism and depuration). Also, other factors, including impact of climate change (seasonal ice and temperature changes, and connection to food web changes, nutrition, etc. in exposed biota), disease, species invasion and the connection to disease resistance will impact toxicant exposure. Overall, further research and better understanding of POP/OHC impact on animal performance in Arctic biota are recommended (Letcher et al 2009).

1.4 Endocrine Disrupting Compounds (EDCs) in Wildlife

Endocrine disrupting compounds (EDCs) are chemicals that may interfere with the body's endocrine system with resulting adverse developmental, reproductive, neurological, and immune effects in both humans and wildlife (NRDC 2009).

Alterations in endocrine development at early stages (or during ontogeny) can have permanent and detrimental effects throughout the life of the organism. Studies of endocrine disruption by pollutant chemicals have focused mainly on alterations in reproductive development and function. For example, beluga whales from the St. Lawrence Seaway, where the waters are highly

contaminated with OCs [e.g. PCBs], showed hermaphroditic qualities where some males had both male organs (epididymis, vas deferens, and testes) and female organs (uterus and ovaries) (Colborn et al 1996). Likewise, Everglades National Park and Big Cypress Swamp in Southern Florida lie downstream from large agriculture areas where pesticides are heavily used. Panthers that reside in this area show signs of sterility, decreased sperm count, and undescended testes (Crain et al 1997). In Lake Apopka in Florida, male alligators have abnormally small penises and defective testes, and females show follicle and ovary abnormalities. The hormone ratios in these male alligators are like those of a normal female and females had estrogen levels above normal (Guillette and Crain 1996). Such observations led to the hypothesis that chemical pollutants bind to hormone receptors for reproductive steroids and either trigger actions similar to those of the native hormone (hormone agonists) or prevent the native hormone from binding but do not trigger cellular action (hormone antagonists) (Gray 1992).

1.4.1 EDCs in Birds

Birds feeding at top of the food chain are exposed to high levels of pollutants due to bioaccumulation and biomagnification of pollutants with trophic level (hunting, opportunistic feeding, etc) (Burger and Gochfeld 2004).

Herring gulls have been an important/keystone study species for the Canadian Wildlife Service since the early 1970s. Specifically, their eggs have been used to measure residue levels of biotoxins as an indication of chemical contamination in

wildlife of the Great Lakes basin (Mineau et al 1984; Hebert et al 1999). Herring gulls are the only species that are year-round residents on the Great Lakes system and therefore represent pollutants accumulated only from the Great Lakes ecosystem.

Braune et al (2007) published data of OCs pesticides; PCBs, total mercury and selenium that were measured in eggs of thick-billed murres, northern fulmars and black-legged kittiwakes collected from Prince Leopold Island in the Canadian High Arctic between 1975 and 2003. The primary organochlorines found were £PCB, p,p'-DDE, oxychlordane, and hexachlorobenzene (HCB). Most of organochlorines analyzed showed either significant declines or no significant change between 1975 and 2003 in all three species. However, significant increases were observed by the authors for £HCH j_n the kittiwakes and fulmars, and B-HCH in the murres and fulmars.

The production and use of nonpolybrominated diphenyl ether (non-PBDE), brominated flame retardant (BFR) alternatives have been on the rise, although their assessment in environmental samples is largely understudied. Gauthier et al (2009) reported several non-PBDE BFRs were found in the egg pools of herring gulls *(Larus argentatus)* from seven colonies in the five Laurentian Great Lakes (collected in 1982 to 2006). Of the 19 BFRs monitored, hexabromobenzene (HBB), 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE), decabromodiphenyl ethane (DBDPE), and a-, B-, y. and 5-isomers of 1,2-

dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH) were present in eggs from all the colonies with the highest detection frequencies of 100%, 54%, 9% and 97%, respectively. The authors concluded that over the past 25 years non-PBDE BFRs have accumulated variably in female herring gulls and have been transferred during ovogenesis to their eggs, indicating that there has been continual exposure and bioaccumulation of several BFRs in the Great Lakes.

Liver concentrations of retinyl palmitate, the principal storage form of vitamin A, have improved in some locations, whereas the depletion of retinyl palmitate has worsened in others. Mild to moderate highly carboxylated porphyria is still a problem, as is the birds' reduced immune competency. Most of the improvements in bird conditions reported by Fox and co-workers occurred before 1985, reflecting the regulation mentioned above of some of the OCs. However, little improvement in bird health has occurred since 1985, which was confirmed with biochemical analyses where was measured dioxin toxic equivalent (TEQs) and monitoring for obvious deformities in chicks (Giesy et al 1994). The lesions frequently found in the chicks from the Great Lakes provide evidence that the problem is still the result of exposure to contaminants during organization prior to birth or hatching (Colborn 2002).

Alteration of endocrine functions mediated by EDC exposure may act through interference with the synthesis, secretion, transport, binding, action, or elimination of endogenous hormones (Damstra et al 2002). The major

explanation evoked for the interaction between EDCs and Gulls from Northern Norway such as certain OHCs —for example, PCBs, PCDDs, DDTs, and hydroxylated metabolites of PCBs (OH-PCBs) and the endocrine system is the structural similarity of EDCs with endogenous hormones (Verreault et al 2004).

1.4.2 Thyroid Hormones and Perfluorinated Compounds

Poly-and perfluorinated organic compounds (PFCs) are a class of substances characterized by a partially or fully fluorinated alkyl chain and a terminal functional group. The C-F bonds result in great stability under extreme heat and chemical stress and give the compound an oleophobic (oil repelling) property, whereas the polar head contributes to the excellent surfactant property of many PFC (e.g., perfluorinated sulfonates and carboxylates). These unique properties contribute to the widespread use of PFCs in a variety of commercial products, such as household surface finishes, food packaging, water- and stain-resistant materials, fire-fighting foams, etc (Kissa 2001).

The toxicology of PFCs has recently been extensively reviewed (Kudo and Kawashima 2003; Lau et al 2008). Among other observations, decreased TH levels after PFC exposure have been found in monkeys and rodents (Luebker et al 2002; Seacat et al 2003; Thibodeaux et al 2003). It has recently been shown that Perfluorooctane Sulfonate (PFOS) compounds does not affect the regulatory functions of the TH system itself, but its competitive binding to transport proteins alters the free thyroxine (T4) levels in blood (Chang et al 2008; Lau et al 2007).

Recently a study by Weiss et al (2009) concluded that the decreased levels of free T4 found in the presence of PFCs in serum and of the bioaccumulation of PFCs in humans and wildlife that may be partly attributed to the affinity of PFCs to bind to TTR in serum. No data related to PFCs effects on avian thyroid hormones or in hormone transport proteins was found.

1.4.3 Organohalogenated compounds and Avian Thyroid Hormones

In the Great Lakes, PCBs are abundant and widespread contaminants and are found in the tissues of fish-eating birds (Fox et al 1993). Until their banning in 1979, PCBs were largely used as fire retardant fluids in capacitors, coolant fluids in transformers, and as wetting agents and surfactants. Due to their long half-life and lipophilic properties, PCBs are incorporated into organisms low in the food chain and are biomagnified; that is, they increase in concentration with each increase in the food chain. Given their high position on the food web, fish-eating (piscivorous) birds, such as herring gulls, potentially are exposed to PCB biomagnification and have been used as sentinel species to monitor the concentrations and biological effects of environmental contaminants in the Great Lakes (Ness et al 1993).

The coplanar PCB congeners are dioxin-like and their toxicity appears to be mediated mainly through the aryl hydrocarbon receptor (AhR). Dioxin-like toxicity is estimated by using 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) potency as the standard, either as toxic equivalency factor (TEF) for individual compounds or as

the TCDD toxic equivalent for mixtures (Safe 1990). The non-planar congeners do not bind to the AhR but are found in high concentrations in the tissues of wild animals (Nessel et al 1992). To date, most of the reports of thyroid disruption in birds have been on wild birds exposed to mixtures of pollutants (Fox 1993; Grasman et al 1996; Murk et al 1994; Van den Berg et al 1994). Since the early 1990s, thyroid alterations in laboratory rodents have been associated with treatments of several specific congeners, both coplanar (e.g. PCB-169, 126, 156, 77) (Grasman et al 1996; Murk et al 1994) and non-coplanar (e.g. PCB-13, 52, 153) (Van der Kolk et al 1992; Morse et al 1993; Van Birgelen et al 1992) and with PCB mixtures (e.g. Arochlor 1254; Bastomsky, 1974; Morse et al 1996).

There are numerous reports of PCBs decreasing plasma T_4 levels in mammals (Byrne et al 1987; van den Berg et al 1988; Ness et al 1993; Li et al 1994) and birds (Van den Berg et al 1994). Significant negative associations between blood levels of a selection of OCs and circulating THs and TH ratios in plasma of male glaucous gulls breeding at Bear Island in the Barents Sea had been documented (Verreault et al 2004). Consequently, It is known that PCBs cause detectable alterations in plasma T4 and/or T3 levels; however, no data about the quantification of the binding affinity of the most relevant compounds (environmental relevant concentrations) was found in the literature for birds.

To date, there is less known about the effects of PCBs on thyroid function in birds than in laboratory mammals or fish. Even so, many developmental and

physiological abnormalities in birds exposed to pollutants are suggestive of thyroid alterations. Most goiters (thyroid gland enlargement) are a compensatory response of the thyroid gland to stimulation by TSH. Thyroid gland enlargement (goiter) and epithelial hyperplasia has been observed and documented since the 70s in herring gulls from polluted Great Lakes sites (Fox et al 1993; Moccia et al 1986) and in PCB-dosed lesser black-backed gulls and guillemots (Jefferies and Parslows 1972). Homing pigeons fed DDT, DDE, dieldrin, and PCBs show signs of goiter and alterations in thyroid histology (Jefferies and French 1972). Lower incubation attentiveness, resulting from nervous system dysfunction, could be due to deficient thyroid hormone supply to the CNS. Wild birds exposed to pollutants [Forster's terns from Green Bay and herring gulls from Lake Ontario (Fox et al 1978)] have lower incubation attentiveness. Likewise, in laboratory studies on ring doves fed PCBs (Peakall and Peakall 1973), and in ring doves dosed with a PCB-organochlorine mixtures (McArthur et al 1983) incubation attentiveness was altered.

THs are also necessary for maintenance of normal metabolic function. Alterations in metabolic rate and oxygen consumption have been observed in PCB-fed morning doves (Mayer and Tori 1981) and pigeons fed dieldrin and DDE (Jefferies and French 1971). More recent studies of laboratory and wild-caught birds have measured thyroid-related variables to access possible thyroid alterations from PCB exposure. In 28-day old Eider ducklings injected with PCB-77 (3,3',4,4'-tetrachlorobiphenyl, a coplanar dioxin-like congener) plasma Total

T_4 and Total T3 concentrations decreased (Murk et al, 1994). Van den Berg (1994) found significant reductions in plasma thyroid hormones in cormorants at 1 of 2 polluted sites studied in the Netherlands. However, though plasma TT_4, FT4 and TT3 levels were reduced, only FT4 concentrations were significantly correlated with site PCB concentrations. Surprisingly, in breeding doves fed PCB mixtures, there was a significant increase in circulating T_4 concentrations (McAuthur et al, 1983). Increases in the T_4 levels were correlated by Peakall and Peakall (1973) on PCB-dosed doves, with total wing-flipping and in-bowl activities of courtship pairs, suggesting a T4-induced hyperactivity.

In contrast, other studies on PCB exposed birds show no alterations in circulating thyroid hormones. In Japanese quail dosed with two mixed PCB preparations, there were no differences in plasma thyroid hormone concentrations despite signs of goiter development (Grassle and Biessmann 1982). Likewise, no changes were found in plasma thyroid hormone concentrations in 3-week old herring gulls injected with two doses of PCB-77 (Brouwer 1991). The herring gulls also showed no signs of thyroid hyperplasia. Some wild-caught birds from polluted sites also show no alterations in circulating thyroid hormones. In herring gull and Caspian tern chicks, plasma T4 levels did not differ in Great Lakes sites with different PCB concentrations (Grasman et al 1996). Murk et al (1994) also found no consistent pattern of a decrease in TH concentrations with an increase in PCB exposure in chicks of the common tern in coastal waters of the Netherlands and Belgium. From these studies, it is difficult to evaluate how PCBs

affect directly the thyroid function in birds. In addition, the lack of obvious alterations in plasma T_4 concentrations in some birds exposed to PCBs suggests that these birds may be compensating for PCB effects by increasing TSH secretion.

Whereas in mammals, brown adipose tissue 5'D type II activity supplies T3 to the circulation even if circulating thyroid hormones concentrations are low, birds lack of BAT, so this source of T3 production is not available in avian systems. Leslie A. Fowler (2000) in her thesis provided experimental evidence that PCBs have direct effects on Brain 5'D type II activity in birds. In her study she examined the regulation of thyroid hormone supply during development in (1) domestic chicken embryos *(Gallus domesticus)* exposed to a specific dioxin like PCB congener (PCB-126) and (2) Herring gull *(Larus argentatus)* embryos and pre-fledglings from Great Like sites with different chemical exposures.

Brominated flame retardants (BFRs) comprise a diverse group of chemical classes, which are used or have been used in an array of commercial and industrial applications for the purpose of fire prevention. The occurrence of several classes of BFRs in the environment has become increasingly evident and presents a potential health risk to organisms exposed to these emerging classes of environmental contaminants (de Wit 2002; McDonald 2002). Several classes of BFRs, namely polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA), hexabromocyclododecane

(HBCD), bis(2,4,6-tribromophenoxy)ethane (BTBPE), and tris(2,3-dibromopropyl)phosphate (Tris), have been identified as environmental contaminants (Hakk and Letcher 2003). PBDEs, TBBPA, and HBCCD are of particular concern due to increasing environmental concentrations and their ubiquitous presence in the tissues of humans and wildlife from Europe, Japan, and North America. BFRs have been shown to be susceptible to several metabolic processes including oxidative debromination, reductive debromination, oxidative CYP enzyme-mediated biotransformation, and/or Phase II conjugation (glucuronidation and sulfation); however, substantially more research on metabolism is necessary to fully assess BFR fate, uptake and elimination kinetics, metabolic pathways, inter-species differences, the influence of congener structure, and the potential health risks to exposed organisms (Hakk and Letcher 2003).

Exposure of wild birds to PBDEs is of concern since these and other structurally similar chemicals (e.g., PCBs) alter blood thyroid hormone homeostasis (Darnerud et al 2001; McDonald 2002) and/or vitamin A stores (Hallgren et al 2001; Rolland 2000). Changes in these parameters may alter development, immunocompetence, reproductive success, and other physiological processes in birds. The mechanism by which PBDE exposure alters thyroid dynamics remains poor studied, but PBDEs are structurally similar to T4 with an halogenated diphenyl / ether backbone structure.

TTR is a plasma protein necessary for the transportation of THs, besides the distribution of THs also transport vitamin A. In birds T_4 is known to have low binding affinity for TTR (Chang et al, 1999) with a short half-life (Astier 1980), which may render them susceptible to organohalogen induce alterations in circulating thyroid hormone concentrations. Studies with laboratory rodents have indicated that some PBDE congeners affect thyroid hormone transport and metabolism (Zhou et al 2001; 2002) as well as vitamin A levels (Hallgren et al, 2001).

More studies are necessary to address the extent to which adaptive thyroid responses may compensate for the effects of organohalogen phenolic mixtures and specific congeners, and the exposure time and concentration of congeners that elicit a thyroid specific biological response. Experiments are needed to be conducted both on field-caught birds and in laboratory assays to understand how environmental relevant organohalogen phenolic compounds may be affecting the TH transportation and delivered to target tissues. Fundamental differences in thyroid function between birds and mammals include: differences in the proportions of T4 and T3 in circulation, affinity of the TH transport proteins, and the lack of BAT in birds. Studies are needed to determine if the EDC effects on birds are comparable to what has been found in mammals.

14.4 *Metabolites from OHCs effects in avian thyroid hormone system*

The THs T_4 and T3 are phylogenetically conserved molecules that affect many aspects of development, growth and metabolism of vertebrates (Edeline et al 2005). THs have multiple effects on metabolism and development, in homoeothermic animals; they regulate basal metabolic rates and are essential for the maintenance of high and constant body temperature. The effect of THs on protein and lipid metabolism is of a biphasic nature: in low physiological concentrations, they are anabolic while at higher concentrations they are catabolic. During development, THs stimulate both growth and differentiation (or maturation). Their action can be direct, indirect or permissive. Most of the actions of THs seem to be dependent on the binding to a nuclear thyroid hormone receptor (TR). Two major isoforms of these receptors are known (TRa and TRB) and both of them preferentially bind T3. The main secretory product of the thyroid gland, the T4 was considered to be a relatively inactive prohormone due to its low binding affinity to TRs. Consequently, the peripheral metabolism of T4 by activating and inactivating pathways is very important in the regulation of the availability of receptor-active T3 and hence of thyroid activity (Decuypere et al 2005).

Several persistent OHCs, among these PBDEs, PCBs and chlorinated paraffins (CPs), have been shown to alter THs levels in experimental animals (Brower et al 1998; Brouwer et al 2001; Collins et al 1980a; Collins et al 1980b; Fowles et al 1994; Hallgreen et al 2001; Wyatt et al 1993). Effects on the thyroid hormonal

system are important to study since THs play an essential role in the development of many organs, e.g. the brain (Dussault and Ruel 1987). Alterations in CNS development, observed or indicated in several species after OHC exposure (Tilson et al 1990), have thus been suggested a result of exposure to T4-modulating chemicals.

The mechanisms involved in the OHC-mediated alteration of plasma THs have been extensively investigated but are still not fully known. Firstly, enzymatic effects where described that could interfere with T4 levels. Bastomsky et al (1974) showed that increased biliary clearance of T4 was associated with an increase in hepatic T_4 glucuronidation due to induction of uridine diphosphoglucuronosyl transferase (UDPGT). McCleary et al (2000) studied the disruption of the UDPGT activity in PCB-treated chicken embryos, and Quinn et al (2002) found that the polychlorinated biphenyl Aroclor 1242 can disrupt T4, estradiol, and consequently the plumage characteristics in the American kestrel *(Falco sparverius).* But, also enzymatic sulphation of THs are reported to be affected by PCB metabolites (Schuur et al 1998).

Secondly, there is morphological evidence for effects of OHCs on thyroid follicles (e.g. Collins et al 1980a; Collins et al 1980a; Saeed and Hansen 1997). These morphological effects, such as follicle epithelial cell hyperplasia, hypertrophy and decreased follicular colloid area, have been correlated with decreased serum levels of T4 and have also been suggested to include disturbances of enzymes directly involved in TH synthesis and release. As a third possible mechanism,

hydroxylated metabolites of OHCs have been found to compete with TH transport proteins for binding THs, but mainly the major T_4 in rats, resulting in a decreased plasma T_4 level (Brouwer et al 2001; Saeed et al 1997). The latter mechanism is dependent on the extent of metabolic conversion as phenolic metabolites of several groups of OHCs have higher affinity to TTR than have their unmetabolised counterparts. A characteristic effect of several OHCs is their potent induction of hepatic detoxification enzymes, i.e. cytochrome P450 (CYP450), and significant induction of 7-ethoxyresorufin-O-deethylase EROD activity during an immunochemically measured CYPIA protein (Eggensl et al 1996).

Attention to analyze metabolites from chlorinated and brominated POPs (hydroxylated-OH- and methoxy-MeO-) has been increasing in the past years. Recently, Kunisue and Tanabe (2009) analyzed plasma of different animals, including black-tailed gull *(Larus crassirostris),* common cormorant *(Phalacrocorax carbo),* and jungle crow *(Corvus macrorhynchos).* The authors detected eighteen known and fifty unknown peaks of OH-PCBs where the major congeners were 4'-OH-CB101/120, 4-OH-CB107/4'-OH-CB108, 4-OH-CB146, 4-OH-CB178, 4-OH-CB187, 4'-OH-CB172, 4-OH-CB202, and 4'-OH-CB199. This biomonitor comparison study showed relatively higher concentrations of OH-PCBs in animal species compared to humans. The authors reported that the OH-PCB levels in black-tailed gull and common cormorant blood were one order of magnitude higher than in humans. In addition, they observe that penta-to hepta-

chlorinated OH-PCB congeners were predominant in human blood, but profiles of OH-PCBs in other animals widely varied by species and taxa. Also, apparent metabolites of PBDEs such as hydroxylated PBDEs (OH-PBDEs), have been shown to have competitive binding affinity relative to T_4 with human transthyretin (TTR) (Hakk and Letcher 2003; Meerts et al 2000).

1.5 Selection of Avian Models

For this study, two birds were selected from two different diets and with exposure to different environments. Herring gulls *(Larus argentatus)* were selected to represent inland freshwater systems like Lake Ontario in Canada and glaucous gulls *(Larus Hyperboreus)* were selected to represent a Marine ecosystem from Svalbard Norway. These two species of sea birds are used as comparative bird models to observe the similarities and/or differences in the aminoacid composition of their TH transport proteins. Then, the identification of their binding affinities values to THs with and without presence of complex chemical mixtures from organohalogens and perfluorinated origins, found in circulating plasma of both species.

1.5.1 Herring gulls in the Great Lakes

The Great Lakes are the world's largest expanse of fresh water; they are Lakes Superior, Huron, Michigan, Erie, and Ontario. They cover over 249,000 km^2 (96,500 mi^2) and form a natural boundary between the Unites States and

Canada. Lake Ontario is the last of the chain of Great Lakes that straddle the Canadian-U.S. border; New York State borders its shoreline on then south and Ontario on the north. Ontario is the smallest of the Great Lakes, with a surface area of 18,960 km^2 (7,340 mi^2), but it has the highest ratio of watershed area to lake surface area. It is relatively deep, with an average depth of 86 m (283 ft) and a maximum depth of 244 m (802 ft), second only to Lake Superior. More than 80% of surface water inputs to Lake Ontario originate from upstream Great Lakes and connecting waters. Lake Ontario discharges to the St. Lawrence River (Nat Aud Soc 2005; Env Canada 2005). The most common birds of the Great Lakes are the herring gull and the ring-billed gull. The common and Caspian tern are regular visitors in spring and summer, when they breed on the hundreds of small islands. In Canada, most seagulls are herring gulls; the adults are about 61cm long from the tip of its bill to the tip of its tail. Its head, body, and tail are white, its bill is yellow with a red spot on the lower tip, and its legs are pink or flesh-colored (Nat Aud Soc 2005; Campbell et al 1990; Gauthier et al 1996).

Herring Gulls regurgitate, or bring up, food remains that they cannot digest, analyses of these pellets and of their feces show that Herring Gulls, like most other gull species, will eat almost anything; from clams, small fish, floating dead animals, small young and adults of other nesting birds, bread, and so on (Godfrey 1986). They have a knack for finding places where food is abundant, such as fish wharves and garbage dumps. Diet studies in the Great Lakes area showed that most pellets in colonies near large urban centers contained remains of garbage as well as various fish species. Pellets in colonies near agricultural

areas often had the remains of small mammals, notably deer mice (Ryckman et al 1997). Herring Gulls are one of the most widespread species in Canada. Indeed, its breeding range includes every province and territory in Canada. Exceptions to their breeding range include the Pacific and Atlantic coasts, the southern United States, the coast of the Gulf of Mexico, and a few Caribbean islands. In the lower Great Lakes area, the species can be found year-round. Of the 43 species of gull found in the world, 16 have bred in Canada, but three have nested only occasionally. Specialized feeding techniques and different ranges prevent, or at least reduce, competition between species (Pierotti and Good 1994).

Herring Gulls are very social birds and prefer to nest in colonies. Once a colony is well established, they are faithful to it and reluctant to settle elsewhere. In the lower Great Lakes area, for example, older, experienced breeding birds usually stay close to their colonies and are the first to reoccupy nesting territories in early spring. Some may use the same nesting site for as long as 10 to 20 years (Pierotti and Good 1994). The year-round residence of this avian model is one of the most interesting characteristics that make herring gulls (Figure 4) a valuable bioindicator of the contamination in Lake Ontario. During this study, bird samples from liver and plasma tissues were obtained from two sites inside Lake Ontario, Canada (Figure 5) .The distribution of possible organohalogen compounds and their metabolites retained in the liver and plasma tissues of Herring gulls *(Larus*

argentatus) from (two different colonies at) Lake Ontario, Canada, will provide data to compare the contaminant exposure with other gull studies.

Figure 4. Herring gull, *Larus argentatus* (www.wikipedia.org).

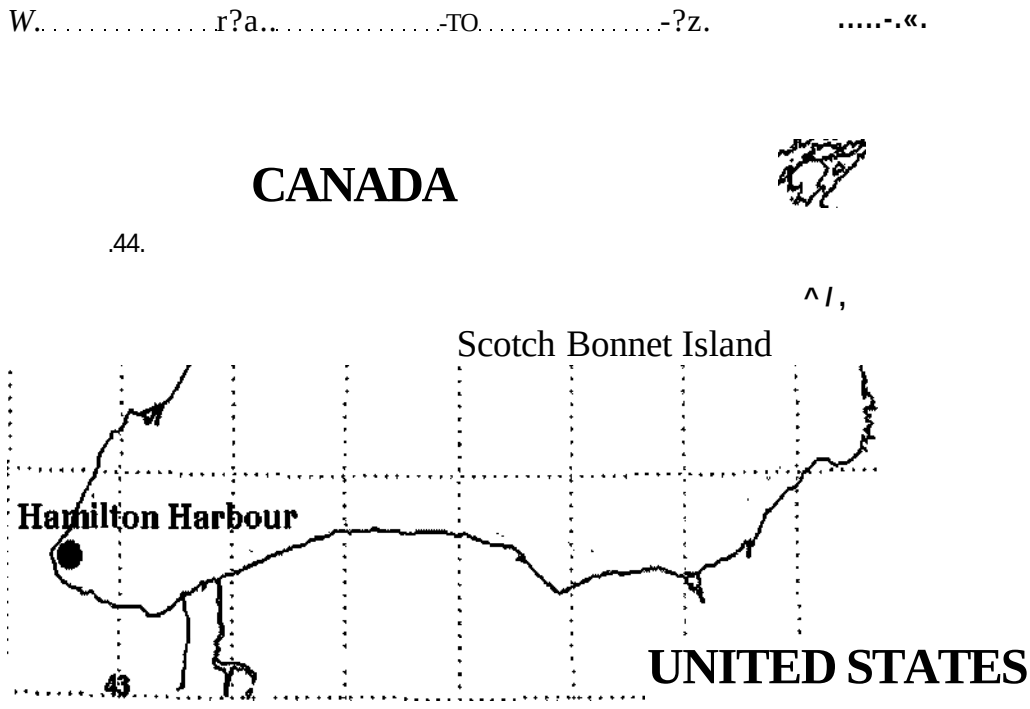

Figure 5. Map of Lake Ontario in Canada and the localization of the two colonies used in this study, Hamilton Harbour and Scotch Bonnet Island.

1.5.2 Glaucous Gull from Svalbard Island, Norway

The Svalbard area is a key site for seabirds. The total breeding population of glaucous gull *(Larus hyperboreus)* on Svalbard has roughly been estimated to 1000 - 10,000 pairs (Boon et al, 1992). In the Svalbard area a considerable number of dead glaucous gulls, with high levels of PCBs have been found (Bruhn et al, 1995). No cause of death has been found, but it was indicated that the contamination levels might be an important problem lowering survival and possibly also reproduction in some areas (Gabrielsen et al, 1995). The glaucous gull is the most important avian predator in the Arctic ecosystem and due to its high trophic levels; it is particularly vulnerable to contaminant exposure. The mobilization of body fat reserves during periods of food limitation may result in the release of accumulated lipophilic contaminants, and a temporary increase in blood contaminant levels (Isaksen and Bakken, 1995). The resulting acute exposure to high contaminant levels imposes a higher risk upon these animals than indicated from tissue concentrations. This gives rise to concern because many contaminants may have a detrimental effect on the health and reproductive performance of animals. Glaucous gulls are omnivores like most *Larus* gulls, and they will scavenge as well as seek suitable small prey. These birds forage while swimming or walking, and also may pick up items off water or catch small birds while flying. They often follow fishing boats. The activity of glaucous gulls is therefore necessary to evaluate the total metabolic capacities of this species, and

to acquire a better understanding of the fate and potential toxicity of environmental pollutants in Arctic seabirds.

1.6 Thesis Objectives

1.6.1 Project summaries

There is increasing evidence that chlorinated and brominated aromatic contaminants, such as polychlorinated diphenyl ethers (PBDEs) and the hydroxylated (OH) metabolites (e.g., OH-PCBs and OH-PBDEs) can interfere with the thyroid hormone system in, e.g. rats, humans and seals. For example, decreased serum levels of thyroxine (T_4) have been correlated with exposure to PCBs and PBDEs both in humans and in rats. THs are lipid soluble and this solubility allows for permeation into membranes. However, the distributions of THs, which are generally hydrophobic, require certain proteins to increase their hydrophilicity.

Therefore, higher evolved vertebrates as mammals and birds possess a number of T4 transport proteins to serve this function. In mammals and birds three T4 transport (carrier) proteins are known to be synthesized in the liver i.e., thyroxine-binding Prealbumin (or transthyretin TTR), thyroxine binding globulin (TBG) and albumin. In avian species, albumin and TTR serve as thyroid hormone carrier proteins.

Selected OH-PCB and OH-PBDE congeners (and other halogenated phenolics) have shown to competitively interact with human TTR. There is a lack of information about halogenated contaminants in thyroid hormones in avian species, specifically in the modulation of TH binding to hormone transporter proteins in birds (Albumin and TTR).

Main Goal: to assess the comparative effects of selected halogenated (phenolic) contaminants and analogues, as well as complex, natural mixtures, on the key thyroid system process of TH transpost, in two differentially exposed avian model species from marine and freshwater ecosystems. This Doctoral research will address four specific aims to test the following hypotheses.

1.6.2 Specific Aims:

1. The first aim is to investigate the distribution of possible organohalogen compounds and their metabolites retained in the liver and plasma tissues of Herring gulls *(Larus argentatus)* from two different colonies at Lake Ontario, Canada. It is possible that the two colonies present differences in their distribution and concentration of contaminants. The objective in this aim is to describe the possible differences between two organohalogen complex mixtures (neutral and phenolic extractions) through *in-vitro* observation and to provide the qualitative and quantitative analysis of the contaminants presents in the mixtures. These complex chemical extract mixtures will have a preliminary environmental approach to assess the

thyroidogenic effects of known circulating levels of organohalogen and perflurinated compounds in plasma of herring gulls. And, the effect the possible disruption of binding affinity between THs and TH transport proteins in gulls. The results will be compared with human TH transport proteins.

2. The second aim is to contribute to the knowledge on thyroidogenic activity and compare the effects of selected halogenated (phenolic) contaminants and endocrine on key thyroidogenic process in avian model species using TH transport protein TTR from glaucous gull *(Larus hyperboreus)* and herring gull. Both species as representative top predators from a seawater ecosystem (Svalbard, Norway) and a freshwater ecosystem (Lake Ontario, Canada), then the finding will be compared with human. It is possible that differences exist in the amino acid composition of TTR between the two gull species examined and with humans. Then, the determination of affinity of TTR with and without presence of thyroidogenic OHCs will be addressed.
3. Since hormone transport proteins are highly likely to be disrupted by organohalogen-metabolite compounds, principally by hydroxyl and methoxy-PCBs / PBDEs, my third aim is based in the hypothesis that the thyroidogenic process in an avian model species are disrupted by chemical-contaminant competition with TH transport protein albumin of herring gulls. The use of a preliminary competitive binding assay using complex chemical mixtures isolated from plasma can guide us to further

experiments and the selection of congeners with higher affinity for displacing TH transport proteins from binding THs. Through this.aim it is possible to assess the differences using cloned, expressed and purified herring gull albumin (ALB) with a selected set of environmental relevant contaminants (PCBs and PBDEs), for the possibility of disruption, and then comparing the affinity values with human albumin to observe possible differences among taxa.

4. In this aim, TTR and albumin from gulls previously cloned, expressed and purified (and commercially available human TTR and ALB) will be used to examine the competitive binding interactions of selected PFCs and PFC - complex mixtures (neutral and acidic extractions) isolated from the livers of Lake Ontario herring gulls as a novel approach to a possible disruption of PFCs in thyroidogenic processes. In this aim, I will examine also the differences or/and similarities of PFCs concentrations in livers from two avian colonies inhabiting Lake Ontario.

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CHAPTER TWO

Material and Methods

2.1 Samples collection, Gull liver and brain tissues

Liver and brain samples were collected in 2003 from herring gulls from Lake Ontario from colonies at Hamilton Harbour (HH) and Scotch Bonnett Island (SBI). Tissue samples were collected in cryogenic vials and immediately frozen in liquid nitrogen (LN2) and stored in LN2 at Environment Canada's National Wildlife Specimen Bank (EC-NWSB) in Ottawa (Carleton University). Since the early 1970's, herring gulls from the Laurentian Great Lakes have been used to monitor trends in levels and effects of OCs (Mineau et al 1984). The present herring gull liver and brain samples were collected as part of the Great Lakes Herring Gull Monitoring Program (GLHGMP) administered by the Canadian Wildlife Service (Environment Canada). All aspects of the sample collections for the GLHGMP have been approved by Environment Canada, and conform to all animal-handling guidelines.

Liver and brain samples from glaucous gulls, collected in 2002 and 2004 at Bear Island (74 °22'N 19 °05'E; Norwegian Arctic) (Verreault et al 2004), were immediately frozen in LN2, and stored in an ultra deep-freezer (-80°C) until

analysis. All field methods employed in this study were approved by the Governor of Svalbard (2002/00483-2 a. 512/2) and the Norwegian National Animal Research Authority (S1030/02). The capture and handling methods of glaucous gulls were approved by the Norwegian National Animal Research Authority (P.O. Box 8147 Dep., NO-0033 Oslo, Norway) and the Governor of Svalbard (Box 633, NO-9171 Longyearbyen, Norway).

2.2 Chemicals and reagents

Trizol® reagent for RNA purification, TA-cloning kit with pCR2.1 vector and InVision™ His-tag In-gel staining were purchased from Invitrogen (Carlsbad, CA, USA). IScript cDNA synthesis kit and iTaq DNA polymerase were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The pET28a expression vector was obtained from Novagen (Madison, WI, USA) and BL21-RIPL *E.coli* cells from Stratagene (La Jolla, CA, USA). His-Trap-FF crude Kit containing 1 ml columns were from GE Healthcare. Human TTR (human pre-albumin, 98% pure) was purchased from Sigma-Aldrich (Mississauga, ON, Canada). Human recALB (human serum albumin protein, 25 mg, 12.5 mg/ml AB7473-25) was purchased from ABCAM PLC. (Cambridge, MA, U.S.A.). These substrates were CB-187 from AccuStandard Chemical Reference Standards (>99% purity).

The PCB, PBDE, OH-PCB and OH-PBDE substrates used in the competitive TTR and Albumin binding studies with T4 and T3 were those found to dominate in

the plasma of Norwegian glaucous gull (Verreault et al 2005a; 2005b). These substrates were CB-187 from AccuStandard Chemical Reference Standards (>99% purity); and BDE-47 and the structurally analogous 4-OH-CB187, 6-OH-BDE47, 4'-OH-BDE49, 4-MeO-CB187 and 6-MeO-BDE47, which were purchased from Cambridge Isotope Laboratories (Cambridge, MA, U.S.A. purity 99% in nonane). All chemicals used were of high purity, HPLC grade.

2.3 TTR

2.3.1 TTR, RNA isolation, cDNA synthesis and PCR

The PCR primers were identified previously as an amino acid sequence set using the BLAST (NCBI) using traces of chicken *(Gallus gallus)* and using Primer3 (v. 0.4.0; http://mit.edu/), and the primer sequence was purchased from Custom Oligonucleotide Synthesis (Biosearch Technologies Inc, Novato CA 94949). Total RNA was purified using frozen (-80°C preserved) brain and liver tissues from herring gull and glaucous gull homogenized in Trizol reagent according to the manufacturer's protocol. The quality of the RNA was determined by agarose-formamide gel electrophoresis, and RNA concentrations were determined using a NanoDrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total cDNA for the polymerase chain reaction (PCR) were generated from 1 ug total RNA using a combination of random and poly-T primers from a iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). PCR was used to generate a 924 bp long product of TTR 3'-end. The 50 ul DNA amplification reaction contained 0.25 ul iTaq DNA

polymerase, 5 pi PCR buffer, 1.5 ul MgCl (20 mM), 1 u! of cDNA and 200 nM of each TTR forward primer (5'-CTCCCATGGCTCTGTTGATT-3') and reverse primer (5'-TTGTCTGAATTTTTGCCAGGT-3'). The three step PCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (1 min), 55°C (1 min), and 72°C (1 min).

One PCR product of 924 bp long representing TTR mRNA (pTTR2 plasmid) for each of brain and liver of herring and glaucous gulls was cloned into pCR2.1 vector and transformed to INVaF in an *Escherichia coli* (Invitrogen) bacteria culture. Each plasmid was sequenced using an ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The aminoacid sequences for each of brain and liver of herring and glaucous gulls were confirmed and compared using NCBI nucleotide BLAST online software (Genbank accession number bankit1047289-EU352211) (http://www.ncbi.nlm.nih.gov/BLAST). The aminoacid sequences of herring and glaucous gull TTR were aligned using ClustalW analysis, and Bootstrap values were obtained after 100 samplings. Positions with gaps were excluded and corrections were made for multiple substitutions.

2.3.2 TTR, Sequence analysis

The 924 bp long PCR products representing TTR mRNA from brain and liver of herring and glaucous gulls was cloned into pCR2.1 vector in an *Escherichia coli* INVaF strain (Invitrogen). TTR containing plasmids were sequenced in both directions using an ABI-prism 3100 Genetic Analyzer (Applied Biosystems,

Foster City, CA, USA). The generated nucleotide sequences were confirmed using NCBIs Basic Local Alignment Search Tool, BLASTx. (http://www.ncbi.nlm.nih.gov/BLAST) and translated into amino acid sequences by the aid of Expasy translation tool (http://us.expasy.Org/tools/#translate). Multiple sequences were aligned for TTR cDNAs from several vertebrates (Crocodile (CAA11129), frog (NP001081349), zebrafish (AAH81488), human (NP000362), rat (NP036813), chicken (NP990666), duck (ABC65926) and gull (EU352211). Phylogenetic analysis of the TTRs was performed using alignment and the neighbour-joining method (Saitou and Nei, 1987) option of the Phylip program (Galtier et al 1996) with 100 bootstrap replicates. For the construction of the phylogenetic tree, the TTR-like protein sequence of *Campylobacter coli* (Accession No. EAL57513) was used as an out-group.

2.3.3 Expression and purification of recombinant TTR (rTTR)

The cloned TTR gene product was transferred from the pCR2.1 vector into the pET28a *E. coli* expression vector that generates a 6-times Histidine tag (His-tag) positioned at the N-terminus of the expressed proteins. An E. *coli* BL21 (RIPL) codon plus strain was used as the expression host. E. *coli* was grown overnight (ON) in 3 ml Lauria Bertani medium (LB-medium; 5 g/L NaCI, 5 g/l Yeast extract and 10 g/l Trypton) containing 50 ug/ml Kanamycin and 34 ug/ml chloramphenicol. Thereafter, the synthesis of rTTR was accomplished by inoculating 250 ml of LB-medium without antibiotics with 2.5 ml of overnight (ON) culture. Cells were grown for 2 hours at 37°C before the temperature was

lowered to 30°C and recombinant protein expression was induced by addition of isopropyl-/3-1-thio-galactopyranoside (IPTG) to a final concentration of 0.5 mM. After 4 hours, cells were harvested by centrifugation (4,200 x g, 20 minutes) before storage at -80°C. Cell pellets were resuspended in column binding buffer (20 mM sodium phosphate, 500 mM NaCI, 5 mM imidazole, pH 7.4) and lysozyme was added to a final concentration of 0.2 mg/ml. After 30 min incubation on ice, Triton-X100 (1% v/v) was added to the lysis solution and DNAse and RNAse (10 mg/ml each) were added for 30 min to reduce the sample viscosity. Finally, the insoluble fraction of the samples was removed by centrifugation (20 min at 20,000 x g).

Before purifying rTTR by affinity chromatography, the sample was filtered through a 0.2 urn filter (Sarstedt). Immobilized metal (Ni^{2+}) affinity chromatography was conducted using His-Trap-FF crude Kit (1 ml column; GE Healthcare) equilibrated with elution buffer (20 mM sodium phosphate, 500 mM NaCI, 20 mM imidazole, pH 7.4). Lysate with recombinant protein was applied to the column and thereafter bound protein was eluted using a stepwise imidazole gradient with 100, 200, 250, 300 and 500 mM imidazole. Aliquots of the eluates were collected and controlled by 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) and InVision™ His-tag In-gel staining (Invitrogen). For each of the liver and brain sourced herring and glaucous gull recombinant TTR, there were two bands (18 and 36 kDa), which eluted at approximately 300 mM imidazole.

The protein concentration of each purified rTTR was quantified using the Bradford method (Bradford 1976).

2.3.4 Competitive rTTR Binding

Competitive ligands, and T4 and T_3 stock solutions were prepared at concentrations ranging from 10^{-3} to 10^5 nM. A stock solution of the purified rTTR protein (5 nM) was prepared by dissolution in equal parts of 0.1 M Tris- HC1, 0.1 mM NaC1, and 1 mM EDTA buffer with a pH 8. As a negative control solutions were prepared with DMSO/ethanol. For the assay volumes, the concentrations of rTTR and buffer were proportionally adapted from previous methods with minor modifications (Meerts et al 2000; Lans et al 1993). For the rTTR competitive binding assays, a volume of 10 ul of stock concentrations (10^{-3} to 10^5 nM) was added to the assay incubation mixture, which had a final volume of 200 ul. For the stocks of concentrations ranging from 10^{-3} to 10^{-1} M, a volume of 50 ul was used for a final incubation volume of 1 ml.

The competitive binding assay was based on previous methods with minor modifications (Meerts et al 2000; Lans et al 1993). For each of the competitive ligand and DMSO control stock solutions, human TTR (30 nM) stock solution (using 3 nM) or gull TTR (equilibrated to 5 nM from an original concentration of 0.77 mg/ml) was incubated with a mixture with each of ^{125}I-T4 (5 nM, 7000 cpm) and unlabeled T_4 (5 nM) in Tris-HCI buffer. The treatment was similar for ^{125}I-T_3 and unlabeled T3. The incubation mixtures were allowed to reach binding

equilibrium overnight at 4°C. After incubation, protein-bound and -free ^{125}I-TH was separated by filtration. Protein-bound and -free ^{125}I-TH was separated on a Biogel-P6DG column (bed volume: 1.2 ml; prepared in a 1-ml disposable syringe) that was equilibrated with 300 pi 10% (w/v) Tris-HCI buffer, and centrifuged for 20 min at 4,200 x g at room temperature. The columns were spin-forced (Jouan C412 centrifuge) again after an additional 200 JJJ of Tris- HCI buffer was added. These first two eluant fractions, containing the protein bound ^{125}I-TH fraction were combined, and the total radioactivity was counted and compared to the control incubations. Protein-bound ^{125}I-TH was quantified in the fractions. Total ^{125}I radioactivity per assay (0.75 KBq/assay) was measured using a gamma counter (Cobra II Auto gamma). Protein-free ^{125}I-TH remained bound to the Biogel matrix (P6DG), and therefore was not present in the first two eluant fractions.

2.4 Albumin

2.4.1 Albumin Cloning and Sequencing

The PCR primers were designed based on the nucleotide acid sequences found in chicken *(Gallus gallus)* using the PCR primer design service of Eurofins MWG Operon (Germany). Total RNA was purified from liver tissues using TRIzol® reagent according to the manufacturer's protocol. The quality of the RNA was determined by agarose gel electrophoresis. Total cDNA for the polymerase chain reaction (PCR) was generated from 1 ug total RNA using iScript cDNA Synthesis Kit with a combination of random and poly-T primers as described by the

manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). PCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (1 min), 55°C (1 min), and 72°C (1 min). The single band obtained by PCR was cloned into pCR2.1 vector in an *E. coli* INVaF strain (Invitrogen), and the plasmids were sequenced at Eurofins MWG Operon (Ebersberg, Germany). The generated nucleotide sequences were confirmed using NCBIs Basic Local Alignment Search Tool, BLASTx. (http://www.ncbi.nlm.nih.gov/BLAST) and translated into amino acid sequences using Expasy translation tool (http://us.expasy.Org/tools/#translate). Since the investigated sequences of glaucous and herring gull were identical, the plasmids obtained from herring gull cDNA were used for the purification of albumin.

In order to investigate the similarity of gull albumin to the albumin of other vertebrates, amino acid sequences from chicken *(Gallus gallus)* human *(Homo sapiens),* rat *(Rattus norvegicus),* frog *(Rana catesbeiana),* gecko *(Hemidactylus frenatus)* and salmon *(Salmo salar)* were aligned using the Multiple Alignment Construction and Analysis Workbench (MACAW). Phylogenetic analysis of the albumin was performed using alignment and the neighbor-joining method (Saitou and Nei 1987) option of the Phylip program (Galtier et al 1996) with 100 bootstrap replicates. For the construction of the phylogenetic tree, the albumin-like protein sequence of 2S seed storage protein 1 from *Arabipdosis thaliana* (Accession No. NM_118848) was used as an out-group as it has low homology to vertebrate albumin protein.

2.4.2 Expression and purification ofrecALB

For albumin expression, the coding sequence were transferred from the pCR2.1 vector into pET28c, and the obtained plasmids were verified by sequencing and then expressed using the BL21 (RIPL) codon plus *E. coli* strain as expression host. *E. coli* was grown overnight in 3 ml Lauria Bertani medium (LB-medium; 5 g/L NaCI, 5 g/l Yeast extract and 10 g/l Trypton) containing 50 ug/ml kanamycin and 34 ug/ml chloramphenicol. Details of cell growth and expression conditions are described in Ucan-Marin et al (2009). Before purification, the insoluble fraction of the samples was removed by centrifugation (20 min at 20,000 x g) and the sample was filtered through a 0.2 urn filter (Sarstedt). Purification of albumin was performed using immobilized metal (Ni^{2+}) affinity chromatography in 1 ml His-Trap-FF crude columns according to the manufacturer's recommendations (GE-Healthcare). Elution was optimized using 500 milliM imidazole buffer (20 milliM sodium phosphate, 500 milliM NaCI, 20 milliM imidazole, pH 7.4). Aliquots of the eluants were controlled by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by InVision™ His-tag In-gel staining (Invitrogen). The protein concentration of each purified recALB was quantified using the Bradford method (Bradford 1976).

2.4.3 Substrate competitive binding with recALB

Competitive ligand binding assays using gull and human recALB were the same as described previously for gull and human recTTR. The preparation of ligand

treatment solutions was as previusly described with the exception that the stock solution of the purified gull recALB was 5 nM.

2.5 Data analysis for Competitive Binding

All TTR and Albumin competitive binding assays were carried out in triplicate, and the triplicate assay set was repeated on separate day, and showed that relative competitive binding results were reproducible. Mean relative competitive binding values were based on n=6 replicates (combined triplicates on two different days). Competitive binding curves for each ligand were made by plotting the relative ^{125}I-T4 or ^{125}I-T_3 protein binding (% of control) against the natural logarithm of the competitor concentration. Competitive binding curves were described by the sigmoidal function y = aO + a1 / (1 + exp ((a2 + x) / a3)) (SlideWrite plus 4.0, Advanced Graphics Software, Carlsbad, CA). The relative potency of the individual competitors was evaluated retrospectively. Using the T3 (or T4) as Relative Potency 1, the value was compared with the competitor ligands with T3 (or T4). Where X (dose) and Y (binding) are independent, the variance of Y/X is V(Y/X) = E[Y^A2] V (1/X) + V(Y) E [1/X] ^{A}2 (V = variance, E = expected value). A single factor ANOVA with a Studentized Newman-Keuls (SNK) test was used to assess the statistical significance ($p < 0.05$) of the differences among competitive binding assays to validate the triplicate response of binding. Inhibitory constants were calculated according to Cheng and Prusoff (1973), where the affinity constant *K*\ (also known as I50) = *Ka* (1+ To/Kd). The I50

is the concentration of inhibitor resulting in 50% inhibition, the Kd is the dissociation constant of inhibitor-binder reaction, Kd* is the dissociation constant of the tracer-binder reaction, and T_0 is the total added concentration of the tracer.

2.6 Organohalogens

2.6.1 Extraction and Cleanup

The extraction and clean up of plasma and liver for OC (1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, o> hexachlorocyclohexane, hexachlorobenzene, B-hexachlorocyclohexane, y-hexachlorocyclohexane, octachlorostyrene, heptachlor epoxide, oxychlordane, t-chlordane, c-chlordane, t-nonachlor, p,p'-DDE, dieldrin, p,p'-DDD, c-nonachlor, p,p'-DDT, photomirex, mirex, TCPM); PCB (PCB-16/32, 17, 18, 22, 28, 31, 33/20, 42, 44, 47/48, 49, 52, 56/60, 64/41, 66, 74, 70/76, 85, 87, 92, 95, 97, 99, 101/90, 105, 110, 114, 118, 128, 130, 137, 138 141, 146, 149, 151, 153, 156, 157, 158, 167, 170/190, 171, 172, 174, 176, 177, 178, 179, 180, 183, 187, 189, 194, 195, 196/203, 199, 200, 202, 206, 207, 208); PBDE (BDE-17, 28, 47, 49, 66, 85, 99, 100, 101, 138, 154/BB153. 153, HBCD, 183, 190, 209), $MeS0_2$-CB (3-$MeS0_2$CB52, 3-$MeS0_2$CB49, 4-$MeS0_2$CB52, 4-$MeS0_2$CB49, 4-$MeS0_2$CB64, 3-$MeSO_2$CB70, 3-$MeSO_2$CB101, 4-$MeSO_2$CB70, 4-$MeSO_2$CB101, $MeS0_2$DDE, 3-$MeSO_2$CB110, 3-$MeS0_2$CB149, 4-$MeSO_2$CB110, 4-$MeS0_2$CB87, 3-$MeS0_2$CB132, 4-$MeS0_2$CB132, 4-$MeS0_2$CB174) and OH-PCB (4-OH-CB79, 4-OH-CB97, 4-OH-CB107/4, 4-OH-CB108, 2-OH-CB114, 4-OH-CB120, 4-OH-CB162, 4-OH-CB163, 4-OH-CB177, 4-OH-CB178, 3-OH-CB180, 3-OH-CB182,

3-OH-CB183, 4-OH-CB184, 4-OH-CB187, 4-OH-CB193, 4-OH-CB198, 4-OH-CB199, 4-OH-CB200, 4-OH-CB201, 4-OH-CB202, 4,4'-diOH-CB202, 3-OH-CB203, 4-OH-CB208), OH-PBDE (6-OH-BDE17, 4-OH-BDE17, 2-OH-BDE68, 6-OH-BDE47, 3-OH-BDE47, 5-OH-BDE47, 4-OH-BDE49, 4-OH-BDE42, 6-OH-BDE90, 6-OH-BDE99, 2-OH-BDE123, 6-OH-BDE85, 6-OH-BDE137), 4-OH-heptachlorstyrene (4-OH-HpCS) and MeS02-DDE and were based on methods described widely in detail for blood and liver, with some modifications (Chu et al 2003; Gebbink et al 2008a, 2008b; McKinney et al 2006; Muir et al 2006; Montie et al 2009; Sandala et al 2004). Other BFRs including pentabromotoluene (PBT), hexabromobenzene (HBB), 2,2',4,4',5-pentabromobiphenyl (BB-101) and total-(a)-hexabromocyclododecane (HBCD) were also measured according to recently published procedures (Gauthier et al 2009).

Briefly, approximately 2.0 g of liver and 1 ml of plasma, were spiked with internal standards [six $^{13}Ci_2$-labeled PCBs (CB-28, -52, -118, -153, -180, and -194), two PBDEs (BDE-30), 3-MeS02-2-CH_3-2',3',4',5,5'-pentachlorobiphenyl, four $^{13}Ci_2$-labeled OH-PCBs (4'-OH-CB120, 4'-OH-CB159, 4'-OH-CB172, 4'-OH-CB187), and 2'-OH-BDE28] and extracted via liquid: liquid partitioning. The extraction and clean up of liver for OCs, PCBs, PBDEs, and $MeS0_2$-PCB and OH-containing compounds were based on procedures described for brain with some modifications (Gebbink et al 2008b).

The quantification of OCs, PCBs, HO-PCBs, $MeS0_2$-PCBs, HO-PBDEs, and the BFRs including PBDEs, PBT, HBB, BB-101 and HBCD using gas chromatography-mass spectrometry (GC-MS) with electron impact (El) or electron capture negative ionization (ECNI) detection was used to determine the various chlorinated and brominated contaminants, respectively, in the isolated chemical fractions. The quantification limit for the contaminants was routinely ca. 0.01 ng/g (lipid weight).

2.6.2 Analysis and Quantification

PCBs analyses were carried out as described previously by Gebbink et al, (2008a); briefly, on an Agilent 6890 gas chromatograph (GC) coupled to an Agilent 5973 mass selective detector (MSD), fitted with a DB-5 column (30 m, 0.25 mm ID, 0.25 urn film thickness, J&W Scientific). The GC oven temperature program for PCB was as follows: 100°C (3 min), 20°C/min to 180°C, 5°C/min to 300°C. The MS was set in El ionization mode, with the ionization voltage set at 70 eV. The source and quadrupole temperature were 230 °C and 150 °C, respectively. In SIM, the [M] + and [M + 2] + were monitored for 51 PCB congeners. For MeS02-PCBs the oven temperature program was 100°C (3 min), 20 °C/min to 220°C (1 min), 3°C/min to 280°C (8 min). The MS was set in the ECNI mode. The source and quadrupole temperature were 180°C and 150°C, respectively. Methane was used as collision gas. Using SIM the [M]- and [M + 2]- ions were monitored for each chlorinated homologue group.

For OH-PCBs determination the oven ramping program was 80°C (1 min), 10 °C/min to 250°C (5 min), 5°C/min to 300°C (10 min). The MS was set in the ECNI mode. The source and quadrupole temperature were 180°C and 150°C, respectively. Methane was used as collision gas. In SIM the [M]-, [M+2]- and [M-15]- {[M-CH3]-} ions of the MeO-containing derivatives of all OH-PCBs were monitored. The PBDEs were determined by GC-MS-ECNI fitted with a DB-5 ht column (15 m, 0.25 mm ID, 0.1 urn film thickness, J&W Scientific). The temperature program was 100°C (2min), 25°C/min to 250°C, 1.5 °C/min to 260°C, 25°C/min to 325°C (7 min). The MS was set in ECNI mode. The source and quadrupole temperature were 150°C and 150°C, respectively. Methane was used as collision gas. Using SIM, the isotopic bromine anions (m/z 79 and 81) were monitored.

2.7 Perfluorinated Compounds

2.7.*1 Sample Preparation for PFCs*

Individual herring gull livers (n=10) were collected from Hamilton Harbour and Scotch Bonnet Island in 2004. The livers were stored at -40°C at Environment Canada's National Wildlife Specimen Bank (EC-NWSB) before chemical analysis. The extraction and cleanup is described in detail by Gebbink et al, (2009) and Chu and Letcher (2009).

Briefly, of each liver, two sub samples of 0.2 g; one sub-sample was spiked with labelled internal standards [PFBS (PFHxA-$^{13}C_2$), PFHxS (PFHxS-$^{18}0_2$), PFOS

(PFOS-$^{13}C_4$), PFDS (PFUA-$^{13}C_2$), PFOSA and N-Me-FOSA (d-N-Me-FOSA), PFHxA (PFHxA-$^{13}C_2$), PFHpA (PFHxS-$^{18}0_2$), PFOA (PFOA-$^{13}C_4$), PFNA (PFNA-$^{13}C_5$), PFDA (PFDA-$^{13}C_2$), PFUnA (FUA-$^{13}C_2$). PFDoA, PFTriA, PFTeA and PFPA (PFDoA-$^{13}C_2$). For 6:2 FTUCA (6:2 FTUCA-$^{13}C_2$), 8:2 FTUCA (8:2 FTUCA-$^{13}C_2$), 10:2 FTUCA (10:2 FTUCA-$^{13}C_2$). For 6:2 FTOH (6:2 FTOH-$^{13}C_2$), 8:2 FTOH (8:2 FTOH-$^{13}C_2$) and 10:2 FTOH (10:2 FTOH-$^{13}C_2$)], used for PFC quantitation. The other sub-sample was not spiked with IS, and was used as a complex mixture (neutral and acidic fractions) in a competitive binding assay, the sub-samples were extracted with 10 mM KOH acetonitrile/water (80/20 v/v). Mainly carbon, but also deuterated and oxygen enriched isotope standards were used as specific internal standards (see Gebbink et al, 2009). A volume of 2 ml of the extract was diluted with 8 ml water and adjusted to pH 4 with 2% aqueous formic acid. The cleanup and fractionation of the overall PFC extract was performed using Waters Oasis WAX cartridges. The cartridges were preconditioned with 3 ml methanol followed by 3 ml of water. The sample was loaded onto the cartridge, washed with 2% aqueous formic acid and then with water. The first fraction was collected by using 1 ml methanol; the fraction contained the neutral PFCs, FTOHs and the FOSAs. The cartridge was washed with methanol before the second fraction was eluted using a solution of 1% ammonium hydroxide in methanol. This fraction contained the acidic PFCs; PFSAs, PFCAs and the FTUCAs. A volume of 0.5ml of fraction 1 was mixed with 20 mg of active carbon. Fraction 2 was dried using a stream of nitrogen and

dissolved in 200 |jl of methanol. Prior to LC-MS/MS analysis, both fractions were filtered using centrifugal filters (modified nylon 0.2 urn, 500 pi).

2.7.2 *Instrumental Analysis*

The separation of the target compounds in both fractions was carried out on a Waters 2695 HPLC equipped with an ACE 3 C-i8 analytical column (50 mm x 2.1 mm I.D., 3pm particle size) and an ACE 3 C_{18} guard column (10 mm x 2.1 mm I.D., 3pm particle size, Advanced Chromatography Technologies, Aberdeen, UK). The mobile phases for fraction 1 were water and methanol; for fraction 2, 2mM ammonium acetate in water and methanol were used (Mobile phase gradients are provided Tables S2 and S3). The flow rate was 0.2 ml/min and the LC column was kept at 40°C. Coupled to the HPLC was a Waters Quattro Ultima triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Nitrogen was used as nebulizing gas and dissolvent gas, argon was used as collision gas when multiple reaction monitoring (MRM) mode was used. For neutral PFCs in fraction 1 atmospheric pressure, photoionization (APPI) was used in negative mode with krypton UV lamp (Chu and Letcher, 2008). Toluene was used a dopant and introduced to the APPI source at 2% of the flow rate of the mobile phase. The source temperature was 150°C, the probe temperature 250°C. Electrospray ionization (ESI) source in negative mode was used for acidic PFCs in fraction 2, with a source temperature of 120°C and a desolvation temperature of 350°C.

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CHAPTER THREE[1]

Recombinant Transthyretin Purification and Competitive Binding with Organohalogen compounds in Two Gull Species *(Larus argentatus* and *Larus hyperboreus)*

3.1 Abstract

Glaucous gulls from Norway and herring gulls from Great Lakes of North America are differentially exposed to chlorinated and brominated contaminants that can perturb thyroid hormone-dependent processes. Environmentally relevant concentrations of selected polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) flame retardant congeners and their hydroxylated (OH) and methoxylated (MeO) analogues were analyzed with competitive assays, to assess their binding affinity with thyroxine (T4) and 3,5,3'-triiodothyronine (T_3) thyroid hormones (THs) on the recombinant albumin and transthyretin transport proteins of humans and gulls. We isolated, cloned, sequenced, purified and expressed the complementary DNA (cDNA) of albumin from liver of herring and glaucous gull. Concentration-dependent, competitive binding curves were generated for T4 and T3 binding alone and for selected substrates using gull and human recombinant albumin (recALB). Albumin amino acid sequences were identical for both gull species, and in phylogenetic comparisons, was ~70%

[1] This chapter is based on a published paper in a peer-reviewed journal. "Ucan-Marin, F, Arukwe A, Mortensen A, Gabrielsen GW, Fox GA, Letcher RJ, 2009. Recombinant transthyretin purification and competitive binding with organohalogen compounds in two gull species *(Larus argentatus* and *Larus hyperboreus).* Toxicol. Sci. 107: 440-450.".

similar to human sequence. Human recALB had high preference for T_4 relative to T3 whereas it was reversed for gull recALB. Binding assays with recALB and recTTR gull proteins showed that relative to 2,2',4,4'-tetrabromoDE (BDE47) and 2,2',3,4',5,5',6-heptaCB (CB187) and the MeO-substituted (4-MeO-CB187 and 6-MeO-BDE47) analogues, 4-OH-CB187, 6-OH-BDE47 and 4'-OH-BDE49 had greater affinity than T3 or T_4. These results indicate that xenobiotic ligand binding to human albumin or TTR cannot be used as a surrogate for gull binding interactions. The combination of TH-like brominated diphenyl ether backbone (relative to the chlorinated biphenyl backbone), and the presence of OH-group produced a more effective competitive ligand on human and gull recALB and recTTR relative to both T3 and T_4. This suggests the possibility that OH-substituted organohalogen contaminants may be exposure concern to the thyroid system in free-ranging gulls as well as for humans.

3.2 Introduction

Transthyretin (TTR), albumin (ALB) and thyroid binding globulin (TBG) are the major hormone transport proteins of thyroid hormones (THs) in all vertebrates (McKinnon et al., 2005). These transport proteins bind to THs, and specifically 3,5,3-triidothyronine (T3) and thyroxine (T_4), and circulate in the blood. In vertebrates, the three thyroid hormone carrier proteins are synthesized by the liver, but only TTR is synthesized in the brain (Dickson et al 1987). Among vertebrate species, there are differences in the relative importance of circulating levels of TTR, ALB and TBG. In avian species, the study of TTR has been mainly in the context of comparative evolution relative to other taxa (Power et al 2002; Richardson et al 1994). In contrast to humans, it has been shown that TTRs from teleost fish (Yamauchi et al 1999), amphibians (Prapunpoj et al 2000; Yamauchi et al 1998), reptiles (Prapunpoj et al 2002), and birds (Chang et al 1999) bind T_3 with higher affinity than T4.

In birds, two main groups of hormones are involved in the growth of birds, growth hormones (GHs) together with the associated insulin-like growth factors (IGFs), and the T4 and T3. Normal growth of post-hatching birds requires GHs, T4 and T_3 (Decuypere et al 2005). THs have been reported to regulate thermogenesis in vertebrates, especially basal metabolic rate and cold-induced thermogenesis (Silva 1995). Therefore, circulating TH levels can be perturbed by temperature stress. For example, Kuhn and Nouwen (1978) reported that in domestic fowl

(Rhode Island Red strain), a gradual decline in ambient temperature from 26.5 to 17.5°C elevated serum T_3 in 40-day-old chicks. Increases in T_4 occurred when the temperature was lowered further to 12.5°C. Physiological processes perturb circulating TH levels and TH-dependent processes. THs are very lipophilic molecules, and in the absence of TH distributor proteins (THDPs), they interact as carriers from serum into lipid membranes (Ekins 1990). The effect of THs on protein and lipid metabolism is a biphasic nature: in low physiological concentrations, they are anabolic while at higher concentrations they are catabolic (Decuypere et al 2005).

A major concern is that TH-dependent processes, such as TH transport, are susceptible to chemical stress and can be disrupted by thyroidogenic, xenobiotic compounds accumulated in an organism (Ishihara et al 2003a; 2003b). Such chemical stressors include polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) flame retardant congeners as well as hydroxylated (OH) analogues such as OH-PCBs and OH-PBDEs. The metabolism of PCBs and particularly PBDE flame-retardants are not well understood in wildlife and particularly in birds (Hakk and Letcher 2003). However, putative OH-PCB and OH-PBDE metabolites have been reported in the tissues of certain avian species. Several OH-PCB and OH-PBDE congeners, and to a much lesser extent methoxylated (MeO)-PBDEs, were recently quantified in the plasma of adult glaucous gulls *(Larus hyperboreus)* from the Norwegian Arctic (Verreault et al 2005a; 2005b), and the plasma of bald eaglets *(Haliaeetus leucocephalus)*

from the west coast of North America (McKinney et al 2006). In the plasma of Norwegian glaucous gulls, 2,2',4,4'-tetrabromo diphenyl ether (BDE-47), 6-OH-BDE47, 4'-OH-2,2',4',5-tetrabromo diphenyl ether (4'-OH-BDE49), 3,-MeO-BDE47, 4'-MeO-BDE49, and most important among the OH-PCBs, 4-OH-2,2',3,4',5,5',6-heptachloro biphenyl (4-OH-CB187), tend to dominate. The presence of OH-PCBs in the plasma of birds and other wildlife is more than likely due to oxidative cytochrome P450 (CYP)-mediated PCB biotransformation. However, both MeO-PBDEs and some OH-PBDE congeners can also bioaccumulate in aquatic food webs as natural products produced by marine organisms such as sponges and algae (Malmvarn et al 2005).

Thyroid system disrupting chemicals may target any of the multiple pathways in a chemical-dependent manner, including TH production, receptor binding, metabolism and interaction with transport proteins such as TTR (Ulrich 2003). Thyroidogenic activity via interaction with human TTR has been reported for such organohalogen contaminants such as congeners of PCBs, PBDE flame retardants, OH-PCBs and/or OH-PBDEs. Several PCB, PBDE, OH-PCB and/or OH-PBDE congeners have been shown to competitively displace T4 from human TTR, which consequently can result in the release of (free) T4, which can enhance T4 metabolism and excretion (Brouwer et al 1998; Meerts et al 2000; Purkey et al 2004). Neurological effects of PCBs and PBDEs have been reported in mammals, and may be partly explained by the ability of these compounds (or their metabolites) to decrease TH levels during a sensitive time

periods, e.g. of neurodevelopment (Zoeller et al 2002; Costa and Giordano 2007). Regardless from natural source accumulation via metabolism of PBDEs, thyroidogenic and estrogenic dysfunction have been also reported in laboratory rats exposed to OH-PBDEs (Meerts et al 2000, 2001).

To my knowledge, the binding affinity of natural T_4 and T_3 ligands to avian TTR transport protein has not been reported, and especially the affinity to anthropogenic contaminants and/or metabolite ligands such as OH-PCBs and OH-PBDEs. In the present study, brain and liver tissues of two gull species from the genus *Larus,* glaucous gull from the Norwegian Arctic and herring gull from the Laurentian Great Lakes of North America were used to isolate TTR cDNA. Isolated TTR cDNA were cloned and sequenced. The recombinant TTR were expressed and purified, then the recombinant TTR protein was expressed and the product used in a competitive binding determination assays. Comparisons were performed on T_3, T_4 with selected PCB, PBDE, OH-PCB, OH-PBDE and analogous MeO-containing congeners previously reported and known for their environmental importance in the plasma of birds.

3.3 Results and Discussion

3.3.1 Cloning and characterization of gull recombinant TTR

The isolated TTR cDNA from brain and liver tissues of herring and glaucous gull were sequenced in both directions and sequence analysis showed identical nucleotide sequences between the species and between liver and brain. High

conservation in aminoacid sequence was expected for the two gulls from the same genus, however divergence was also speculated due to their different ecosystem habits; depending in seawater and freshwater. The feeding patterns and metabolism was considered as a main factor for a probable different homeostasis and consequently differences in TH transport proteins nucleotide constitutence. However, TTR was a much conserved protein, and confirm his use as a protein to observe evolution in vertebrates.

The achieved nucleotide sequence was translated and the deduced gull TTR translation showed 126 amino acid residues with a calculated molecular mass of 13.8 kiloDalton (kDa) (mass without his-tag labeling) and a theoretical isoelectric point (pi) of 5.1. A multiple alignment analysis was performed using the gull, duck, chicken, crocodile, human, rat, frog and zebra fish TTR variant (Figure 6) (Schuler et al 1991). The alignment showed that TTR is highly conserved among vertebrate species, but the N-terminal region presented a low sequence homology. To define the relationship of gull rTTR (where the common "gull" rTTR from liver and brain is referred to as gullTTR) to TTRs from other vertebrate species, a phylogenetic tree was constructed using the neighbour-joining method and bootstrapped 100 times. This tree clearly grouped the gull rTTR with avian (chicken and duck) TTR variants (Figure 7).

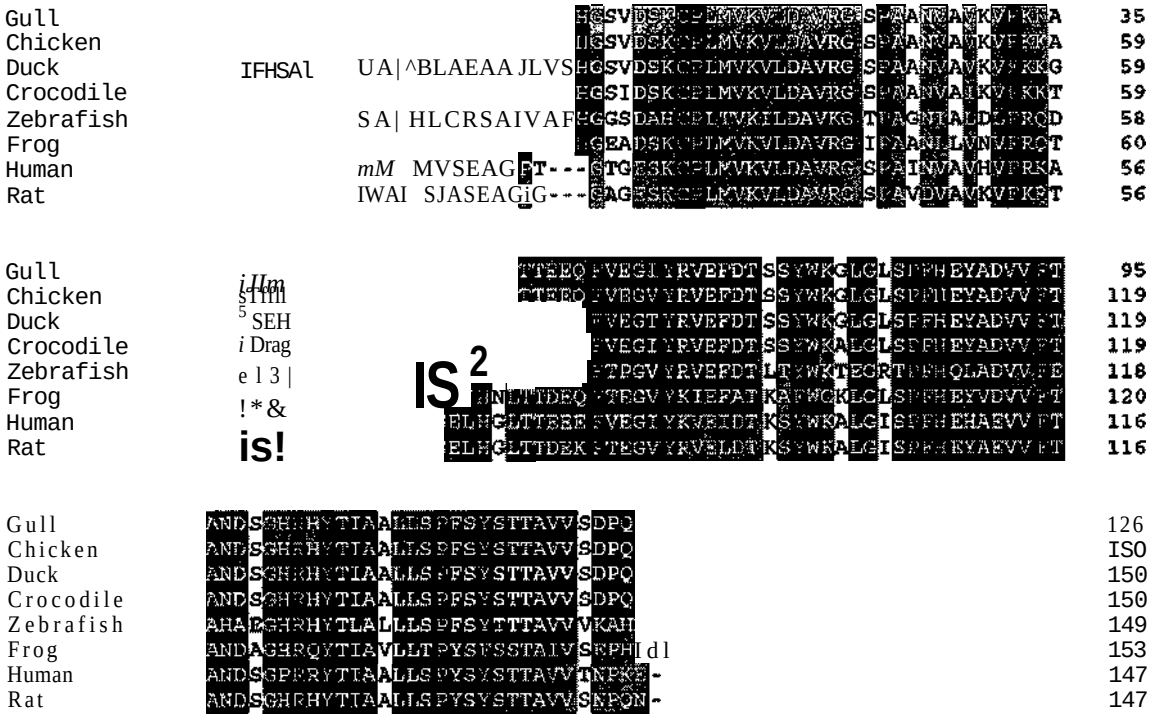

Figure 6. A multiple alignment of the predicted gull rTTR translation with TTRs from chicken, duck, crocodile, zebrafish, frog, human and rat was generated using MACAW. Identical and similar residues are indicated, darker shade corresponds to the most similar and no shade denotes any sequence homology. Accession numbers are: crocodile (CAA11129), frog (NP001081349), zebrafish (AAH81488), human (NP000362), rat (NP036813), chicken (NP990666), duck (ABC65926) and gull (sequence reported herein; EU352211).

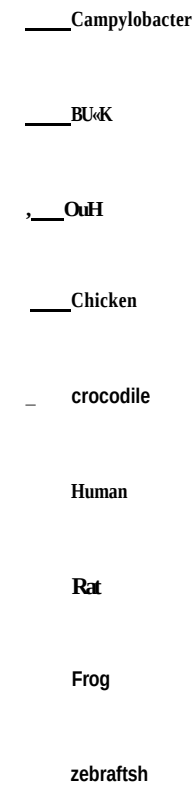

Figure 7. Phylogenetic analysis of the amino acid sequences of TTR from several vertebrate species and Campylobacter coli. The tree was constructed using the neighbour-joining method and bootstrap values from 100 replicates. The sequence accession numbers of analysed genes are: Crocodile (CAA11129), frog (NP001081349), Zebrafish (AAH81488), human (NP000362), rat (NP036813), Chicken (NP990666), duck (ABC65926) and gull (EU352211).

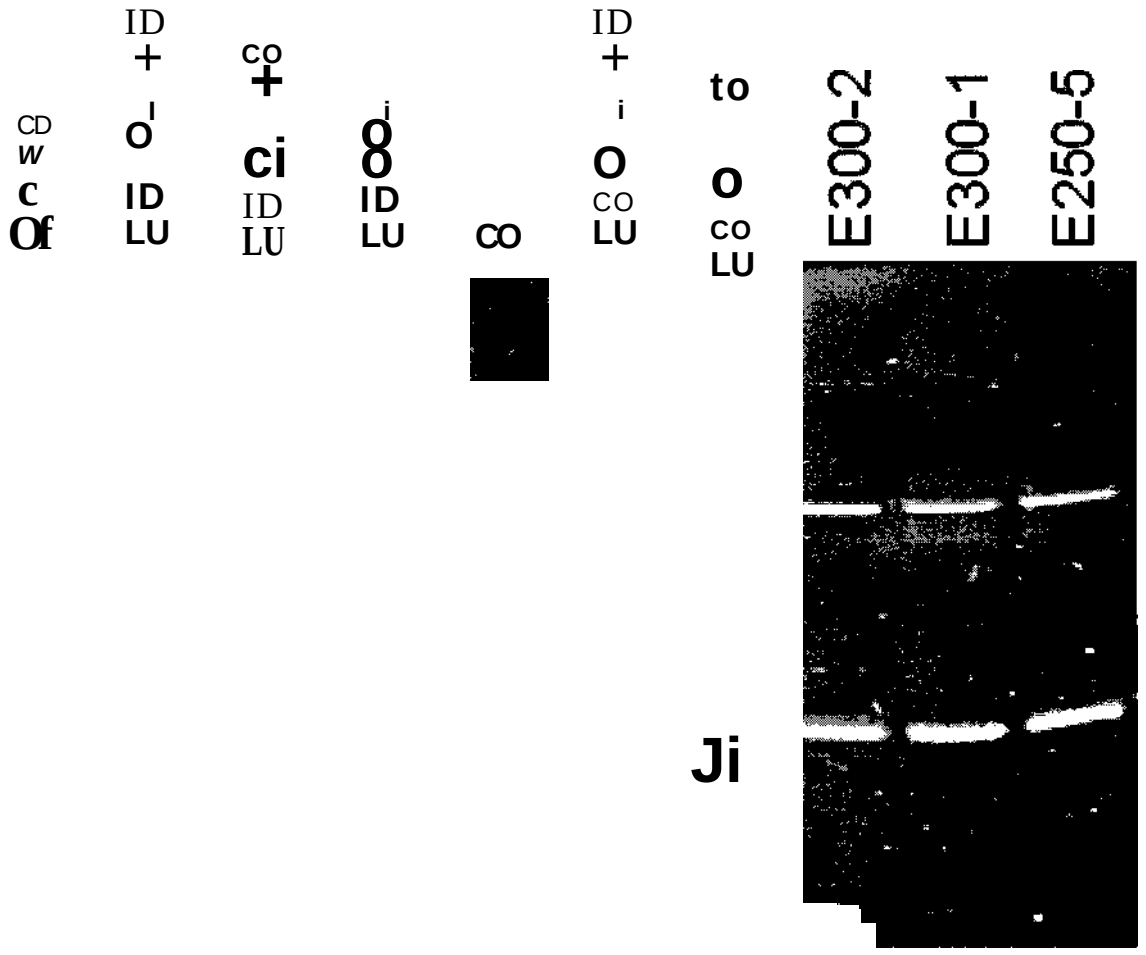

Figure 8. Purification of gull recombinant TTR (rTTR) expressed in E.coli BL21-RIPL. The electrophoretic gel shows eluants from a step-wise imidazole gradient with (from left to right) 250, 300 and 500 mM imidazole concentrations, and the his-tag labelled gull rTTR protein is observed as a monomer of 18 kDa and homodimer of 36 kDa that is comprised of the two-monomer forms.

Avian TTR is more similar to crocodile TTR than those from human, rat, frog or zebrafish. After transfection of the E.coli expression host, rTTR was expressed and purified using affinity column chromatography. SDS-PAGE analysis was performed to determine the expression of gull rTTR in the E.coli expression host (Figure 8). Gel analysis of the eluates showed a step-wise imidazole gradient with 100, 200, 250, 300 and 500 mM and the his-tag labelled gull rTTR protein was observed as a protein monomer of 18 kDa and homodimer of 36 kDa that putatively comprises of the two protein monomers (Figure 8). Eluates containing >250 mM imidazole contained rTTR of high purity and were used for competitive binding assays.

3.3.2 Competitive TTR binding assays

Competitive T3 and T_4 binding assays with the selected exogenous contaminant ligands were performed with purified human and/or gullTTR (Figure 9, Table 1 and 2), and showed that both T_3 and T4 more competitively bind for gullTTR relative to human TTR. Concentration-dependent competitive binding curves were generated for the natural ligands T3 and T4, and comparatively for the exogenous ligands under study (Figure 10 and 11, Table 1 and 2). As shown in Figure 10, in comparison to CB187 and 4-MeO-CB187, 4-OH-CB187 was the most potent as a competitive ligand for the displacement of both T3 and T4, which were comparatively displaced by 4-OH-CB187. As shown in Figure 11, in comparison to BDE47 and 6-MeO-BDE47, 6-OH-BDE47 and 4'-OH-BDE49 was comparatively more potent in the competitive displacement of T_3 and T4.

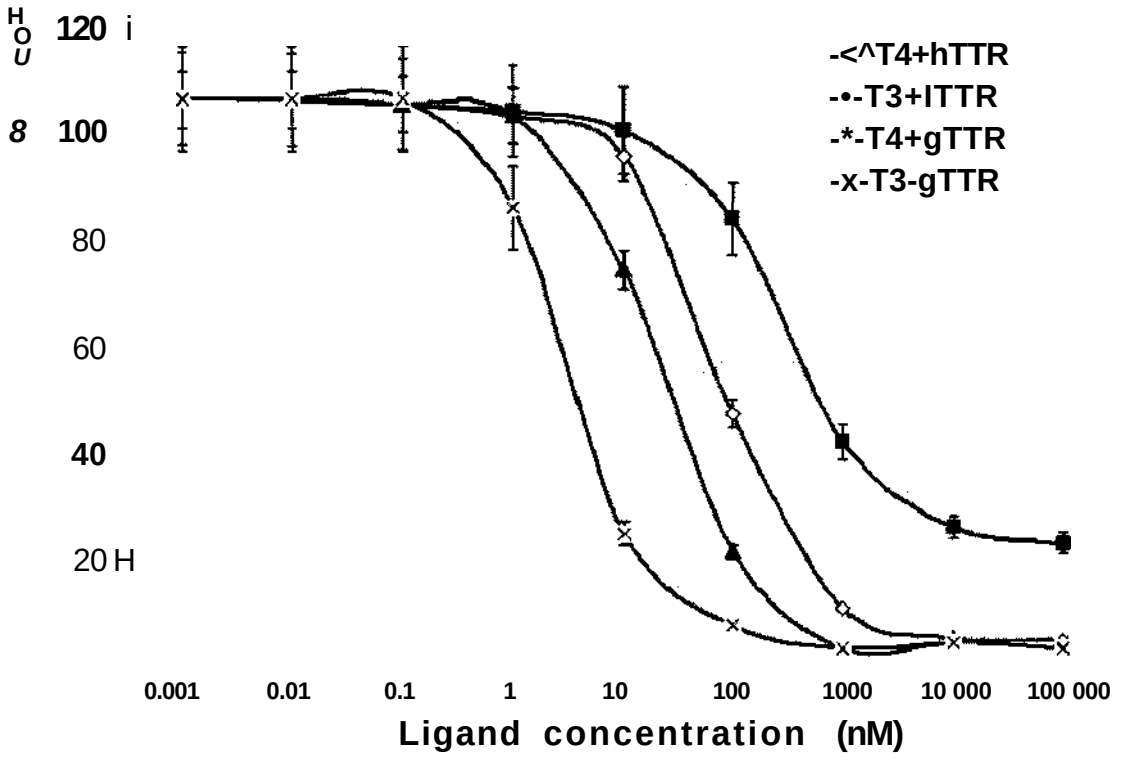

Figure 9. Concentration-dependent, competitive binding curves of gull transthyretin (gTTR) and commercially available human transthyretin (hTTR) with T_3 or T_4. The error bars denote the standard deviation of n=6 replicated (two n=3 replicate sets performed on different days).

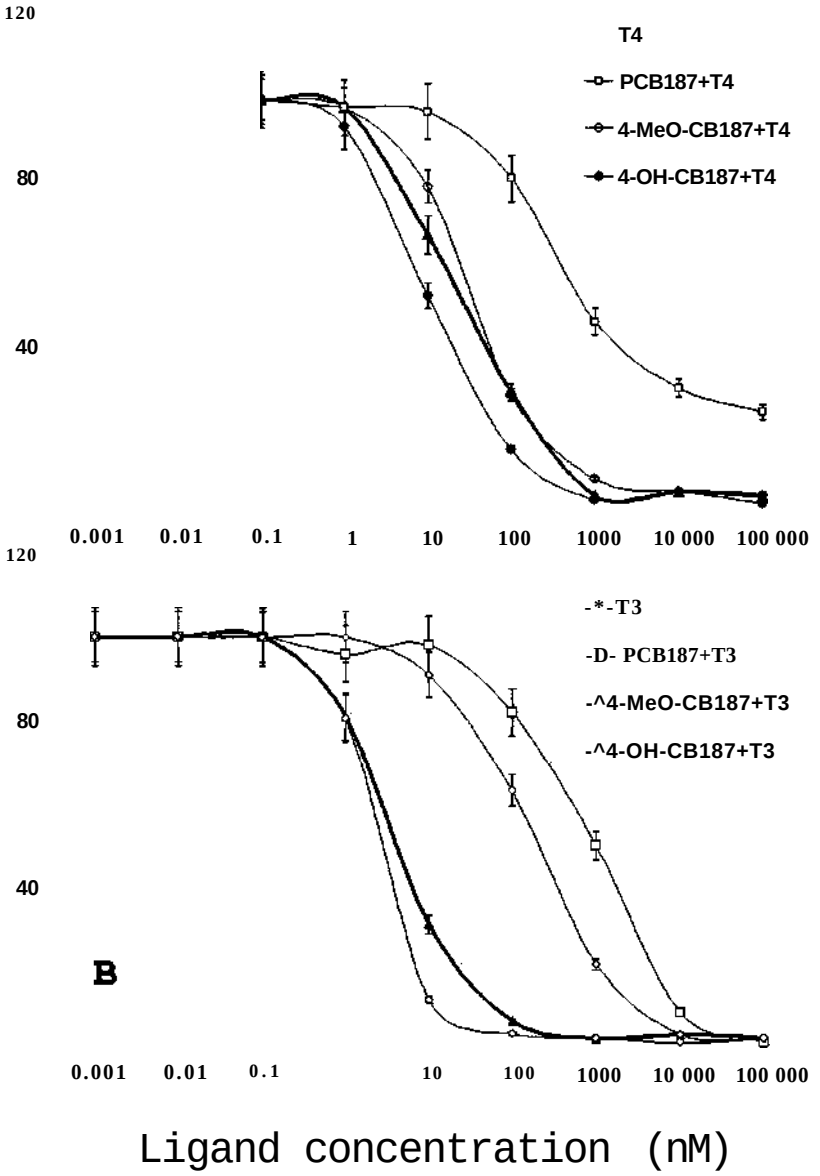

Figure 10. Concentration-dependent, competitive binding curves of (A) T4 and (B) T_3 displacement from gull transthyretin by 2,2',3,4',5,5',6-heptaCB (CB-187), 4-hydroxy-CB187 (4-OH-CB187) or 4-methoxy-CB187 (4-MeO-CB187). The competitive binding parameters are listed in Table 1. The error bars denote the standard deviation of n=6 replicated (two n=3 replicate sets performed on different days).

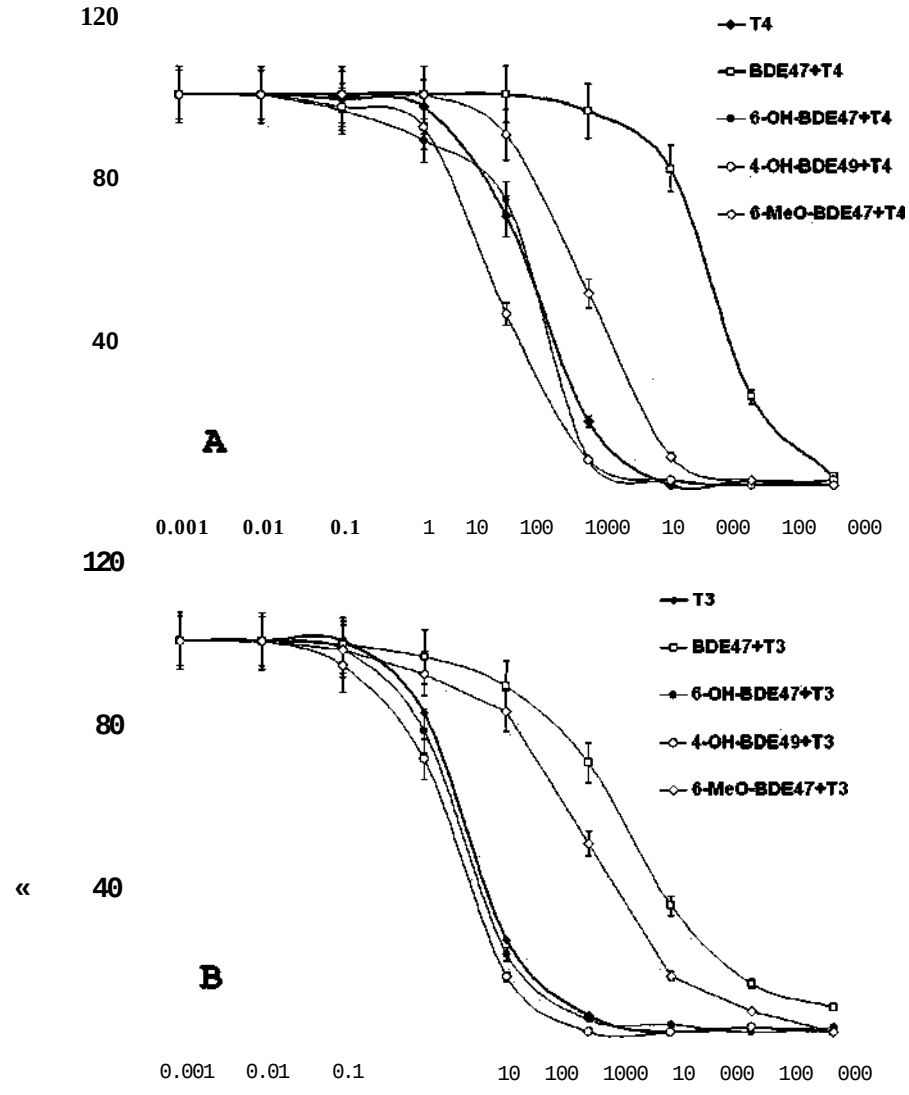

Figure 11. Concentration-dependent, competitive binding curves of (A) T4 and (B) T3 displacement from gull transthyretin by 2,2',4,4'-tetrabromoDE (BDE-47) flame retardant and 6-hydroxy-BDE47 (6-OH-BDE47), 6-methoxy-BDE47 (6-MeO-BDE47) or 4-OH-2,2'|4,5-tetrabromoDE (4-OH-BDE49). The competitive binding parameters are listed in Table 2. The error bars denote the standard deviation of n=6 replicated (two n=3 replicate sets performed on different days).

Table 1. Competitive binding parameters for gull transthyretin protein and T_3 or T4 in the presence of model PCB and structural analogue congener ligands, and human TTR values for T3 and T_4 as reference.

Compound	K (nM) [a]	Relative potency [b]	Kd [c]	Maximum % competition [d]
T_3	4.48	1	13.1	87±4.3
CB187	771	6.02x10" ^{3}±2.9x10"^{4}	195	89±3.7
4-MeO-CB187	62.67	0.08±4.5x10-[a]	23.5	90±2.6
4-OH-CB187	1.1	3.77±0.55	11.1	91±2.7
T_4	10.31	1	27.5	91±3.7
CB187	71.3	0.139±0.01	36.04	76±5.8
4-MeO-CB187	941	0.010±1.35x10"^{3}	432	86±2.6
4-OH-CB187	12.5	0.8±0.05	10.8	90±1.8
Human TTR				
T_4	50.89	1	14.9	98±2.1
T_3	214	0.19±0.02	60.1	77.3±3.7

Note: Results presented as the mean of individual measurements, and based on n=6 replicates (combined triplicates on two different days). The ±SD for relative potency and maximum % competition is based on the standard deviation of the two values comprising the ratio. See Figure 6 for competitive binding curves.

[a] Ki (or I_{50}) = Kd (1+ T_0/Kd*) (Cheng and Prusoff, 1973). See Materials and Methods section for further details.

[b] Calculated as ratio of IC_{50} for a chemical (Y/X) = E[Y^{A}2] V (1/X) + V(Y) E *[MX]* ^{A}2

[c] The Kd values (mean ± SD) were determined from the slope of the linear regression line of Scatchard plots

[d] Percentage of competition reached at highest tested concentration (1x10^5 nM).

Table 2. Competitive binding parameters for gull transthyretin protein and T3 or T4 in the presence of model PBDE and structural analogue congener ligands.

Compound	K| (nM) [a]	Relative potency [b]	Kd [c]	Maximum % competition [d]
T_3	4.48	1	13.1	87±4.3
BDE47	671	6.6914.4X10"[4]	201	88±3.8
6-OH-BDE47	78	0.07±3.91x10"[3]	7.1	91 ±2.7
4-OH-BDE49	57	0.81±0.013	6.3	90±2.2
6-MeO-BDE47	233.7	0.01912.3x10-*	29.6	90±3.5
T_4	10.31	1	27.5	91±3.7
BDE47	89.1	0.13±0.02	213	94±3.1
6-OH-BDE47	4.91	2.17±0.173	14.9	96±2.6
4-OH-BDE49	4.68	2.20±0.161	13.8	95±2.7
6-MeO-BDE47	54.32	0.189±0.032	139.3	96±2.8

Note: Results presented as the mean of individual measurements, and based on n=6 replicates (combined triplicates on two different days). The ±SD for relative potency and maximum % competition is based on the standard deviation of the two values comprising the ratio. See Figure 7 for competitive binding curves.

[a] Ki (or I_{50}) = K_d(1+ T_0/Kd*) (Cheng and Prusoff, 1973). See Materials and Methods section for further details.

"Calculated as ratio of IC_{50} for a chemical (Y/X) = E[Y^A2] V (1/X) + V(Y) E [1/X] ^{A}2

[c] The Kd values (mean ± SD) were determined from the slope of the linear regression line of Scatchard plots

[d] Percentage of competition reached at highest tested concentration (1x10^5 nM).

3.3.3 Phylogenetic analysis and comparison to other vertebrates

Herring gulls from Lake Ontario (Laurentian Great Lakes of North America) and glaucous gull from Bear Island, in the Norwegian Arctic are top avian predators in their respective aquatic ecosystems. Herring gulls in this study inhabits a freshwater system (Lake Ontario), and are distributed within a highly urbanized area such as the Greater Toronto Area, while the Bear Island glaucous gulls inhabit a marine system, and inhabit a more remote Arctic location where contaminant exposure is a manifestation of contaminant input through atmospheric transport and subsequently into the marine food web. The two gulls populations studied are exposed to different organohalogen profiles, and if they possess contrasting TTR protein structure (and consequently, ligand interactions); this factor may have a differential effect on the homeostasis of the circulating THs (McNabb 2003). In the present study, I compared the binding parameters of commercial human TTR and recombinant gullTTR. The recombinant gullTTR protein resembles the human TTR; however there is a truncation of approximately 26 amino acids in the N-terminal end of gull TTR. In addition, the recombinant TTR contains 6 histidine-tag residues. In the present attempt to further understand the similarities or differences of the present gullTTR with human TTR; one confounding factor to consider is that minor differences may have occurred in the post-translational modification of the recombinant gullTTR.

This is the first report describing the TTR nucleic acid and amino acid sequences as well as the expression and purification of recombinant TTR from any gull species. Previously in birds, the synthesis, expression and secretion of TTR in the liver and choroid plexus has been demonstrated in chicken, quail and pigeon (Zanotti et al 2001). For this reason, it was presently decided to evaluate the expression of TTR mRNA in brain and liver of both gull species to identify possible differences or similarities. The TTR sequences in the liver and brain of both herring and glaucous gull were found to be identical, and thus it was possible to use one tissue source of TTR protein that was representative of *Larus* genus and for use in competitive binding assays. TTR is a major, circulating TH-binding protein in birds, herbivorous marsupials and small eutherians (Richardson et al 1996). TTR is considered the only TH-binding protein that is synthesized in the cells of the blood-cerebro espinal fluid barrier, in addition to its synthesis in the liver (Schreiber and Richardson 1997). Comparison of the gull sequences with other vertebrate species showed the evolutionary conservation of TTR nucleotide and amino acid sequences (Figure 6 and 7). The deduced TTR amino acid sequences indicated that the protein is highly conserved among avian species. However, it was presently observed that the N-terminal region of TTR was less homologous among species. This is not surprising since the non-involvement of this region in and/or influencing TH binding was previously proposed by Chang and colleagues (1999). Overall, when grouped based on similarities in the alignment of genome sequences, the amino acids in the N-terminal regions of the TTRs in marsupials, birds, reptiles, amphibians and fish,

differ from that of the order insectivora (Schreiber and Richardson 1997). The basic structure of TTR (four identical subunits, central channel, T_4-binding sites in mammals) evolved while this protein was an extra-cellular brain protein, long before the initiation of its synthesis in the liver (Hamilton and Benson 2001). Although the TH binding region of TTR is highly conserved among vertebrate species including birds, other TH binding factors can affect the binding affinity among species and in different stages of development (Richardson et al 1994).

3.3.4 Natural T3 and T4 ligand binding to gull rTTR versus human TTR

The results obtained on this study showed that gullTTR is more effective at binding T3 relative to T_4, while for human TTR T_4 more effectively binds relative to T_3. This suggests that in gulls T3 binding relative to that of T_4 would be less susceptible to competitive displacement by other endogenous or exogenous ligands present in the blood stream and targeting liver or brain. The thyroid gland synthesizes and releases into the circulation primarily T_4 (95%, 5% T3), which is the less active precursor that is subsequently deiodinated to T3 at the target tissues. Besides the differences among avian and mammalian TTR binding T_3 and T_4, in mammals about 12% of total TH transport protein is synthesized by the choroid plexus, where about 50% of the protein secreted is TTR (Schreiber and Richardson 1997). The present results suggest that since much more T_4 (relative to T3) is associated with circulating TTR, in the gulls relative to humans exogenous ligand competition on circulating levels of T_4 may present disrupting affinity. The relative $T3/T_4$ binding affinity for human and gull TTR may be

partially explained from an evolutionary perspective, where different taxon levels have differing specificity with respect to transport proteins and the hormones transported.

For the present gull TTR, where T3 is more effectively bound than T_4, there is a consistency with other reports in the literature, where it has been reported that chicken TTR has about twice the affinity for T_3 relative to T4 (Schreiber 2002). TTR in birds, reptiles, amphibians, and teleost fish have generally been found to preferentially bind T_3 over T_4 (Kawakami et al 2006). The preference of avian TTR for T_3 relative to T4 could be related to the N-terminus of the TTR subunits that are more than merely changes in the primary structure, but are manifested in the relative affinity of the TTR homotetramer for T4 and T_3. Prapunpoj et al (2006) recently demonstrated in a reptile model the causal relationship between the N-terminal region of the TTR subunits and the affinity of the homotetramer for THs. They showed that removing the N-terminus of the *Crocodylus porosus* TTR subunit or replacing it with the N-terminus of the human subunit resulted in a homotetramer with increased affinity for T4. The present human TTR binding results are also consistent with that for humans, where mammalian TTR preferentially binds T4 relative to T_3. In addition, in humans, TTR has higher affinity for THs than albumin, and TBG has higher affinity for THs than TTR. Schreiber and Richardson (1997) suggested that the onset of hepatic TTR synthesis correlated with the development of homeothermy and the increase in lipid volume to body mass ratio.

3.3.5 Competitive gull rTTR binding of T_3 and T4 with exogenous contaminant ligands

PBDEs are used as a flame retardant. Like other brominated flame retardants, PBDEs have been used in a wide array of products, including building materials, electronics, furnishings, motor vehicles, plastics, polyurethane foams, and textiles. They are produced as commercial mixtures, and congeners such as BDE-47 are persistent and bioaccumulative in wildlife such as Great Lakes herring gulls and Svalbard glaucous gulls and/or their eggs (Gauthier et al 2008). Several OH-PCB and OH-PBDE congeners, and to a much lesser extent methoxylated (MeO)-PBDEs, were recently quantified in glaucous gull adult plasma from the Norwegian Arctic (Verreault et al 2005a; 2005b), where 6-OH-BDE47, 4'-OH-BDE49, 3,-MeO-BDE47, 4'-MeO-BDE49, and most important among the OH-PCBs, 4-OH-CB187 were dominant. The persistence of OH-PCB and OH-PBDE congeners in the blood of birds and other wildlife has been postulated as being via competitive binding to TH transport proteins and specifically TTR.

Better understanding of OH-PCB and OH-PBDE binding with TTR (and other TH transport proteins) is important with respect to effects on the thyroid system and target organs, e.g., in the brain because the binding of OH-PCBs and OH-PBDEs to TTR may be an avenue for these chemicals to reach TH receptors. OH-PCB congeners (e.g., 4-OH-CB106) have been shown to bind to the human thyroid

hormone receptor (TR) (You et al 2006). Kimura-Kuroda et al (2005) reported that 4'-OH-CB106 and 4'-OH-CB159 significantly inhibited T_3-dependent extension of Purkinje cell dendrites extracted from mouse cerebellum *in vitro.*

In the present study, the gull rTTR binding affinity or potency was exceptionally low for BDE-47 relative to T4 or T_3 and as compared to the MeO-BDE and especially the OH-BDE ligands. This is consistent with a previous study on the PBDE competitive binding with T4 and human TTR, where no substantial TTR binding was observed for 17 PBDE congeners, including BDE-47, at maximum concentrations of 0.25 uM (Meerts et al 2001). Morgado et al (2007) also reported a lack of competitive PBDE congener binding with sea bream TTR but even at higher concentrations. The present results are also consistent were the results reported by Hamers et al (2006) who screened a test set of 27 individual BFRs, including 19 PBDE congeners and 6-OH-BDE47, for their relative potency to compete with T4 for binding *in vitro* with human TTR. With T4 and human TTR, Meerts et al (2001) reported high human TTR binding competitive with T4 for 6-OH-BDE47, but was of lesser potency than for other brominated phenolic substances and used as flame retardants such as tetrabromobisphenyl A (TBBPA) and 2,4,6-tribromophenol (2,4,6-TBP). The TTR-binding potencies of TBBPA and 2,4,6-TBP exceeded that of the native prohormone T4. For both OH-PBDEs and OH-PCBs, it has been proposed that for optimal human TTR competitive binding (with T4), hydroxylation should exist at a para position (relative to the aromatic ring linkage), and that there be at least one, but

preferably two, halogen substituents on carbons adjacent to the OH-group (Lans et al 1993; Harriers et al 2006; Morgado et al 2007). TTR is a homotetramer consisting of a dimer of dimers. The binding channel for TH is at the interaction site between the two dimers. In this study it is also possible that the high differences found in the K_d and Kj values comparing gull TTR with human TTR binding could be attribute to the fact that I studied the interaction between THs and organohalogen compounds with the dimer form.

The present results demonstrate profound differences in the binding affinity of T_4 and T_3 and several environmentally relevant PCB, PBDE, OH-PCB, OH-PBDE, and MeO-PCBs and MeO-PBDEs for both human and/or gull TTR. Regardless, the OH-containing forms of PCBs and PBDEs are strong binding competitors for gull TTR. Depending on the circulatory quantities of OH-PCBs and OH-PBDEs circulating on plasma, it is possible an influence and/or change on the circulating THs, both levels and relative proportions of T3 and T4. Such TTR interactions could affect the circulatory TH homeostasis, and perhaps the TH-dependent function and health of exposed birds. OH-PCB binding affinity to TTR in mammals has been linked to alterations of TH and vitamin A levels in OH-PCB exposed laboratory rats (Schuur et al 1998). Thyroidogenic contaminants such as PCBs, PBDEs, OH-PCBs and OH-PBDEs that can displace T_4 from TTR, would subsequently release free T4, which may enhance T4 metabolism and excretion (Brouwer et al 1998). A sufficiently increased excretion of T4 could result in a decrease in circulating T4, leading to potential hypothyroidism.

To my knowledge, there are no reports on circulating OH-PCB, OH-PBDE, PBDE or possibly MeO-PBDEs in herring gulls from the Laurentian Great Lakes. Recently it was reported that the mean concentrations of 6-OH-BDE47 and 4'-OH-BDE49 in the plasma of male and female glaucous gulls from the Norwegian Arctic was up to 0.32 ng/g (wet weight) (Verreault et al 2005b), which is ~0.0006 nM. The competitive potency (IC_{50}) was ~10 nM for both OH-PBDE congeners with T_3 and T_4 on gullTTR, and therefore in free-ranging Svalbard gulls OH-PBDEs are not likely to substantially affect circulating T_3 or T_4 levels.

In contrast to OH-PBDEs, the mean concentrations of 4-OH-CB187 in the plasma of male and female glaucous gulls from the Norwegian Arctic was up to 17.5 ng/g (wet weight) (Verreault et al., 2005a), which is ~0.04 nM. The competitive potency (IC50) was 5 to 10 nM for 4-OH-CB187 with T3 and T4 on gullTTR, and thus 4-OH-CB187 levels in free-ranging Svalbard gulls may be high enough to effect circulating T3 or T4 levels. It is highly probable that the patterns and levels of exposure to these OH-containing contaminants differ in Great Lakes herring gull blood, and thus the potential effects on circulating T3 and T4 with respect to TTR interaction.

3.3.6 Conclusions

The present gullTTR molecular characterization and competitive binding studies clearly showed that human TTR cannot be used as a surrogate to assess the effects on circulating THs in birds (or at least gull species). The present results also demonstrate that there are potential physiological consequences of the competitive binding of OH-containing organohalogens to gull TTR. In birds and other wildlife, displacement of T4 from TTR would provide more T_4 to target tissues and would increase the amount of substrate for deiodinase enzymes, including the 5' deiodinases Dl and DM, which can convert T4 to its active form T3 (Verhoelst et al., 2005). Like other bird species, gulls not only have TTR but also ALB, which also bind and are involved in the transport of THs. However, in the context of overall TH binding in birds, TTR may be of lesser importance. For example, in birds the proportion of circulating TH-binding transport proteins is low for TTR. McNabb et al (1984, 2003) reported that in chicken the circulating T4 is bound 75% to ALB, 17% to TTR and 7.5% to a a-globulin.

Ligand binding parameter assessments for TH transport protein in gull species, and in wildlife in general, are necessary to more fully understand the potential effects in reproductive, nutritional, physiological and environmental (e.g., temperature) factors that can influence circulating T4 and T3 and subsequently on TH-depending processes.

3.4 References

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CHAPTER FOUR[2]

Recombinant albumin transport protein from gull species *(Larus argentatus* and *hyperboreus)* and human: Chlorinated and brominated contaminant binding and thyroid hormones.

4.1 Abstract

Glaucous gulls from Norway and herring gulls from Great Lakes of North America are differentially exposed to chlorinated and brominated contaminants that can perturb thyroid hormone-dependent processes. Environmentally relevant concentrations of selected polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) flame retardant congeners and their hydroxylated (OH) and methoxylated (MeO) analogues were comparatively examined with respect to competitive binding with thyroxine (T_4) and 3,5,3'-triiodothyronine (T3) thyroid hormones (THs) on recombinant human and gull albumin and transthyretin transport proteins. I isolated, cloned, sequenced, purified and expressed the complementary DNA (cDNA) of albumin from liver of herring and glaucous gull. Concentration-dependent, competitive binding curves were generated for T_4 and T3 binding alone and for selected substrates using gull and human recombinant

[2] This chapter is based on an accepted paper in a peer-reviewed journal. "Ucan-Marin F, Arukwe A, Mortensen A, Gabrielsen GW, Letcher RJ, 2009. Recombinant albumin transport protein from gull species *(Larus argentatus* and *hyperboreus)* and human: Chlorinated and brominated contaminant binding and thyroid hormones. Environ. Sci. Technol. Article ASAP Publication Date (Web): December 3, 2009).

albumin (recALB). Albumin amino acid sequences were identical for both gull species, and in phylogenetic comparisons, was ~70% similar to human sequence. Human recALB had high preference for T_4 relative to T_3 whereas it was reversed for gull recALB. Binding assays with recALB and recTTR gull proteins showed that relative to 2,2',4,4'-tetrabromoDE (BDE47) and 2,2',3,4',5,5',6-heptaCB (CB187) and the MeO-substituted (4-MeO-CB187 and 6-MeO-BDE47) analogues, 4-OH-CB187, 6-OH-BDE47 and 4'-OH-BDE49 had greater affinity than T3 or T_4. These results indicate that xenobiotic ligand binding to human albumin or TTR cannot be used as a surrogate for gull binding interactions. The combination of TH-like brominated diphenyl ether backbone (relative to the chlorinated biphenyl backbone), and the presence of OH-group produced a more effective competitive ligand on human and gull recALB and recTTR relative to both T_3 and T_4. This suggests the possibility that OH-substituted organohalogen contaminants may be an exposure concern to the thyroid system in free-ranging gulls as well as for humans.

4.2 Introduction

Disruption of the endocrine system by xenobiotic compounds continues to be reported in humans and wildlife. More specifically, growing evidence is being reported on the (potential) effects and impacts of organohalogen exposure on the thyroid system of vertebrates (Miller et al 2009; Tan and Zoeller 2007). Thyroid hormones (THs) are crucial for many biological processes and thyroid disruption can compromise normal development and physiology of exposed humans and biota (Boas et al 2006; Chan and Kilby 2000). THs include l-thyroxine (T_4) and its biologically active metabolite, 3',5,3-triidothyronine (T_3). THs perform important roles via multiple pathways and molecular signaling in vertebrates including birds, e.g., development and differentiation of several tissues, energy balance and metabolism, and modulation of cellular metabolic rate (Bernal et al 2003; Miller et al 2009).

The thyroid gland produces and releases T4 in response to the thyroid-stimulating hormone (TSH) released by the pituitary. Once T4 enters the blood stream, it is bound to TH-binding proteins (THBPs) and distributed to the target tissues. T4 is specifically transported through the cell membrane into intracellular compartments in different tissues and deiodinated to T3 (Friesema et al 2005). In vertebrates, THBPs include transthyretin (TTR), thyroxine-binding globulin (TBG) and albumin (Morgado et al 2007). Albumin is one THBP that is relatively non-

selective for hormones and found at high concentrations in the blood of all vertebrates including birds (Baker 2002). Albumin is the major circulating THBP in birds, herbivorous marsupials and small eutherians (Richardson et al 1994, 1996).

Thyroid system disrupting chemicals, e.g., those structurally and chemically resembling T_4 and T3, may target multiple TH control pathways in a chemical-dependent manner. Thyroid disruption may be via a variety of mechanisms depending on the different levels that a chemical interferes with the hypothalamic-pituitary-thyroid axis (Boas et al 2006). The mechanism of interference can include TH production and metabolism (e.g., TH degradation and interconversion), thyroid receptor (TR) binding and interaction with THBPs (Miller al 2009; Ulrich 2003). A major concern is that TH-dependent processes such as TH transport are susceptible to chemical stress and can be disrupted by thyroidogenic, xenobiotic compounds accumulated in humans and wildlife (Ishihara et al 2003; Letcher et al 2009; Miller et al 2009).

Polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) flame retardant congeners are environmentally relevant organohalogen contaminants (OHCs), and as well as some of their hydroxylated (OH) analogues they have been reported to have effects on TH-dependent processes, including TTR-TH binding in *in vitro* and *in vivo* studies with non-avian species (Hakk and Letcher 2003; Legler and Brouwer 2003; Letcher et al 2000; Meerts et al 2000; 2001).

Ishihara et al (2003) examined the effects of industrial, medical and agricultural chemicals on ^{125}I-T3 binding to TTRs and TH receptors in chicken and bullfrog. I recently reported on contaminant competitive binding with THs and gull TTR protein (Ucan-Marin et al 2009). However, to my knowledge, there are no reports on competitive binding interactions between halogenated organic contaminant and THs on albumin for any vertebrate species including birds.

The presence of OH-PCBs in avian plasma and other wildlife it is likely due to oxidative cytochrome P450 (CYP)-mediated PCB biotransformation (Letcher et al 2000; Park et al 2009). The metabolism of PCBs and particularly PBDE flame retardants are not well understood (Hakk and Letcher 2003). However, Stapleton et al (2009) recently reported on BDE-99 metabolism to OH-PBDEs *in vitro* in human hepatocytes. Nevertheless, OH-PCBs and OH-PBDEs have been reported in diverse tissues (mainly in blood) of wildlife species, including a few gull *(Land)* species. Several OH-PCB and OH-PBDE congeners, and to a much lesser extent methoxylated (MeO)-PBDEs, has been quantified in the plasma of adult Arctic seabirds from the Norwegian Arctic (Letcher et al 2009; Verreault et al 2005a, 2005b).

In the present study, liver tissues of two gull species and populations, *L. hyperboreous* from Arctic Svalbard and *L. argentatus* from the Laurentian Great Lakes of North America, were used to isolate, clone and sequence albumin cDNA, with a subsequent expression and purification of recALB protein. Given

the known environmental relevance of certain PCB, PBDE, OH-PCB and OH-PBDE congeners in e.g. glaucous gulls, and the strong competitive binding interaction with THs for gull recTTR (Ucan-Marin et al 2009), I investigated the competitive binding (with T_3 or T_4) with the isolated gull recALB, and compared to commercially available human recALB, and relative to human and gull recTTR.

4.3 Results and Discussion

4.3.1 Cloning and characterization of gull albumin

As I reported previously, cDNA sequences of herring and glaucous gull TTR from both liver and brain were identical (Ucan-Marin et al 2009). Thus, I isolated gull albumin cDNA from the liver and from the same herring and glaucous gulls. Albumin was sequenced in both directions and observed to have identical nucleotide sequences between the species analyzed. Translation of nucleotide sequence showed 490 amino acid residues (Figure 12) with a putative molecular weight of 55.3 kiloDalton (kDa) and theoretical isoelectric point (pi) of 6.34. The sequence of the present gull albumin was compared with albumin sequences from other species, i.e. chicken, humans, rat, gecko, frog, salmon (Figure 13). The phylogenetic tree indicates that gull albumin shares more homology with mammalian (-70%) than amphibian and fish albumin.

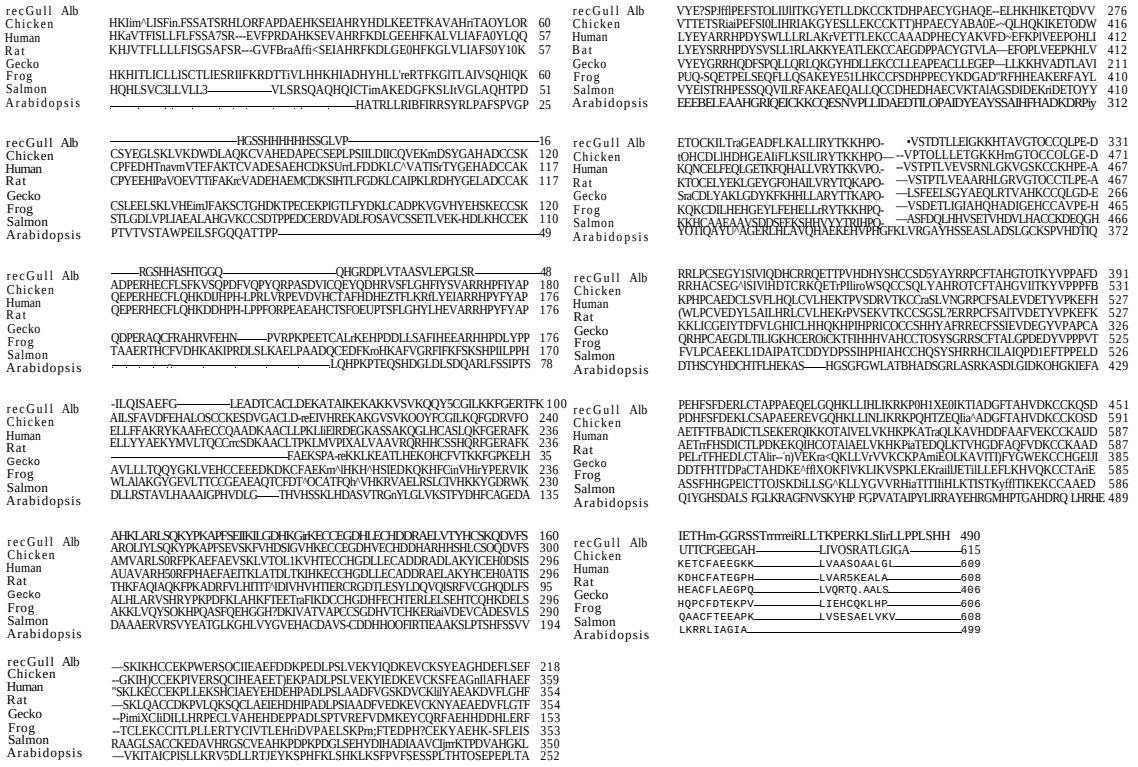

Figure 12. Multiple alignments of recombinant gull albumin sequence with serum albumin sequences from chicken, gecko, rat, human, frog and salmon was generated using MACAW. GenBank accession numbers are: *Larus sp.* (temp access # lcl|50720), *Gallus gallus* (NP_990592), *Hoplodactylus maculatus* (AF375972), *Homo sapiens* (AAX63425), *Rattus norvegicus* (NP599153), *Xenopus laevis* (NP001081244) and *Salmo salar* (P21848).

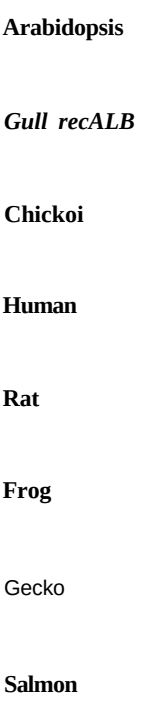

Figure 13. Phylogenetic analysis of the amino acid sequences of albumin of vertebrate species. The amino acid sequences of albumin were aligned using ClustalW analysis, and Bootstrap values based on 100 samplings. The osmotic stress-induced proline dehydrogenase sequence of *Arabidopsis thaliana* (accession no. AAB40615) was used as outgroup. The other protein accession numbers used; *Larus sp.* (temp access # lcl|50720), *Gallus gallus* (NP_990592), *Hoplodactylus maculatus* (AF375972), *Homo sapiens* (AAX63425), *Rattus norvegicus* (NP599153), *Xenopus laevis* (NP001081244) and *Salmo salar* (P21848).

4.3.2 Substrate competitive binding with thyroid hormones

To establish a standard competitive binding curve, in the absence of binding competitors human recALB was observed to have a greater binding affinity for T4 than for T_3, whereas for gull recALB T_3 was much greater than T4 (Figure 14). As summarized in Tables 3 and 4, for gull recALB the *K*\ was lower for T3 (1.12) relative to T4 (5.11), and the *K*\ for gull recTTR was also lower for T_3 (5.99) relative to T_4 (15.74) and thus greater binding affinity of T_3 to both recALB and recTTR. In contrast, the reverse was found for human recTTR and recALB. That is, the *K,* for human recTTR was higher for T_3 (207.67) relative to T_4 (56.88), and similarly for human recALB the *K*\ was higher for T_3 (23.88) relative to T_4 (7.91), and thus greater binding affinity of T_4 to both recALB and recTTR.

Competitive T_3 and T_4 binding assays in presence of selected exogenous contaminant substrates were performed with purified human and gull recALB (Figures 15 and 16). CB187 and 4-MeO-CB187 had lower affinity than 4-OH-CB187 (Figure 15), and 4-OH-CB187 was the most potent as a competitive substrate for the displacement either T_3 or T_4 (Table 3). However, one exception was that for T_3 with gull recTTR, 4-MeO-CB187 had a higher binding affinity than CB187, whereas the reverse was true for competition with T_4. The gull recALB affinity of the selected brominated compounds (Table 4) shows that BDE47 and 6-MeO-BDE47 had a lower binding affinity for both T_3 and T_4 relative to 4'-OH-

BDE49 and 6-OH-BDE47. Also, 4'-OH-BDE49 and 6-OH-BDE47 had comparable but greater binding affinity for both $T_3$and T_4 (Figure 16).

4.3.3 Phylogenetic comparison of gull albumin to other vertebrates

To my knowledge, this is the first report describing the albumin nucleotide and amino acid sequences as well as the expression and purification of THTP recALB from any gull species. In the present study, the albumin amino acid sequences from the liver of both herring and glaucous gull were found to be identical in their composition. This finding allowed us to use only one tissue as a source of a "standard" albumin protein that was representative of the *Larus* genus and for competitive binding. It is known that albumin is a major circulating THTP in birds, herbivorous marsupials and small eutherians (Richardson et al 1994, 1996).

Comparison of the obtained gull amino acid sequences with other vertebrates showed an evolutionary conservation of albumin nucleotides and their deduced amino acid sequences. The amino acid composition obtained for gulls in this study showed very similar sequences of >85% with chicken albumin, which strongly suggested that the protein is highly conserved for avian species. High amino acid sequence similarities (-70%) were observed between birds and mammals (humans), and both the mammalian and avian sequences were lower than amphibians, reptiles and fish. An exception was found with the Tuatara, a New Zealand endemic reptilian *Sphenodon punctatus,* which showed a 64%

positive amino acid match with gull albumin. This finding is contrary to gull TTR that I previously demonstrated was closer in homology to reptilians than to mammalian species (Ucan-Marin et al 2009). Therefore, the use of human albumin protein to assess the T_3 or T_4 competitive binding with xenobiotic substrates is not suitable to assess T_3 or T4 transport via albumin in avian species, but specifically in gulls *(Laridae).*

4.3.4 T3 and T4 ligand binding to gull recALB versus human recALB

I observed that gull and human recALB were clearly contrasting in the binding affinity for T3 or T_4. In gull recALB, T3 had a ~5-fold greater binding affinity than T4, while in human recALB had greater affinity for T_4 (~ 4-fold greater) relative to T_3. Avian recALB had a ~20-fold greater binding affinity than human recALB relative to binding T_3. However, binding affinity was comparable for T_4 with recALB for human and gull. It has been reported that at equimolar amounts, T3 possesses several orders of magnitude higher binding affinity than T_4 in avian species (Tritsch and Tritsch 1965). It was also reported that in avian species, TRs have the highest affinity for T3 relative to T_4, although T_4 can trigger TH effects despite lower binding affinity (Decuypere et al 2005; McNabb and Wilson 1997).

Table 3. Competitive binding parameters for recombinant gull albumin (ALB) and transthyretin (TTR) hormone transport proteins (THBPs) and T_3 or T_4, and in the presence of model PCB and substituted structural analogue ligands, and compared to recombinant human TTR and ALB with T3 and T4 for comparison.

Compound		Ki (nM) [a]	Relative potency[b]	Kd[c]	Maximum % competition [d]
T_3	TTR	5.99	1	17.63	88±4.1
	ALB	1.12	1	3.77	90±2.6
CB187	TTR	791.05	1.2x10-*	188.05	89±3.7
	ALB	15.1	7.4x10-*	56.09	91±2.8
4-MeO-CB187	TTR	55.08	0.108	23.5	90±3.6
	ALB	4.5	0.248	5.63	90±2.8
4-OH-CB187	TTR	1.9	3.152	11.17	89±1.7
	ALB	0.89	1.258	2.99	91 ±2.8
T_4	TTR	15.74	1	27.5	91±3.7
	ALB	5.11	1	18.32	90±2.6
CB187	TTR	91.33	0.171	36.04	76±5.8
	ALB	89.1	0.057	24.09	83±4.3
4-MeO-CB187	TTR	981.15	0.016	432	86±2.6
	ALB	15.81	0.322	58.8	89±3.1
4-OH-CB187	TTR	13.9	1.19	10.8	90±1.8
	ALB	4.19	1.246	4.63	91±2.7
Human THTPs					
T_4	TTR	56.88	1	14.9	98±2.1
	ALB	7.91	1	2.77	91±3.9
T_3	TTR	207.67	0.27	60.1	77.3±3.7
	ALB	23.88	0.331	8.01	89±3.5

Note: Results presented as the mean of individual measurements, and based on n=6 replicates (combined triplicates on two different days). The ±SD for relative potency and maximum % competition is based on the standard deviation of the two values comprising the ratio. See Figure 19 for competitive binding curves.

[a] *K* (or I_{50}) = K_d (1+ *To/Ka)* (Cheng and Prusoff 1973). See Materials and Methods section for further details.

[b] Calculated as ratio of IC_{50} for a chemical (Y/X) = E[Y^A2] V (1/X) + V(Y) E [1/X] ^{A}2

[c] The K_d values (mean ± SD) were determined from the slope of the linear regression line of Scatchard plots

[d] Percentage of competition reached at highest tested concentration (1x10^5 nM).

Table 4. Competitive binding parameters for recombinant gull albumin (ALB) and transthyretin (TTR) hormone transport proteins and T3 or T_4, and model PBDE and substituted structural analogue ligands.

Compound		Ki (nM)[a]	Relative potency[b]	Kd[c]	Maximum % competition[d]
T_3	TTR	5.99	1	17.6	88±4.3
	ALB	1.12	1	3.75	91±2.8
BDE47	TTR	529.14	0.011	189	88±3.8
	ALB	119.8	9.3x10"*	67.9	90±1.8
6-OH-BDE47	TTR	69.08	0.07	8.06	91±2.7
	ALB	1.02	1.09	2.9	92±2.9
4-OH-BDE49	TTR	4.89	1.12	10.01	90±2.2
	ALB	0.79	1.41	1.98	89±4.3
6-MeO-BDE47	TTR	233.7	0.025	39.6	90±3.5
	ALB	109.8	0.010	33.21	89±3.9
T_4	TTR	15.74	1	33.7	90±2.7
	ALB	5.11	1	18.32	90±3.5
BDE47	TTR	89.1	0.176	221.8	92±4.1
	ALB	95.12	0.053	251.29	92±3.1
6-OH-BDE47	TTR	11.91	1.32	19.9	95±3.6
	ALB	4.99	1.02	15.43	94±3.9
4-OH-BDE49	TTR	7.68	2.049	11.57	96±3.9
	ALB	4.11	1.2433	5.98	92±2.9
6-MeO-BDE47	TTR	54.32	0.289	117.7	94±3.7
	ALB	42.15	0.12	108.09	94±3.1

Note: Results presented as the mean of individual measurements, and based on n=6 replicates (combined triplicates on two different days). The ±SD for relative potency and maximum % competition is based on the standard deviation of the two values comprising the ratio. See Figure 20 for competitive binding curves.

[a] $K\backslash$ (or I_{50}) = Kd(1+ T_0/K_d*) (Cheng and Prusoff 1973). See Materials and Methods section for further details.

[b] Calculated as ratio of IC_{50} for a chemical (Y/X) = E[Y^A2] V (1/X) + V(Y) E [1/X] ^{A}2

[c] The Kd values (mean ± SD) were determined from the slope of the linear regression line of Scatchard plots

[d] Percentage of competition reached at highest tested concentration ($1x10^5$ nM).

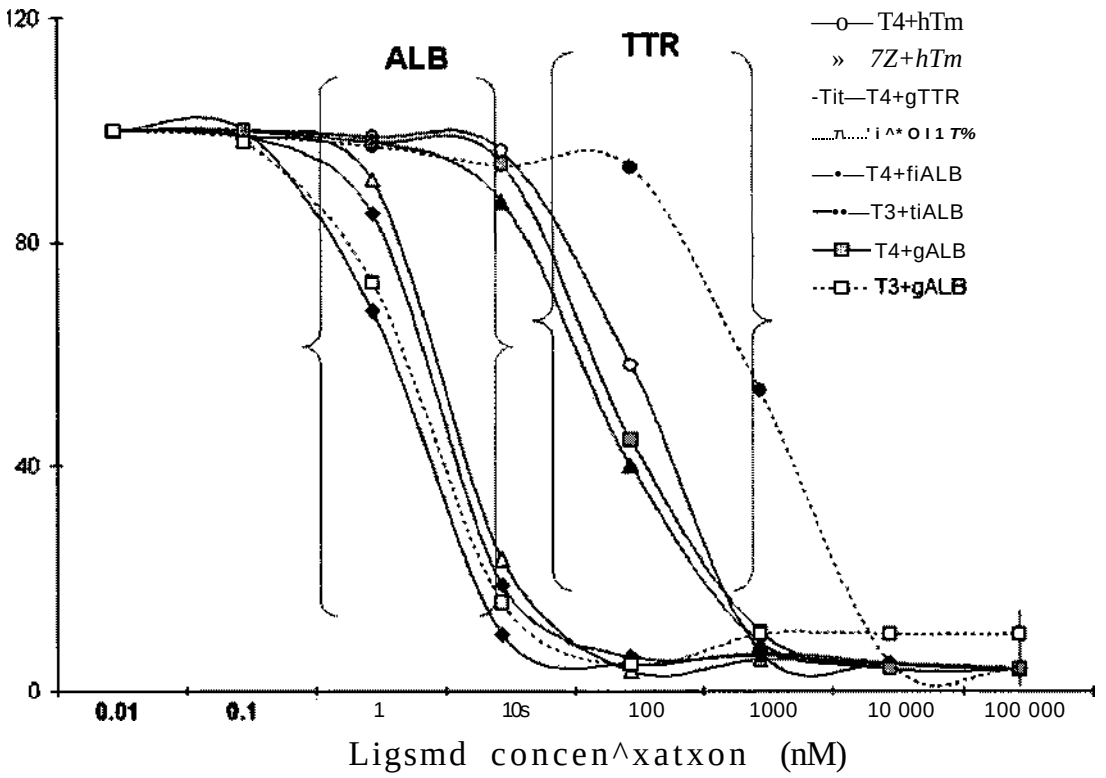

Figure 14. Concentration-dependent, competitive binding curves for recombinant gull albumin (gALB) and transthyretin (gTTR), and recombinant human hALB and hTTR, with T3 or T4 Areas in brackets denote contrasting competitive binding concentrations and parameters (Table 3) for TTR and ALB.

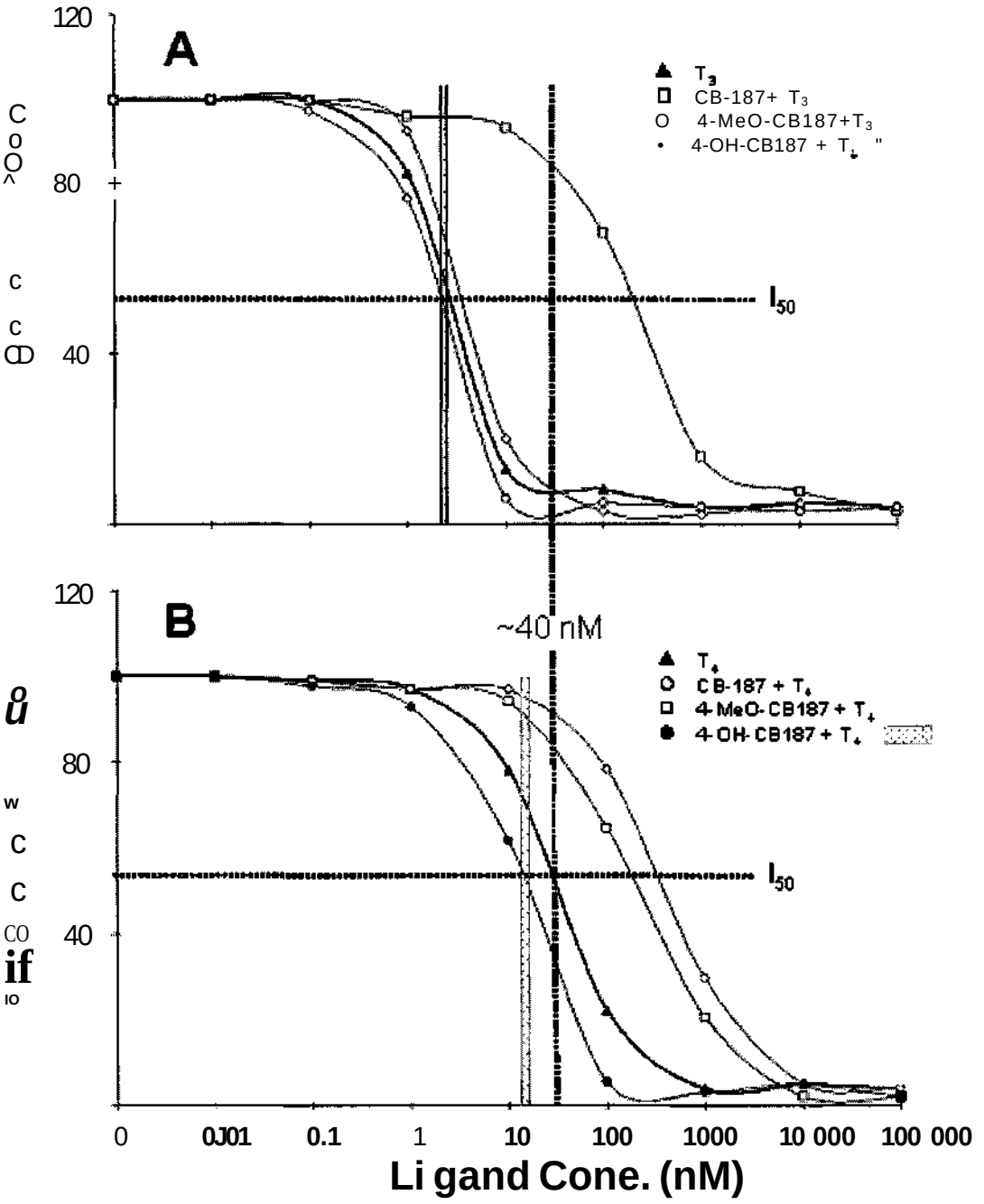

Figure 15. Concentration-dependent, competitive binding curves (percent relative to controls; n=9 replicates, three sets of n=3 triplicate sets performed on different days) for (A) T_3 and (B) T_4 displacement from gull recALB by 2,2',3,4',5,5',6-heptaCB (CB-187), 4-hydroxy-CB187 (4-OH-CB187) or 4-methoxy-CB187 (4-MeO-CB187). The competitive binding parameters are listed in Table 3. Concentration ranges in brackets denote blood plasma concentrations recently reported in Norwegian (Svalbard) glaucous gulls (Letcher et al 2009; Verreaultetal. 2005a).

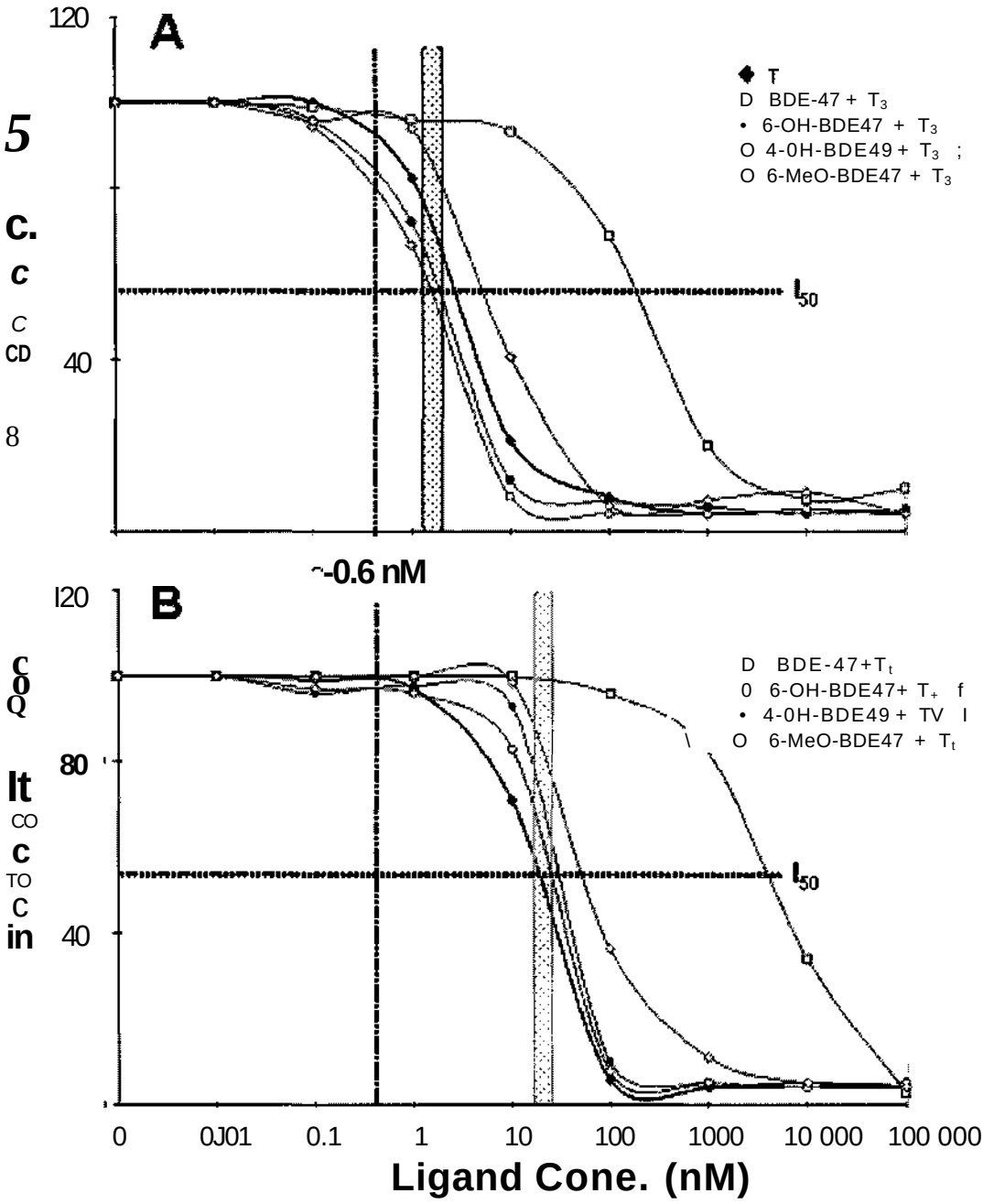

Figure 16. Concentration-dependent, competitive binding curves (percent relative to controls; n=9 replicates, three sets of n=3 triplicate sets performed on different days) for (A) T_3 and (B) T_4 displacement from gull recALB by 2,2',4,4'-tetrabromoDE (BDE-47) flame retardant and 6-hydroxy-BDE47 (6-OH-BDE47), 6-methoxy-BDE47 (6-MeO-BDE47) or 4-OH-2,2',4,5-tetrabromoDE (4-OH-BDE49). The competitive binding parameters are listed in Table 4. Concentration ranges in brackets denote blood plasma concentrations recently reported in Norwegian (Svalbard) glaucous gulls (Letcher et al 2009; Verreault et al 2005b).

There are also differences among avian and other taxonomic groups with respect to TH binding interaction with albumin. For example, T3 is the primary TH bound to receptors *in vivo* in the few cases where this has been investigated and the physiological potency of T3 is much higher than that of T4 in mammals (Engler and Burger 1984). Also, it was reported by Bellabarba et al (1988) that avian TRs also have the highest affinity for T3 and is virtually identical to mammalian TRs. In this study, the differences in the binding affinity of THs and recALB either from gulls or human could be attributed to the complex process interaction of THs with albumin, and relative to *in vivo* interactions where THs are subject to a wide variety of enzyme-dependent pathways such as via deiodinases, uridine diphosphate glucuronyl-transferase UDPGT and thyroperoxidases (Miller et al 2009).

4.3.5 Competitive T3 or T4 binding of contaminant substrates with gull recALB

To my knowledge, no scientific reports presently exist on xenobiotic interactions and binding affinity with recALB from a vertebrate species including birds. In fact, in the context of overall TH binding in birds, albumin is reported to be the most important THBP in chicken with circulating T4 being bound 75% to albumin, 17% to TTR and 7.5% to an a-globulin (McNabb et al 2003). However, research reports on THBP binding interactions with xenobiotics have predominantly been with TTR and mainly with human TTR protein. Results obtained in this study with

the OH-PCBs and -PBDEs showed higher affinity to human and gull recALB compared with MeO-substituted analogues and unsubstituted PCB and PBDE congeners. This was consistent with studies on BDE47 binding with gull recTTR (Ucan-Marin et al 2009). This is also consistent with results reported by Hamers et al (2006) who screened a set of 27 individual BFRs, including 19 PBDE congeners and 6-OH-BDE47, for their relative potency of competition with T_4 for binding *in vitro* with human TTR. Meerts et al (2001) reported no substantial human recTTR binding for 17 PBDE congeners, including BDE47, at maximum treatment concentrations of 0.25 uM. Furthermore, Meerts et al (2001) showed high binding affinity by 6-OH-BDE47 in competition with T4, but was of lesser potency than for other brominated phenolic substances such as tetrabromobisphenyl A (TBBPA) and 2,4,6-tribromophenol (2,4,6-TBP). Consistent with this finding, for gull and human recTTR and recALB, 4'-OH-BDE49, which is para-hydroxylated was a more effective competitor than 6-OH-BDE47 or BDE47. In one rare fish example, Morgado et al (2007) reported that there was moderate competitive binding between several PBDE congeners and 6-OH-BDE47 and the displacement of [^{125}I]-T3 binding to sea bream recTTR, although with a significantly higher ($p<0.001$) IC_{50} value (700.6 ± 40.4 nM) than for THs and at a concentration (10x) higher than the highest concentration used in the present study with human and gull recTTR. For both OH-PBDEs and OH-PCBs, it has been proposed that for optimal THBP competitive binding (with T4), hydroxylation should exist at a *para* position (relative to the aromatic ring linkage), and that there be one, but preferably two, halogen substituents on

carbons adjacent to the OH-group (Lans et al 1993; Hamers et al 2006; Morgado et al 2007; Ucan-Marin et al 2009). It is also possible that the ligand binding differences observed in the present study, and reflected in the K_d and *K*\ values for gull versus human recALB, may be attributed to differences in the amino acid sequence in the TH binding domain. It could also be attributed to an evolutionary divergence of human and gull albumin. Albumin also has an important role in the binding and transport of a wide variety of small molecules, and especially organic anions (e. g. bilirubin, haem, and fatty acids), which is likely species-dependent (Wallace and Wilson 1972).

The environmental relevance of the IC50 concentrations and *K,* and *Ka* values of competitive binding of OH-PCB and OH-PBDE ligands under study, with respect to gull recALB, is emphasized by comparisons to known circulating levels of these contaminants in free-ranging gulls. To my knowledge, there are no reports on circulating OH-PCB, OH-PBDE, PBDE or possibly MeO-PBDEs in herring gulls from the Laurentian Great Lakes. However, it has been reported that the mean concentrations of 6-OH-BDE47 and 4'-OH-BDE49 in the plasma of male and female glaucous gulls from the Norwegian Arctic was up to 0.32 ng/g (wet weight) (Verreault et al. 2005b), which is -0.0006 nM. In the present competitive binding with gull recALB, the same ligands effectively displaced T3 or T4 at substantially higher concentrations. Therefore, environmentally relevant OH-PBDE concentrations are not likely to substantially affect circulating T_3 or T4 levels in wild glaucous gulls. In contrast, 4-OH-CB187 was reported at a plasma

concentration of 17.5 ng/g ww (-0.04 nM) in the same glaucous gulls (Verreault et al., 2005b). Furthermore, 4-OH-CB187 competitively displaced T3 and T_4 from gull recALB at 5 to 10 nM. Therefore, 4-OH-CB187 levels in free-ranging Svalbard gulls may be high enough to effect circulating T_3 or T_4 levels. This is consistent with my recent *in vitro* binding studies with gull recTTR from the same samples (Ucan-Marin et al 2009).

4.3.6 Implications ofxenobiotic modulation of thyroid hormone transport

It is possible that the same levels of contaminants circulating in gull blood (e.g., Norwegian glaucous gulls) could effectively disrupt TH homeostasis due in part to the fact that albumin has a higher binding affinity than TTR and is thus more susceptible to displacement competition by hydroxylated compounds such as OH-PCBs or OH-PBDEs. It is also possible that an influence and/or change on the cellular TH levels and the ratio of T3 and T4 could affect TH homeostasis (Mendel 1989), and perhaps the TH-dependent function and affected health of exposed individuals. In birds and other wildlife, an implication of the competitive xenobiotic displacement of T_4 from albumin or TTR would result in less T4 available to target tissues, and a subsequent decrease in the amount of T_4 substrate for deiodinase enzymes, including the 5' deiodinases Dl and DM, and thus T_4 conversion to active T3 and subsequently the decreased availability of T3 to TRs (Brouwer et al 1998; Verhoelst et al 2005). In mammals for example, OH-PCB (4-hydroxy-2',3,3',4',5-pentachlorobipheny) binding affinity using rat liver

cytosol and T_2 suggested that the nature of the T_2 sulfation inhibition is competitive (Schuur et al 1998).

T_4 and particularly the primary metabolically active T_3 are considered the prime controllers for the regulation of metabolic functions and thermogenesis in mammals and birds (McNabb and Fox 2003). T4 and T_3 are suspected to be involved in a partial dissipation of the mitochondrial proton electrochemical gradient that would uncouple phosphorylation from oxidation and hence produce heat. High concentrations of contaminants in mammals and birds may alter circulating TH status, basal metabolism and capacity for adaptive thermogenesis. Verreault et al (2007) recently reported for breeding glaucous gulls (from the Svalbard area), negative associations between basal metabolic rate and concentrations of PCBs, DDTs and particularly chlordanes. However, levels of THs were not associated significantly with variation of basal metabolic rate or concentrations of any blood residue levels on OH-PCBs or OH-PBDEs.

Further studies are necessary to observe effects e.g., *in vitro* sub cellular and OHC correlations at the *in vivo* (whole organism) level in free-ranging wildlife where TH-dependent effects are suggestive (Letcher et al 2009). There is a dearth of studies examining complex OHC exposures, and virtually none have addressed competitive binding with THs and THBP interaction. More experiments are needed to address the differences on species and population specifics such as TH-related effects with THBPs and confounding factors: (i)

physiological status and timing (e.g., reproductively active); (ii) sensitivity as a function of other stressors (e.g., climate change), (iii) deleterious (chronic) effects and risks at organism or population levels.

4.3.7 Conclusions

The present gull albumin molecular characterization and competitive binding studies clearly showed that, although there are high amino acid sequence similarities among human and gull albumin than was observed for human and gull TTR, human albumin cannot be used as a surrogate to assess the effects on circulating THs in wildlife. Differences between human albumin and TTR for TH binding are several fold compared with gull albumin and TTR. The present study contributes with novel values of binding affinities of gull recALB binding T3 and T4, and also through a set of environmentally relevant contaminants found in circulating plasma of gulls. It was observed that OH-metabolites either chlorinated or brominated had higher binding affinity than THs to albumin and TTR. This indicates that the combination of the more TH-like brominated diphenyl ether backbone (relative to the chlorinated biphenyl backbone), and in combination of having an OH-group, results to more effective competitive ligand on gull hormone transport proteins relative to both T3 and T4. A ligand-binding parameter assessment for TH transport protein in gull species, and in wildlife in general, are necessary to fully understanding the potential effects in reproductive, nutritional and physiological processes that could be influenced by changes in the circulating T4 and T_3, and subsequently TH-dependent processes.

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CHAPTER FIVE[3]

Organohalogens and Metabolites in the Blood and Livers of Lake Ontario Herring Gulls *(Larus argentatus)* and Competitive Binding of Complex Mixture Fractions With Gull Thyroid Hormone Transport Proteins *In Vitro*

5.1 Abstract

Polychlorinated biphenyl (PCB) and polybrominated diphenyl ether (PBDE) flame retardant congeners are environmentally relevant organohalogen contaminants (OHCs), and as well as some of their hydroxylated (OH) analogues they have been reported to have effects on thyroid hormones (THs)-dependent processes. The Great Lakes herring gulls *(Larus argentatus)* are a suitable bio-monitor of health ecosystems, due to their natural range, diet condition, and opportunistic feeding patterns. The main objective of this study was to examine if two selected chemical complex fractions (phenolic and neutral fractions extracted from plasma samples), were able to disrupt the affinity of TH transport proteins binding THs. In this study two colonies of Herring gulls: one from Hamilton Harbour (HH) and from Scotch bonnet Island (SBI) where sampled, analyzed and compared. Liver and plasma (n=10, 5 females, 5 males) were screened for OC (21 compounds), PCB (71 congeners), PBDE (15 congeners), $MeSO_2$-CB (16 congeners) and HO-

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PCB (33 congeners), HO-PBDE (14 congeners), 4-HO-heptachlorstyrene (4-HO-HpCS) and $MeS0_2$-DDE. Other BFRs including pentabromotoluene (PBT), hexabromobenzene (HBB), 2,2',4,4',5-pentabromobiphenyl (BB-101) and total-(a)-hexabromocyclododecane (HBCD) were also measured. PCA observed show a positive relation between OH-PCB/PBDE and plasma of herring gulls from HH. This is the first report on circulating £OH-PCB, £OH-PBDE, £PBDE and £MeO-PBDEs in herring gulls plasma and livers from the Laurentian Great Lakes. During the plasma preparation to analysis, complex mixtures samples from acidic and neutral fractions were isolated; then used in competitive binding assays in presence of recombinant albumin (gALB, hALB), and transthyretin from gulls and humans (gTTR, hTTR) for the binding of T_4 and T3. The results showed a higher concentration of OHCs from HH than SBI herring gulls. Competitive binding assay shows that the neutral fraction did not have significant impact in the binding; but the phenolic fraction (containgin OH-PCBs and OH-PBDEs) was able to disrupt significantly ($p<0.001$) the binding of hTTR with T_4, hALB with T_4, and the gTTR with T3. Concluding that human T_4 and T3 differ in their affinity for thyroidogenic environmental pollutants.

5.2 Introduction

Modern industrial and agricultural practices in the Great Lakes basin began in the early 1940s. Since that time, thousands of chemicals and synthetic compounds have been discharged into the environment. Over 400 different man-made chemicals have been detected in Great Lakes biota; many of these are toxic, bioaccumulative and persistent (Environment Canada, 2009). Research and monitoring have focused on heavy metals such as mercury, organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), dieldrin and mirex, and other chlorinated organics such as polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), dioxins and furans.

Endocrine disrupting compounds (EDCs) are compounds that alter normal hormone regulation (Waring et al 2005). They may be naturally occurring, such as the antioxidant flavonoids, which are found in fruits and vegetables, or may be industrial chemicals, such as some types of plasticizers, which act as environmental contaminants. Wide ranges of species, from crustaceans, fish, birds through to mammals and man, have been reported as being deregulated by EDs (Waring et al 2005). The biological actions of hormones, including oestrogens, androgens, progesterone, thyroxine and the neurosteroids pregnenolone and dehydroepiandrosterone (DHEA), are mediated via high affinity protein receptors within the target cells. DDT and other organochlorines

carried some of the earliest work on ED effects out in bird populations affected. These compounds are now banned over most of the world but their use in the 1960s coincided with a decrease in reproductive ability of birds, particularly those at the top of the food chain such as raptors and gulls (Halldin et al 2003).

Since the early 90s it has been suggested that PCBs may be causing disruption of thyroid function in wild birds (Fox 1993). Herring gulls *(Larus argentatus)* exposed to pollutant loads in the Great Lakes have exhibited increased ontogenic and immunological problems compared to reference populations (Moccia et al 1986; Grasman et al 1996). Thyroid gland histopathology in gulls with high environmental PCB exposure (Moccia et al 1986) and developmental effects in chicken embryos exposed to PCBs *in-ovo* (Powell et al 1996) are suggestive of thyroid abnormalities. Herring gulls are considered important indicators of habitat quality because they are mostly piscivorous top predators (and commonly opportunistic feeders) that are exposed to concentrated pollutant loads through bioaccumulation in the food chain. Studies had published a strong relation between high organohalogen concentrations and depletion in thyroid hormone stores in their thyroid glands compared to gulls from reference sites (McNabb et al 2001 •).

Recent work in Canada has measured contaminants present in eggs from Herring gulls from highly polluted sites in the Great Lakes, with novel reports on the PBDEs (including BDE-209 and nonabromoDE and octabromoDE gradation

products) and several non-PBDE Brominated Flame Retardants, including BTBPE and DBDPE, from egg pools from diverse colonies (Gauthier et al 2008, Gauthier et al 2009).

However, no reports in hydroxyl- (OH) and methoxyl (MeO)-containing analogues (i.e., 4-OH-CB187, 6-OH-BDE47, 4'-OH-BDE49, 4-MeO-CB187 and 6-MeO-BDE47) reported in other gulls, had been reported for Herring gulls residing in the Great Lakes. OH-PCB compounds had been observed to disturb the competitive binding of thyroid hormones (THs) *in-vitro* using human TH transport proteins (Meerts et al 2000; Ucan-Marin 2009a; 2009b). This study will address if these compounds are present in detectable levels, and if they vary from two bird colonies (Hamilton Harbor (HH) and Scotch Bonnet Island (SBI)) at Lake Ontario.

A selected extraction of complex chemical fractions from plasma will be also addressed through competitive binding studies to observe the possibility of disruption of TH transport proteins when binding THs. Attention to the effect of EDCs complex chemical mixtures (Levin et al 2005; 2007) in modulation of different physiology process has increased recently. Since TH transport proteins are disrupted in presence of selected OH-PCBs (Purkey et al 2004) and OH-PBDEs (Meerts et al 2000; Ucan-Marfn et al 2009a).

This study intend to answer if the binding of THs to TH transport proteins can also be disrupted by the presence of a chemical complex fractions [phenolic (OH-

PCB/PBDE) and neutral (PCB and PBDE) containing], and the differences among human and avian TH transport proteins.

5.3 Results and Discussion

5.3.1 Contaminants in blood and liver of herring gulls from Lake Ontario

The North American Great Lakes are an example of an ecosystem impacted by both local sources and by atmospheric deposition of contaminants transported over hundreds or thousands of kilometers (Eisenreich 1981, Baker 1997). Avian wildlife has frequently been used as an indicator of chemical pollution exposure and toxicity for assessing the health of aquatic ecosystems. PCB levels in the Great Lakes had been reported in previous reported on birds to be up to £PCBs of 59,000 ng/gr ww (Weseloh et al 1990, 1994; Haffner et al 1997). In the Great Lakes, considerable success has been achieved using fish-eating birds such as double crested cormorant *(Phalacrocorax auritus)* and Herring gull *(Larus argentatus),* as representative of contaminant exposure dynamics and in the upper trophic levels (Fox et al 1991a). Furthermore, Fox et al (1991b) noted that this species is not only representative of chemical exposures, but also of toxicological stress. Historically, herring gulls have provided an integrated measure of chemical bioavailability because of their diverse feeding habits (Weseloh et al 1990; Norstrom et al 1991; Herbert et al 1994).

5.3.2 Organochlorine compounds

Plasma and liver samples were examined during this study for major organohalogens compounds and metabolites, and assessed the inter-population and -tissue of concentrations and congener patterns, for two populations of herring gulls at the far east end of Lake Ontario (SBI) and the far west end of Lake Ontario (HH). No significant ($p<0.05$) differences in contaminant levels and patterns bettwen males versus females were found among sites, when organizing data from SBI and HH was addressed.

Differences in the concentrations ($p<0.005$) were observed for the two bird colonies in Lake Ontario; HH had significant higher levels of contaminants in livers and plasma than SBI herring gulls. The major organochlorine group found in herring gulls livers and plasma were ZDDT (predominantly p,p'-DDE) followed by ZPCB, ZCHL (c- and t-nonachlor) and ZCBz (mainly HCB). This pattern is consistent with results from other studies on Great Lakes Biota (Baumann and Whittle, 1988) including herring gull eggs (Gibertson, 1974; Gilman et a, 1977; Waseloh et al, 1979; Mineau et al, 1984: Weseloh et al, 1989). This is the first report of the pattern of organochlorines in the Lake Ontario using liver concentration levels of herring gulls.

The arithmetic mean concentration of the principal classes of chlorinated and brominated contaminants (and metabolites) for plasma and liver of HH and SBI

herring gulls are listed in Table 6. I found that IPCB concentrations in plasma of HH gulls were 1.7-fold and significantly, higher ($p<0.005$) than the herring gull colony at SBI; while, in the livers analyzed from HH (6279±1025 ng/g) were 4.5-fold ($p<0.003$) higher than SBI. Differences in the concentration among colonies in the Lake Ontario were found, consistent with traditionally levels of organochlorine contaminants in herring gull eggs reported from 1970s and 80s had been higher in Lake Huron and in Lake Superior compared to Lake Erie or the lake Ontario (Mineau et al 1984). Differences in the feeding habits of the two colonies sampled in this study can be attributed to the higher concentrations of IPCB found in HH compared to SBI. Hebert et al (1999) reported that stable nitrogen ($d^{15}N$) and carbon ($d^{13}C$) isotope measured in lipid-free homogenates of herring gulls eggs collected from the Laurentian Great Lakes from 1974 to1995 had significant differences in gull trophic positions found among diverse colonies. The authors attributed the results to the differing proportions offish in the diets of gulls from the various colonies; where aquatic foods available to gulls had greater $d^{15}N$ values than terrestrial foods, concluding that terrestrial foods, particularly waste and urban garbage, were more enriched in ^{13}C than aquatic feeding patterns. As the proportion of fish in the diet decreased, the fraction consisting of terrestrial food increased, resulting in an increase in $d^{13}C$ values and a decline in $d^{15}N$ values. Fish availability besides the stability physicochemical properties of compounds and water surrounding the colonies of gulls are possibly main factors in the higher concentration found in HH site.

This is the first report of concentrations of ZPCBs for livers inLake Ontario, then comparison of levels found are not comparable iwht published data, however levels found in this study are similar to the data published for arctic birds. Mallory et al (2005) analyzed liver tissues of glaucous gulls, *(Larus hyperboreus)* from Qikiqtarjuaq, (Nunavut, Canada) with values of 8,013 ng/g ww.

In this study, the dominant congeners were PCB 138, 153, 180 and 118, trend that repeats the dominant congeners of PCBs in plasma and liver of herring gull from Lake Ontario. The dominant congeners PCB 153 was found in the samples of this study in concentrations of 169 ng/g ww for plasma and 1619 ng/g ww for liver in HH and herring gull. Concentrations for SBI site were 29.02-ng/g ww for plasma and 325 ng/g ww for the liver. The most dominant congeners reported in herring gulls at Lake Ontario during 1981 (Haffner et al, 1997) were PCB 138, 180, 153 and 118 and during 1992 with a decrease in the concentrations, but with the same pattern of PCB congeners (118, 153, 180 and 138).

Concentrations observed for PCB-138 were 162 ng/g ww on plasma and 1638 ng/g ww on liver of Herring gulls at HH site. Gulls at SBI location had 29.3 ng/g ww on plasma and 278 ng/g ww in livers. PCB-180 in plasma of herring gulls from HH had concentrations of 94.12 ng/g ww and 929 ng/g for the liver, while samples from SBI were 19.06 ng/g ww on plasma and 173 ng/g ww in liver. The next PCBs in order of concentration detected were PCB118, 187, 170/190 and 70/76.

Since the early 1970s, the Canadian Wildlife Service has been monitoring organochlorines, including DDE and PCBs, in wildlife (Norstrom, 1988). Eggs of fish-eating birds have been the main monitoring tissue, and in 1974, the herring gull was adopted as the main avian species for monitoring organochlorine contaminants in the Great Lakes (Mineau et al 1984). £DDT j_n this study was observed 3.2-fold higher on the plasma concentration of herring gulls from HH site than SBI. Comparing the livers of herring gulls from HH with SBI, they had 3.4-fold higher concentrations (Table 6).

The compounds in order of concentration detected were: p,p'-DDE with 296.3 ng/g ww for plasma samples from herring gulls at HH site and 76.01 ng/g for plasma on SB gulls. Livers from HH site had 2486 ng/g ww, and from SBI had 623.5 ng/g ww. These concentrations detected are very low compared to previous report for Herring gulls eggs from Hamilton Harbour in 1989 (Weseloh et al, 1995) where the authors registered DDE cocentrations of 56,000 ng/g ww.
For the *p,p* -DDT concentrations of 19.72 ng/g ww for plasma in gulls from HH site was observed, and 20.5 ng/g ww for SB gulls. The livers from HH site had levels of 477 ng/g ww and SBI had 225 ng/g. Regarding the p,p'-DDD found in plasma, were detected levels of 1.91 ng/g ww on HH site and 0.37 ng/g ww for herring gulls residing at SBI. Liver concentration levels of p,p'-DDD were 15.55 ng/g ww for Hamilton site and 2.38 ng/g ww for the herring gulls at Scotch Bonnet Island.

Table 6. Arithmetic mean, Standard Error (SE) and Data Range of concentrations of ^compounds identified in plasma and liver of Herring gulls from Hamilton Harbour (HH) and Scotch Bonnet Island (SB).

ng/g ww	**Plasma**		**Liver**	
	HH	SB	HH	SB
£PCBs[a]				
Mean± SE	787±275	4651192	6279±1025	1537±387
Range	68-3402	83-2013	1121-45568	617-8174
IOH PCBs[b]				
Mean± SE	39.3±9.4	22.7±6.5	38.3±11.8	16.7±6.1
Range	ND-86.5	ND-74.3	ND-91.5	ND-116
IPBDEs[c]				
Mean± SE	166±37.2	58.95±11.9	1857±198.5	665±181.7
Range	4.9-314	2.85-219	21.8-3451	19.8-2178
ZOH PBDEs[d]				
Meant SE	1.1 ±0.62	0.34±0.12	0.66±0.21	0.56±0.17
Range	ND-5.9	ND-6.8	ND-10.1	ND-6.4
IMeO PBDEs[e]				
Mean± SE	4.1±1.7	2.87±0.92	37.8±5.2	22.5±4.8
Range	0.68-15.4	0.83-12.01	10.2-45.5	6.1-38.7
£CHL*				
Mean± SE	8.3±2.4	1.62±0.65	79.5±18.8	18.2±4.1
Range	ND-26.5	ND-14.3	ND-151.5	ND-56.7
ICBz[9]				
Meani SE	21.6±4.2	3.31±0.96	16.45±5.5	3.4±1.7
Range	ND-41.2	ND-21.9	ND-45.1	ND-23.8
IDDTs[h]				
Mean± SE	316.43±61.1	98.13±23.68	2978±521	855±247
Range	10.23-915	12.23-438.56	95.68-5213	89.6-2489
£ Mirex'				
Mean± SE	187±39.13	42.01±9.21	1477±352	373.2±97.45
Range	15.9-356	13.8-102	23.6-2563	29.4-856

[a] IPCB: sum of PCB 18, 17, 16/32, 31, 28, 33/20, 22, 52, 49, 47/48, 44, 64/41, 74, 70/76, 95, 66, 56/60, 92, 101/90, 99, 97, 87, 85, 110, 151, 149, 118, 146, 153, 105, 141, 130, 176, 137, 138, 158, 178, 187, 183, 128, 167, 174, 177, 202, 171, 156, 200, 157, 172, 180, 170/190, 189, 199, 196/203, 208, 194, 195 and 206, 207; [b] IOH PCBs: Sum of 4'-OH-CB79, 4-OH-CB146, 4'-OH-CB177, 4-OH-CB187, 4'-OH-CB199, 4'-OH-CB201, 4'-OH-CB202; [c] IPBDE: sum of PBDE 47, 99, 100, 138.154/BB153, HBCDD, 153, and 183; [d] £OH PBDEs: Sum of 6'-OH-BDE49, 6-OH-BDE47, 3-OH-BDE47, 4'-OH-BDE49; [e] £MeO PBDE: Sum of 6-MeO-BDE47and 5-MeO-BDE47 3-MeO-BDE47, 4-MeO-BDE49; [f] ICHL: Octachlorostyrene, Heptachlor.epoxide, Oxychlordane, t-Chlordane, c-Chlordane, t-Nonachlor; [9] £CBz: Sum of 1,2,3,4-Tetrachlorobenzene, 1,2,4,5-Tetrachlorobenzene, Pentachlorobenzene, a-Hexachlorocyclohexane, Hexachlorobenzene; [h] £DDTs: p,p'-DDE, p,p'-DDD and p,p'-DDT;' £Mirex: Photomirex and Mirex

There are other recent new data of DDT in herring gulls livers and plasma from Lake Ontario, so to compare the concentration levels found in plasma of herring gulls a comparison was made with recent publications overseas. Concentrations detected by Verboven et al (2009) in plasma of gulls (Glaucous) were of 310.1 ±50.8 ng/g ww from Bjornoya Norway. Verboven et al (2009) values were similar to the data obtained from Hamilton Harbour site. In another study, Mallory et al (2005) reported a £DDT $_0$f 4263 ng/g ww for livers of glaucous gulls from Qikiqtarjuaq (Nunavut Canada). These values are 1.5-fold higher than the values for HH herring gulls livers, and 5-fold higher than the livers from SBI. The same pattern repeats in this data, agreeing to previous data published for herring gull eggs by Weseloh et al (1995) that published that Cis-nonachlor and T-nonachlor as the main contaminants of £CHL Differences were observed when comparing values, because data in this study was significantly lower than the levels reported from eggs at Hamilton Harbour in 1989 where values for t-Nonachlor were 840 ng/g ww and c-Nonachlor had 620 ng/g ww. In addition, data obtained for £CHL were similar with recently reported concentrations overseas by Verboven et al (2009); where the analysis of £CHL in plasma of glaoucous gulls from Bjornoya, Norway was 15.9±2.7 ng/g in females and 39.0±12.0 ng/g for males.

Comparison of £CBz, for gulls from HH and SB showed that plasma of herring gulls from HH was 6.5-fold higher than SBI and concentrations from herring gull livers from HH were 4.8-fold higher than SBI. The dominant congeners analyzed in this group of chemicals where hexachlorobenzene and pentachlorobenzene.

Concentrations of hexachlorobenzene in plasma were 19.6 ng/g ww for herring gulls at HH and 2.97 ng/g ww for gulls from SBI. Liver concentrations were 14.56 ng/g ww for HH site, and 3.12 ng/g ww for herring gulls from SBI. Values of pentachlorobenzene found in this study are significant low compared to the values (520 ng/g ww) reported in 1989 for herring gulls eggs at Hamilton Harbour by Weseloh et al (1995). Data of £CBz, from Mallory et al (2005) in glaucus gull livers from Qikiqtarjuaq; (Nunavut Canada) reported concentrations of 495 ng/g ww; data that is 30-fold higher than the values that I observed for herring gull livers at HH, but similar to the concentrations reported in 1995 for Hamilton Harbour herring gull eggs (Weseloh et al 1995).

Levels of £Mirex $_{w}$here mostly conformed for Photomirex and Mirex, where XMirex on plasma of HH gulls was 4.5-fold higher than the levels found in SBI gulls. Level values in livers of HH gulls were 4-fold higher than the gulls sampled from SB. Mirex was the dominant compounds with the highest concentration in liver and plasma in both sited sampled followed by Photomirex. Mirex concentration found in this study differs from Mirex concentrations reported in herring gull eggs at Hamilton Harbour with 8800 ng/g (Weseloh et al 1995). The concentration found in this study of photomirex were significantly lower than concentrations for herring gull eggs reported for HH site in 1989, where Weseloh et al (1995) had values of 3590 ng/g ww. However, £Mirex concentration levels in this study in livers from SBI were similar to values reported by Mallory et al, (2005) for the livers of glaucus gulls (324 ng/g ww) at Qikiqtarjuaq, Canada. Data

from herring gull livers from HH in this study were 4.5-fold higher than SBI gulls and also higher than gulls analyzed by Mallory et al, (2005) from Qikiqtarjuaq.

Some studies had reported declines in most of the legacy persistent organic pollutants (e.g. PCBs, DDT), with data over the recent decades that have been documented a number of seabird species throughout the marine environment of the northern hemisphere; e.g. gannets *(Sula bassana)* from western Scotland (Alcock et al 2002), common terns *(Sterna hirundo)* from the Wadden Sea (Becker et al 2001), guillemots/common murres *{Uria aalge)* and little terns *{Sterna albifrons)* from the Baltic Sea (Thyen et al 2000), black guillemots *(Cepphus grylle)* from Iceland (Olafsdottir et al 2005), common and thick-billed murres from Alaska (Vander Pol et al 2004), and double-crested *(Phalacrocorax auritus)* and pelagic *(Phalacrocorax pelagicus)* cormorants from the Canada's west coast (Harris et al 2005).

The decrease of the OCs contaminants in lake Ontario differ from other pattern found in avian species from emerging industrial economies, recent data published by Chen et al (2009) shows new data of organochlorine contamination in Chinese terrestrial birds of prey, where the authors reported the presence of PCBs, DDTs and other organochlorine pesticides in various raptors from northern China in extremely high quantities (mg/kg wet weight basis). One example of the top food-chain bioaccumulation is the Eurasian sparrow hawks *(Accipiter nisus)* where DDE levels exhibited the highest concentrations among

targeted compounds (23.5-1020 mg/kg lipid weight). This concentration was observed even the bird does not reside in China, Chen et al (2009) related this level due to their stopover in southeastern China, where high DDT and dicofol applications have been recently documented.

Low levels of OCs were observed in this study of plasma and liver of Herring gulls compared to historical levels detected in eggs from Lake Ontario in the 70s and 80s, however, has been documented that herring gulls are strong to be resistant and with high tolerance to contaminants. Ewins et al (1992) found little evidence of impaired reproduction attributable to organochlorine contamination in Lake Huron in 1980. Weseloh et al (1990) reported that productivity in Lake Erie in 1979 was normal at widely separated colonies, despite widely differing levels of organochlorine contaminantion. There was not a significant correlation between increased PCB loading and decreased reproductive output in Lake Superior Herring gulls in 1983 (Weseloh et al 1994).

5.3.3 Brominated Compounds

For the brominated concentrations found in this study, it was observed that ZPBDEs in plasma of herring gulls HH site was 2.8-fold higher than SBI (Table 6, appendix I), with levels of 166 ng/g ww in plasma for HH site and 58.95 ng/gr ww for SBI. Livers analyzed from herring gulls collected at HH had 2.1-fold higher than SBI, the concentrations found were 1857 ng/g ww for livers of HH and 665 ng/g ww for SBI. These concentrations are higher than previous data published

by Norstrom et al (2002) with values of 755 ng/g ww in eggs of herring gulls from Hamilton Harbour, and levels of 1003 ng/g ww for Toronto Harbour; and 530 ng/g ww in Snake Island; all three sites inside the Lake Ontario, Canada. Values in this investigation are similar with Norstrom et al (2002) where the authors analyzed the trend of £PBDEs increasing concentration without interruption since 1981 to 2000. Where in 1981 the average concentration was 9.4 ng/g ww, and by 2000 the concentration was 530 ng/g at Snake Island in Lake Ontario.

The most dominant PBDE congeners observed in this study in importance of concentration detected were BDE 153 with concentrations in plasma of 49.29 ng/g ww for gulls at HH and 6.51 ng/g ww of SBI. Liver concentrations found in HH were 510 ng/g ww and 71.52 ng/g for SBI. This differs from values reported by Norstrom et al (2002) where the most dominant congener was BDE-47 for Hamilton Harbour and for Toronto Harbour; however, for the Snake Island in the Lake Ontario these authors reported the most dominant congener to be BDE-99.

The next PBDE congener of relevance due to its concentration was BDE-99 where levels in plasma found in herring gulls from HH site had 43 ng/g ww, and 11.41 ng/g ww for gulls at SBI. The BDE-99 concentration in livers was 510 ng/gr ww for HH gulls and 122 ng/g ww for herring gull liver from SBI. These values are consistent with a previous trend published by Norstrom et al (2002), which reported PBDE-99 as the second most dominant congener in Hamilton Harbour and Toronto Harbour after analyzing eggs of Herring gulls.

The compound BDE-154/BB-153 was detected with 32.17 ng/g ww at plasma samples from HH site, and 4.31 for SBI. Liver concentrations were 317 ng/g ww on HH site and 52.23 ng/g ww for SBI herring gulls. The next congener in order of importance and concentrations was BDE-100 and -47. BDE-100 had 31.71 ng/g ww on plasma from HH site and 8.9 ng/g ww for SBI. In livers, the BDE-100 had 332 ng/g ww for HH site and 97.63 ng/g ww for SBI.

BDE-47 was the only compound detected with higher concentrations in plasma and liver from SBI than HH site (Table 6, Appendix I). This value is different that the previous data published in eggs from Hamilton Harbour, where commonly, the sites of Hamilton and Toronto Harbour had higher BDE-47 levels (Norstrom et al 2002). In this study, it was also observed that SBI site had higher concentrations of BDE-47 than HH, in liver and in plasma.

Concentrations of 28.12 ng/g ww for SBI herring gull plasma, and 20.01 ng/g ww for HH site were observed. Liver concentrations from SBI were 312.54 ng/g ww and 268.63 ng/g ww for HH. The pattern of the five most dominant congeners found in this study were BDE 153>99>154>100>47; contrary to a traditional pattern found in Hamilton and Toronto Harbour of BDE 47>99>100>153>154. Observing that the pattern of congener varies among tissues analyzed, in this case liver and plasma from this study with eggs from previous reports.

Sellstrom et al (2003) mention that concentrations of BDE-47, BDE-99, and BDE-100 declined after peaking in the mid- to late-1980s. But also PBDE concentrations have increased exponentially in wild birds in the North American Great Lakes from 1981 to 2000 (Norstrom et al., 2002) The PBDE congeners found in wild herring gulls (Larus argentatus) were predominantly the 2,2',4,4'-tetrabromoDE (BDE-47), 2,2',4,4',5-pentabromoDE (BDE-99), 2,2',4,4',6-pentabromoDE (BDE-100), 2,2',4,4',5,5'-hexabromoDE (BDE-153), 2,2',4,4',5,6'-hexabromoDE (BDE-154), and 2,2',3,4,4',5',6-heptabromoDE (BDE-183) (Norstrom et al 2002). In addition to these congeners, in this study was observed detection levels of decabromoDE 209 in liver and plasma in SBI and HH sites, compound previously thought not to accumulate in living organisms (Lindberg et al 2004). A swith in diet containgin BDE-47, probably in fish. In a European study by Jaspers et al (2005) BDE-47 was reported the most abundant congener in fish that mainly feed on fish such as heron *(Ardea cinerea)* and grebe *(Podiceps cristatus),* suggesting that BDE 47, 99 and 153 were equally important in the terrestrial species; while BDE-183 and BDE 209 were only measured in the terrestrial birds. These results indicate that terrestrial birds may be more exposed to higher brominated BDE congeners than aquatic species. A different feeding pattern among colonies of herring gulls at HH and SBI, and a decrease in fish diet, could be the differences in the patterns observed in this study.

Despite the high concentrations of PBDEs found in wildlife tissues, brief information is available related the possible toxic effects of these chemicals in

birds and in wildlife in general. Current reviews provide some wildlife toxicity data but there have been no studies to date investigating possible PBDE -induced immunomodulation or other physiological effects on birds (de Wit 2002; Darnerud 2003). Immunological effects have also been suggested for PBDE exposure, which were used as constituents in commercial flame retardants (Lipson, 1987). Modulation effects on endocrine systems in wildlife may also occur because of PBDE exposure and or subsequent OH-PBDE metabolite formation (Darnerud 2003; Legler and Brouwer 2003).

5.3.4 Novel compounds detected in Herring gulls at Great Lakes

There are non previous reports of the presence of OH-PCBs and OH-PBDEs in Great Lakes Herring gulls. Concentrations of OH PBDEs/PCBs were found in plasma and liver from both sites sampled at Lake Ontario. The ^OH-PBDEs and -PCBs detected in the liver and blood from HH and SBI gulls are listed in Table 6 and Appendix I. In reference to the chlorinated compounds the two most important compounds detected in this study were 4-OH-CB187 and 4-OH-CB146. This is the first report of ZOH-PCBs in herring gulls at the Great Lakes. Hydroxylated metabolites from brominated compounds in this study had lower concentrations than the PCBs. According to their concentrations detected it was observed that the most dominant congeners were 4-OH-BDE49 and 6-OH-BDE47, followed by 6-OH-BDE49 and 6-OH-BDE49.

Levels detected in this study are lower than values published by Verreault et al (2007) where the ZOH-PCB for glaucous gull from Svalbard had 52.5 ng/g ww in plasma and 28 ng/g ww in livers. In addition, data in this study was higher than recent values reported by Verboven et al (2009) for glaucous gulls from Bjornoya Norway where the authors reported 4.2 ng/g ww for females and 14.9 ng/g ww in males. For glaucous gull plasma from Bjomoya Norway (Verboven et al 2009) 2-OH-BDE concentration were 1.7±0.8 ng/g ww for females and 2.2±0.8 ww ng/g for males.

Methoxylated brominated diphenyl ethers *feMeO-PBDEs)* observed in this study was higher in plasma from HH than SB, and concentrations in livers of HH were 1.6-fold higher than SB (see Table 6 and Appendix I). The two dominant methoxylated metabolites observed in this analysis in order of importance by their concentration were 6-MeO-BDE47 and 5-Me- BDE47.

Current understanding of PCB biotransformation and detoxification mechanisms suggests that the compounds found in the phenolic fraction such as OH-PCBs are retained in vertebrates mainly are derived from enzyme-mediated processes (e.g., cytochrome P450 (CYP) monooxygenases), whereas the OH-PBDEs may be formed via metabolism of major PBDEs and/or accumulated as naturally-occurring compounds (e.g., via formation in algae and sponges) (Hakk and Letcher 2003; Letcher et al 2000; Malmberg et al 2005; Stapleton et al 2009).

5.3.5 Competitive binding of Complex Mixtures

To address if the concentrations of OH-PCBs/PBDEs contained in the phenolic fraction (Table 6) are able to disrupt normal competitive binding among hormone transport gull (and human rTTR and rALB). *In vitro* assays were performed to evaluate the competitive binding of the chemical extractions (neutral and phenolics) to observe if there was an increase or decrease in the natural affinity of T_4 and T_3 to bind albumin and TTR. The results obtained show that neutral fraction (Figure 17), did not present significant differences ($p>0.06$); however, an apparent high binding was observed among T4 and the neutral fraction in binding competition with recombinant human albumin.

The phenolic fraction (Figure 18) had more competitive binding affinity differences with TH transport proteins. Phenolic fraction with human TTR was significantly able to disrupt ($p<0.001$) the T4 binding increasing to 143% the binding affinity of the assay, and the same phenolic fraction with T3 in presence of human TTR had 108% count per minute. Phenolic complex mixtures competing with Gull TTRs had no significant differences ($p>0.06$) in the binding of TH; however, an apparent increase in the displacement of T4 was observed compared to control. Recent in vitro studies have shown that hydroxylated metabolites of PBDEs bind with high affinity to thyroid hormone transport protein (i.e., transthyretin) (Meerts et al 2000) and bind to thyroid hormone receptors TR-a1 and TR-(3 (Marsh et al 1998), although the latter binding is with low affinity.

During the competitive binding of the selected chemical fractions was observable that the fractions containing the hydroxylated congeners of PCBs and PBDEs had the higher affinity, with differences among human and gull TH transport proteins. Where humans TTR was disrupted greatly towards binding T_4, while gull were more affected in albumin binding T_3, not surpringly since had been demonstrated the natural affinity of bird albumin for T3 and human TTR for T_4 (Ucan et al, 2009a)

5.3.6 Comparison of contaminants

ZPCB, ZOH-PCB, £PBDE, £OH-PBDE, £MeO-PBDE, £CHL, £CBz, $£_{DDT}$ and XMirex had potential associations between the concentrations of POPs and tissues/sites contained in the interaction area and concentrations (Figure 19 and 20). A positive relation of plasma from Hamilton Harbour and the livers of herring gulls from Scotch Bonnet Island were found (Figure 19). Also a direct relation between plasma HH and liver SB, and negative with Liver HH and Plasma SB was observed (Figure 20). £OH-PCB and XOH-BDE containgin fractions had the closer association with plasma, then liver, than £PBDE, £CBz, XCHL, £DDT, XMeO-PBDE and £Mirex. The relation observed in this study between OH-PCBs and PBDEs, suggest that not all OH-PCBs are excreted; several circulate in blood (Park et al 2007) and may exert toxicological effects, particularly on the thyroid system. OH-PCBs compete with and replace T4 on TTR, a possible indication of endocrine disruption associated with hypothyroidism (Lans et al 1993; Meerts et al 2002; Ucan Marin et al 2009a).

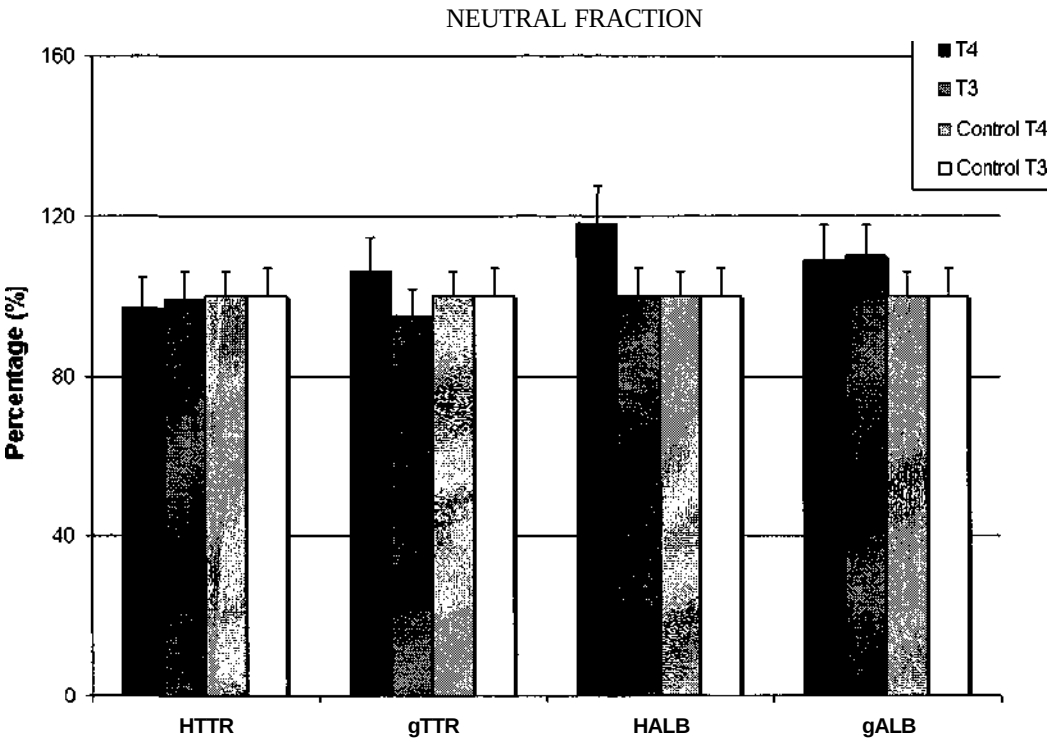

Figure 17. Extracted fraction-dependent, competitive binding assay for recombinant gull albumin (gALB) and transthyretin (gTTR), and recombinant human hALB and hTTR, with T3 or T4 in presence of Neutral Fraction (SE±). Percentage of competitive binding is based in control ^{125}I-T4 and ^{125}I-T_3.

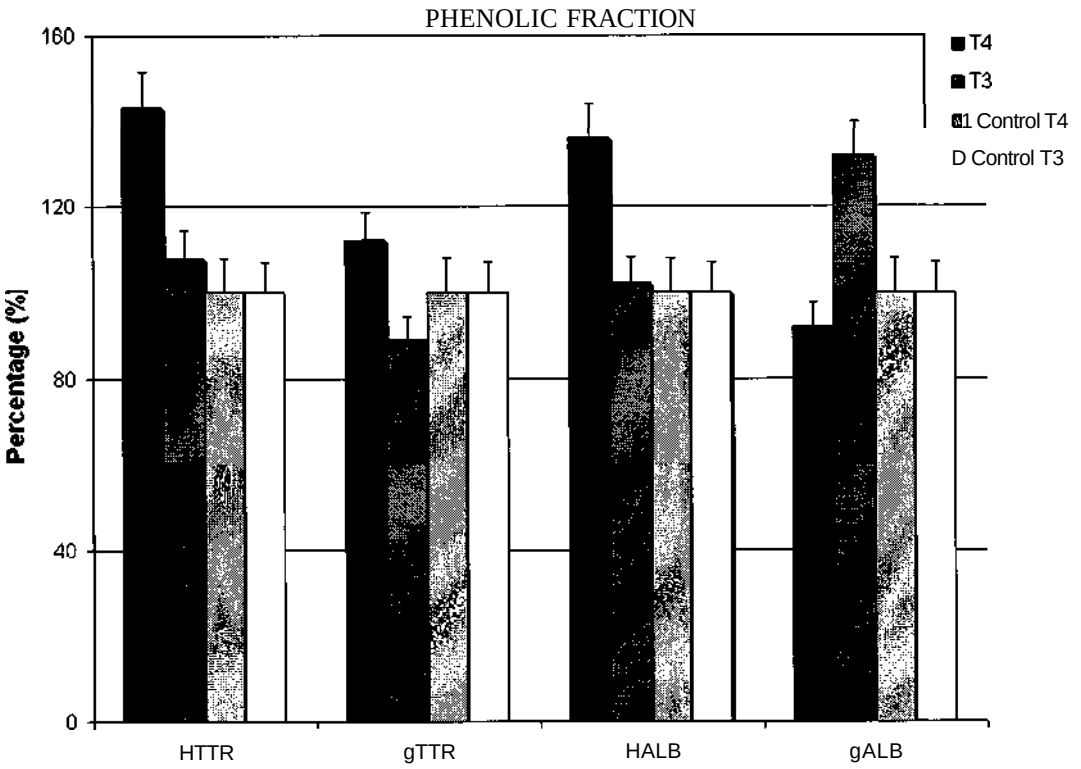

Figure 18. Extracted fraction-dependent, competitive binding assay for recombinant gull albumin (gALB) and transthyretin (gTTR), and recombinant human hALB and hTTR, with T3 or T4 in presence of Phenolic fraction (SE±). Percentage of competitive binding is based in control ^{125}I-T4 and ^{125}I-T3-

Recent evidence suggests that some of the hydroxylated metabolites of PCBs (OH-PCBs) may be more toxic than their parent compounds. These metabolites are formed in vivo by cytochrome P450 enzymes which oxidize PCBs to more water-soluble forms (e.g., OH-PCBs) via a 1,2 shift (NIH shift) or direct oxygen insertion (Letcher et al 2000).

Association were observed for selected major PCB and PBDE compounds with phenyl group substituents (i.e., OH) that modify physicochemical characteristic such as polarity, with circulating plasma. Substances typically shown as having lipophilic properties such as PCBs and CHLs, had negative values but a correlative association with livers of herring gulls from Hamilton Harbour. These data suggests that exist a tissue-specific relation and this play an important role, together with numerous other biochemical processes, in the toxicokinetics and fate of chlorinated and brominated contaminants (and metabolic) and natural products. Further studies are necessary to investigate the mechanisms of macromolecule selection and binding interactions with hydroxylated organohalogen accumulation in avian species.

TH binding to Human recombinant albumin showed significant differences ($p>0.003$) when compared to control T4, and T_3 did not show any difference. Human albumin in presence of the phenolic fraction extraction was disrupted significantly compared to T3, but recombinant albumin from gulls did not have

differences. For the gull albumin, the presence of the phenolic fraction and T_4 had a small decrease of 8% in the binding of T_4; and T3 had 32% higher binding than control. Meaning that albumin cloned from gulls in presence of the phenolic mixture extraction was significantly disturbed by more than 30% ($p<0.001$); concluding the higher sensitivity of T_3 to the phenolic fraction compared to T4 in herring gulls.

In the phenolic fraction used in this assay, I include compounds such as OH-PCBs which are known to be driven by the biotransformation of PCBs in the body, external sources such as fish intake and the abiotic environment possibly contribute to the body burden of OH-PCB metabolites (Campbell et al 2003; Hasegawa et al 2007; Ueno et al 2007). Due to their chemical structure that involves a hydroxyl group in either the para- or meta-position of a biphenyl ring, adjacent to chlorine atoms on both sides (Lans et al 1993; Letcher et al 2000, Ucan Marin et al 2009a, 2009b) is a suitable compound to bind to TH transport proteins TTR and albumin. The phenolic fraction isolated in this study contain the most dominant OH-PCB congeners, 4-OH-CB187, that was found in the present study, and has been considered as a biologically active compound (Meerts et al 2002; Negishi et al, 2007; Otake et al 2007; Park et al 2009). This is the first report on circulating £OH-PCB, ZOH-PBDE, £PBDE and £MeO-PBDEs in herring gulls from the Laurentian Great Lakes.

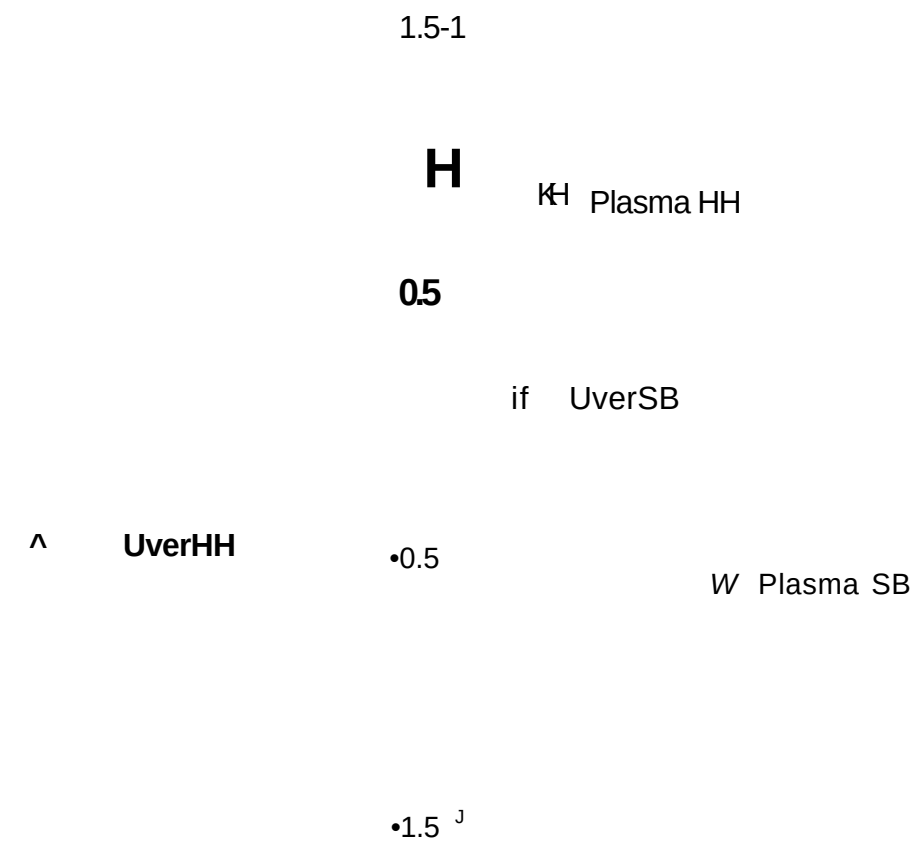

Figure 19. Proportions of the four tissues compared and their colony location plotted using the two first principal components (PCs), PC 1 and PC 2. Mean (±1 standard error) factor scores (right biplot) are showed for Hamilton Harbour and Scotch Bonnet Island.

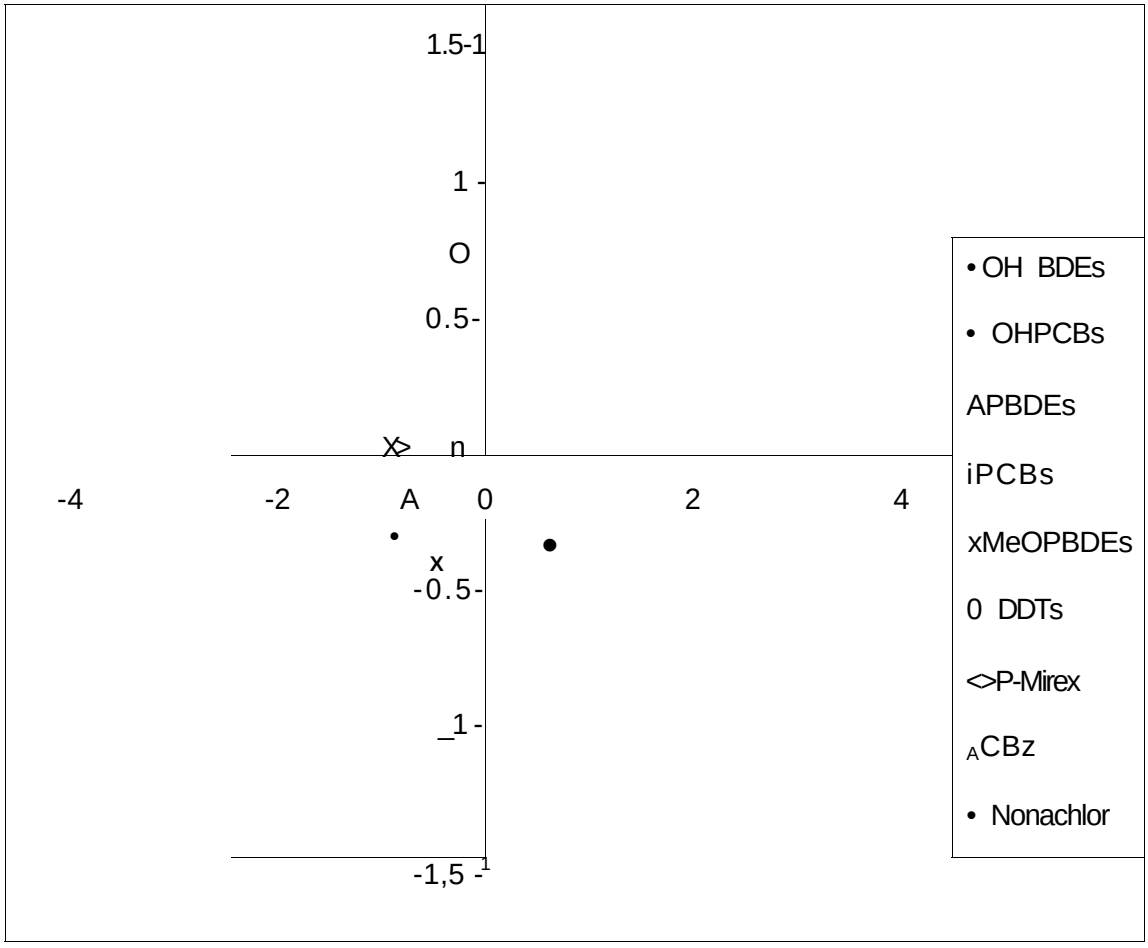

Figure 20. Proportions of nine major chlorinated and brominated contaminant classes or individual compounds plotted using the two first principal components (PCs), PC 1 and PC 2. Mean (±1 standard error) factor scores (right biplot) are showed.

With the results and data obtained in the competitive binding assays with phenolic and neutral fractions, was observable that concentrations of OH-PCB/PBDE found in the chemical phenolic fraction were able to affect normal TH binding (compared to control). Even, it has been demonstrated that some OH-PCBs (Purkey et al 2004, Ucan Marin et al 2009a) and OH-PBDEs (Meerts et al., 2000, Ucan Marin et al 2009a), are able to disrupt thyroid hormone binding. With these results, it was confirmed that hydroxylated contaminants contained in a complex chemical fractions were able to disrupt competitive binding of THs with TH transport proteins, as if they were tested individually. In this study it was confirm that the differences among human and avian TTR and albumin in presence of exogenous complex mixtures are significant and important.

The development and validation of complex mixtures through in vitro assays, is reliable and relevant to *in vivo* exposures, will provide an attractive and economical alternative to the in vivo assays for regulatory purposes. It will provide an opportunity for some studies in species (such as humans, marine mammals and diverse endangered species) for which controlled *in-vivo* exposures would be impractical for logistic, economic as well as ethical reasons. Another significant outcome for preliminary complex mixture screening is the opportunity to address some clarification of the interactions (agonistic/antagonistic properties) of individual compounds in immunotoxicity of mixtures of OCs. This will provide directions and help define research hypothesis

for further mechanistic studies. The expected results should also shed some light on the difference of susceptibility of different species of birds to the immunotoxic effects of mixtures of OCs. This could be a useful tool for wildlife conservation as well as population and habitat management. Finally, a better understanding of avian immunotoxicology and physiological response (using thyroid system model) may provide a good model for predicting the risk associated with life-long exposure to low concentrations of environmental contaminants in complex mixtures, such as experienced by human beings.

5.3.7 Conclusions

Polychlorinated biphenyls (PCBs) concentrations levels in plasma found in this study were lower compared with historical data reported for the Great Lakes. This study contributes with novel concentration data from two colonies of herring gulls from the Lake Ontario (Hamilton Harbour and Scotch Bonnet Island). It appears that polybrominated diphenyl ethers (PBDEs) trend follow the historical increase of the concentrations levels previously reported for the Great Lakes. This study also contributes with the first report of hydroxylated-PCBs/PBDEs (OH-PCBs and OH PBDEs), and Methoxylated brominated diphenyl ethers (XMeO-PBDEs) in plasma and liver of Herring gulls at Lake Ontario (and any avian in the Great Lakes). Differences were also observed in the affinity of TH transport proteins between humans and herring gulls (and between fractions), where the phenolic chemical complex fractions had higher affinity, and consequently higher capacity to disrupt TH binding from TTR and albumin.

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CHAPTER SIX[4]

Perfluoroalkyl Compounds (PFCs) and PFC-Containing Complex Mixtures Isolated From Herring Gull Liver (Lake Ontario): Comparative *In Vitro* Competitive Binding With Thyroid Hormones on Gull and Human Albumin and Transthyretin Transport Proteins

6.1 Abstract

Herring gulls *(Larus argentatus)* from the Great Lakes are top avian predators in the aquatic food web, but have also been shown to have a terrestrial component to their diet. There is a dearth of information, especially for wild avian species, on the PFC-mediated perturbation of thyroid hormone (TH)-dependent processes; particularly on TH transport. Cloned, expressed and purified herring gull recombinant transthyretin (rTTR) and albumin (rALB) and commercially available human TTR and ALB were studied in a competitive binding assays (CBA) to assess the interactions between complex chemical fractions isolated from gull livers that inhabit in two colonies of Lake Ontario. The £PFSA chemical neutral fraction containing e.q perflurooctane sulafanomide (PFOSA) and the acidic

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fraction containing (PFBs, PHxS, PFOs, PFDA, PFUdA and PFTrDA) £PFCA. A representative compound from each complex mixture: FOSA and PFUdA were examined in competition with recTTR and recALB for 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4). PFUnA was the dominant PFCA and PFOSA was also present in livers samples analyzed; and PFOS were the most abundant compound of the PFCs both colonies examined. CBA analysis show that relative to control in human and gull TTR, T_4 and not T3 were displaced by PFUdA and the (acidic), PFCA- and PFSA-containing fractions. PFOSA was able to disrupt T4 binding of human TTR and the (neutral), PFOSA-containing fractions was able to disrupt T4 -human TTR binding; and T_3 and T4 on human and gull albumin. Neutral and acidic PFCs chemical fractions differ in their affinity for T_3 and T_4, depending if were human/ gull and TTR/ALB. This study suggests a strong disruption possibility of TH transport proteins binding THs at environmentally relevant levels, caused hipotethicaly by the strong polar head of PFCs (mainly carboxylated) with a possible effect in TH homeostasis in free-ranging gulls.

6.2 Introduction

Perfluorinated compounds (PFCs) represent a large group of chemicals which are characterized by a fully fluorinated hydrophobic linear carbon chain attached to various hydrophilic heads. The chemical structures of some important PFCs are given in (Figure 21). PFCs have been produced since the 1950s and are widely used for many industrial purposes and consumer-related applications (Prevedouros et al 2006). This is due to their unique physico-chemical characteristics such as chemical and thermal stability, low surface free energy and surface-active properties (Hekster et al 2003; Lehmler 2005).

In recent years, polyfluorinated chemicals (PFCs) have increasingly been used as surfactants in various industry- and consumer products, because of their unique properties as repellents of dirt, water and oils. The most well-known PFCs are perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA) and their derivatives belonging to the group of perfluoroalkylated substances. The PFCs are very persistent in the environment, and some of them have been discovered as global pollutants of air, water, soil and wildlife and even found in remote polar areas (Ensen et al 2008).

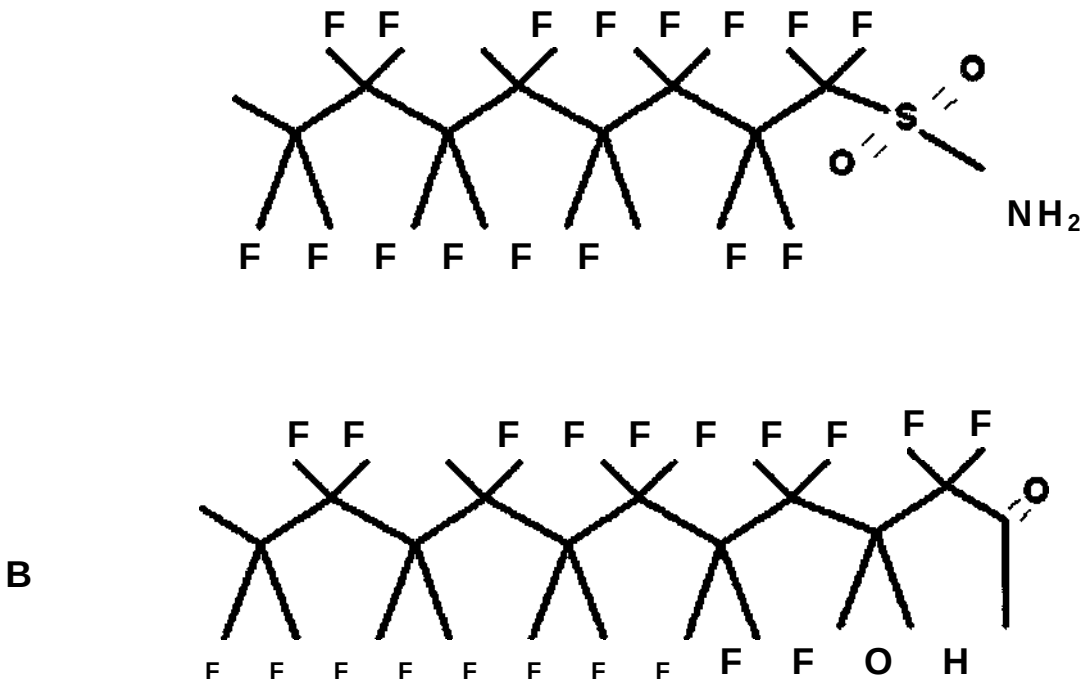

Figure 21. Chemical structure of two typical perfluorinated substances. (A) Perfluorooctane sulfonamide (PFOSA) $CF_3(CF2)7S02NH_{2)}$ (B) Perfluoroundecanoic acid (PFUdA) $CF3(CF_2)9C0_2H$.

The C-F bond is particularly strong, and is resistant to various modes of degradation, including reaction with acids and bases, oxidation, and reduction (Kissa 2001). This resistance contributes to the extraordinary stability of PFCs. The most commonly studied PFC substances as environmental contaminants are the perfluorinated sulfonates and the perfluorinated carboxylates. Among these, PFOS and PFOA are of greatest concern; because both persist in humans and the environment (Fromme et al, 2009). PFOS, its precursors (e.g., PFOSA), and related compounds are used in many applications ranging from oil and water repellant coatings for carpets, textiles, leather, paper, cardboard, and food packing materials; electronic and photographic devices; and surfactants in diverse cleaning agents, cosmetics, and fire-fighting foams (OECD 2002; Kissa 2001). The more persistent PFCs, such as PFOS and PFOA, can also be formed in the environment from abiotic and biotic transformation of commercially synthesized precursors. PFOA, as its ammonium salt, is principally used as an essential processing aid in the manufacture of certain fluoropolymers such as polytetrafluoroethylene (PTFE) and to a lesser extent in industrial applications as an antistatic additive and in the electronic industry (OECD 2005).

During synthesis of PFCs via electrochemical fluorination (ECF) and subsequent commercial reactions, numerous substances such as perfluoroalkyl-sulfonamide alcohols were unintentionally produced, or remained as by-products in commercial products. Degradation of these substances has been observed in the

ecosystem and in living organisms to produce persistent PFCAs or PFOS. For example, it has been demonstrated that perfluorooctane sulfonamides (PFOSA) can be metabolized to PFOS (Xu et al 2004; Tomy et al 2004). It has to be noted that PFOS may therefore be the final degradation or metabolic product of many perfluorooctylsulfonyl substances (Hekster et al 2003).

Bioaccumulation occurs also in humans, and everybody in our society has traces of these PFCs in their blood and internal organs such as the liver, kidneys, spleen, gall bladder and testes. In the blood, PFOS and PFOA are bound to serum proteins. The acute toxicity of the polyfluorinated substances is moderate but some substances can induce peroxisome proliferation in rat livers and may change the fluidity of cell membranes. Some of these PFCs, such as PFOS and PFOA, are potential developmental toxicants and are suspected endocrine disruptors with effects on sex hormone levels resulting in lower testosterone levels and higher oestradiol level. Other PFCs have oestrogenic effects in cell cultures (Ensen et al 2008). The persistence of PFCs in the environment, plus their potential to accumulate in organisms and to biomagnify in the food chain is of particular toxicological concern. Several PFCs have been detected in nearly all environmental media and biota reflecting the widespread global pollution in all parts of the ecosystem (Giesy and Kannan 2001). The toxicity of PFOS and PFOA has been studied extensively, mainly in rodents. Several reviews are available that discuss results from these studies (OECD 2002; Kennedy et al,

2004; US EPA, 2005; Harada et al, 2005b; Andersen et al, 2008; Lau et al, 2007).

Top predators are sensitive to persistent and bioaccumulative contaminants such as PFCs, due to their high trophic position in the food web (Martin et al 2004; Haukas et al 2007). Bird eggs had been traditionally used as indicators of environmental contamination and most of the current information on PFCs concentrations comes from this biological indicator collected widely in Northern Hemisphere (Kannan et al 2001a; Van de Vijver et al 2005; Ishibashi et al 2008b; Gebbink et al 2009). Little is known about the status and temporal trend of PFC contamination in tissues of avian top food-chain models from Lake Ontario in the Great Lakes.

It has recently been shown that PFOS does not affect the regulatory functions of the thyroid hormone system itself, but it is the competitive binding to transport proteins that alters the free thyroxine (T4) levels in blood (Chang et al 2008; Lau et al 2007). THs have been show to be associated (not covalently bound) to transport proteins such as transthyretin (TTR). This TTR-TH complex functions as a circulating reservoir to buffer changes in thyroid hormone levels. TTR is not only a highly conservative plasma protein and the main T4 carrier in cerebrospinal fluid (CSF) but also important in serum of birds (Weiss et al 2009).

The aim of this study was to screen the affinity of perfluorinated complex mixtures (acidic and neutral) obtained from liver extraction and to test two representative compounds found in the two mixtures, as single congeners for binding affinity. Binding studies using commercially available human TTR and PFCs had been reported recently by Weiss et al (2009). The intend of this chapter is to contribute with novel knowledge to a better understanding of PFCs affinities for TH, and the possibility of a toxic mode of action; using selected relevant complex chemical mixtures (extracted from livers). In this examination, cloned, expressed and purified TH transport proteins TTR and albumin from Herring gull *(Larus argentatus)* will be used. The set of PFCs representing the complex mixtures will be selected for testing according to their presence in livers and their environmental relevance.

6.3 Results and Discussion

6.3.1 Comparison of sites and contaminants

The C6, Cs and C10 PFSAs were detected in the herring gull livers and dominated by PFOS (Cs), which comprised >90% of the £PFSA. This finding is consistent with recent reports of £PFSA j_n herring gulls eggs from the Great Lakes (Gebbink et al 2009), also similar result was reported for the contribution of PFOS to IPFSA concentration livers of glaucous gulls *(Larus hyperboreus)* from the Norwegian Arctic and herring gull eggs from northern Norway (>97%) (Verreault et al 2005, 2007).

The concentrations of PFCs found in the livers of herring gulls from the Hamilton Harbour colony in this study are >2-fold compared to the concentration found by Gebbink et al (2009) in eggs (319±32 ng/g ww) who also used colonied of birds from the Great Lakes. This concentration it is consistent with the chemical pattern recently reported in fish [the alewife *(Alosa pseudoharengus)* and rainbow smelt *(Osmerus mordax)]* (Martin et al 2004) where PFSA patterns were similar to those observed here. The alewife and smelt are a major component in the aquatic diet of the herring gulls from the Great Lakes (Hebert et al 1999).

No significant differences among sites were observed for £PFSA concentration levels; Scotch Bonnet Island had 709.05 ng/g ww and Hamilton Harbour 686.95. A small increase in the £PFSA concentration was observed, however, the concentration levels of PFDS where in SBI 0.001% and in HH 0.48% from the total £PFSA. Perfluorodecanoic acid (PFDA) is a breakdown product of stain- and grease-proof coatings on food packaging, couches, and carpets, including Stainmaster. The chemical is part of a family of perfluoroalkyl carboxylates, all with structures similar to the well-known chemical contaminant PFOA, but with carbon chain lengths ranging from 4 to 15 carbons. PFDA is the 10 carbon version of PFOA. All of these perfluoroalkyl carboxylates are highly persistent. But those with carbon chain lengths of at least 8 carbons are of particular concern because they are known to be bioaccumulative, globally distributed pollutants. These chemicals have been found in human and wildlife blood and tissues from around the globe, even in remote locations such as the arctic (e.g.

Common Guillemot *(Uria aalge)* (Holmstrom and Berger 2008). It is possible that this compound is highly persistent in biota, consequently the highest urban exposure of Hamilton Harbour, make the difference in concentrations compared to SBI.

XPFCA concentrations in the other hand, presented differences ($p<0.05$) between SBI and Hamilton Harbour. In this analysis PFUdA in SBI was 57.4% of the total £PFCA and HH registered 25.5%. Considering that PFUdA is a breakdown product of stain- and grease-proof coatings on food packaging, couches, carpets. This 11-carbon version of PFOA is considered to be very persistent; and bioaccumulative, is surprising to found that the higher urban exposed bird colony had half of the concentration of herring gull livers at SBI. Asociations among feeding patterns could be also added to this discussion, while birds in urban areas tend to have a more diverse diet oriented to human waste, in SBI agricultural and dump near vicinity bird range of feeding could be a factor.

Total concentrations levels of PFNA (that is another breakdown product of stain- and grease-proof coatings on food packaging, couches, carpets; a 9-carbon version of PFOA; persistent; bioaccumulative) in SBI were lower (7%), than HH (39.6%) related to the £PFCAs. SBI concentration of PFTrDA were 01.5%, while HH had not detectable levels registered.

Hickey et al (2009) recently analyzed five of the PFCAs here detected (PFNA, PFDA, PFUnDA, PFDoDA, and PFTrDA) and reported that this compounds

induced liver fatty acid-binding protein (L-FABP) at concentrations of 10 or 50 uM; the strongest inducer of L-FABP was PFUnDA, with a 23-fold level of induction at a concentration of 10 uM. Also, at concentrations of 10 or 50 uM, six of the PFCAs (PFPeA, PFHA, PFNA, PFUnDA, PFTrDA, and PFTeDA) induced EROD activity (a measure of the catalytic activity of CYP1A4 in chicken) mRNA. The authors also mention that the only PFCAs that affected Cytochrome P450 4B1 is a protein that is encoded by the CYP4B1 gene (CYP4B1) expression (regulator of xenobiotic metabolism) were PFNA (induced) and PFTrDA (repressed)

XPFCAs with chain length of Cg-Cn and C13 were measurable in the herring gull livers, and PFUdA, PFDA and PFNA dominated the £PFCA, this differs from Gebbink et al, (2009) publication who analyzed eggs from Hamilton Harbour and where PFUnA and PFTrA dominated the PFCA pattern in the eggs for the same site. This could be related to the mechanistic excresion of the liver compared to eggs, where female birds excret high amount of contaminants attached to the albumin of the egg, due to the lipophylic characteristics of PFCAs. The £PFCAs found in this study, are also lower compared to the values described by Gebbink et al (2009) in eggs of herring gulls (where bioaccumulation is very possible), and the authors reported that £PFCA was 56±8 ng/gr ww..

There are many similarities among the concentration found in livers, with previous data published with PFCs in eggs of herring gulls at the Great Lakes by

Gebbink et al (2009). However related to the main differences observed among the two bird colonies, the source of the contaminants present are attributed mainly to that the areas represented a high populated, urban and industrialized parts of the Great Lakes (HH), and a more rural inside lake bird colony (SBI).

6.3.2 Complex chemical mixtures from neutral and acidic extraction

Complex mixture of neutral fraction (including PFOSA, values in table 7) was observed to have highest affinity and displacement of the binding of T4 and human TTR, but a small effect was observed when binding gull TTR. Also gull TTR have a small interruptio of T3, however, human TTR had a brief binding disruption with T3 binding TH transport proteins in presence of the neutral complex mixture. Albumin was the TH transport protein more affected by the complex mix, repeating the same effects in human and herring gulls (Figure 22). This could be related that albumin is a relatively non-selective transport protein found in all vertebrates (in this study birds and mammals). As the most abundant plasma protein, albumin's blood concentration often far exceeds even the flavonoids that flood the bloodstream after sugars are is metabolism. Although albumin is not strongly attracted to particular molecules, there is a complex molecular interaction in the blood and the PFCs are transported due to their lipophilic molecular characteristics and similar chemical structure with fatty acids (FAs).

The structure of many PFCs and their behavior within the body of organisms are comparable to free FAs, and as such they bind to liver FA-binding protein, and the protein albumin, which is mainly present in blood, liver, and eggs (Jones et al 2003; Luebker et al 2002; Martin et al 2003). It is suggested that the polar hydrophobic nature of fluorine containing compounds can lead to increased affinity for proteins, despite the relatively weak dipolar interactions that characterize the hard C-F dipole. The polar hydrophobic concept can explain some of the protein-binding data characteristics of fluorinated compounds (Biffinger et al 2004).

PFOSA is a known precursor to PFOS and was measurable in all herring gull livers in this study. Environmental research initially focused on the compound perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), the perfluorinated contaminants predominantly analyzed and found in the environment. Recently, a variety of other PFCs are being found worldwide in the environment, animals, and humans from urban to remote areas in all trophic levels (Houde et al 2006; Kallenborn et al 2004; Kannan et al 2004; Lau et al 2007; van Leeuwen et al 2006).

PFOSA as single congener in competitive binding assay (Figure 24) disrupt human TTR binding T4, but a difference of what was observed in their correspondent chemical fraction, PFOSA does no have effect in albumin, independently is was from human or bird when binding T4. Therefore, other

factors of unknown affinity are affecting the THs and albumin, and an increased disruption of T_3 -albumin appears to take place when albumin had a competitive interaction with PFOSA.

The Acidic fractions (including PFBs, PHxS, PFOS, PFDA and PFudA; table 8) was assayed with T3 and T4 and binding interaction with gull and human TTR and ALB. Relative to control group the acidic fraction displaced T_4 from binding human TTR (Figure 23). The affinity observed among human and herring gull albumin exposed to the chemical neutral fraction competitive binding (Figure 23) differs from acidic extraction; where neutral fraction had a higher affinity for T4, affecting human and gull albumin TH transport protein.

Biomonitoring surveys analyzing fish-eating birds demonstrated that the most elevated PFCA and PFSA besides PFOA and PFOS were the longer chained perfluoroundecanoic acid (PFUdA) and perfluorodecane sulfonate (PFDS), respectively (Bustnes et al, 2008; Butt et al, 2007; Holmstrom and Berger, 2008; Houde et al, 2006; Lofstrand et al, 2008; Tao et al, 2006; Verreault et al, 2005, 2007; Wang et al, 2008). PFUdA was selected in relation to an acidic fraction to examine the competitive binding assay affinity with TH and TTR and albumin (Figure 25). PFUdA in this study had binding affinity effects on T_4 in both human and herring gull TH transport proteins. Also the possibility of the longer chain of PFCs, the most involve of cytochrome P450 (CYP102) can be inlvove since branched chain fatty acids are substrates for CP450.

Table 7 Arithmetic Mean Concentration of £PFSA (ng/g wet weight) in individual Herring gull livers collected from two colonies in the Great lakes; Hamilton Harbour (HH) and Scotch Bonnet Island (SBI).

Site SBI	Average	SE	Min	Max
PFBS	0	0	0	0
PFHxS	0	0	0	0
PFOS	707.801	58.036	341.585	1151.703
PFDS	0.014	0.009	0	0.117
FOSA	1.241	0.0903	0.691	1.671
IPFSA	709.055	58.084	342.275	1153.153
Site HH	Average	SE	Min	Max
PFBS	0	0	0	0
PFHxS	0	0	0	0
PFOS	681.502	70.801	337.249	1116.881
PFDS	3.324	1.941	0	19.088
FOSA	2.131	0.232	0.841	3.021
IPFSA	686.957	70.787	338.089	1119.882

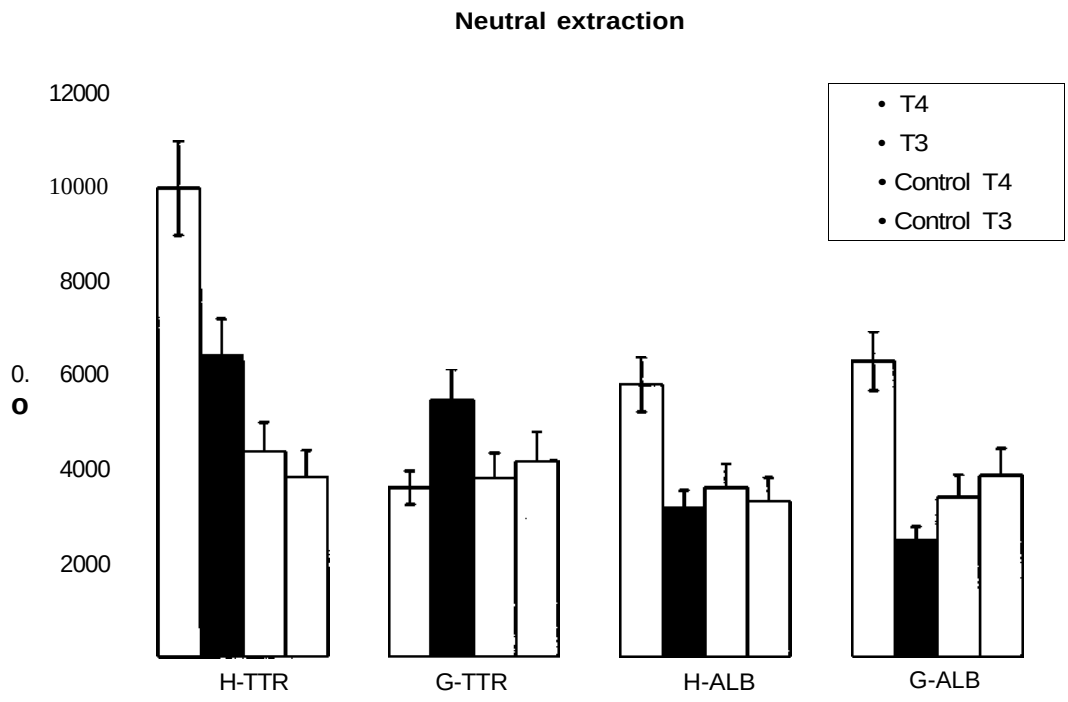

Figure 22. Human or Gull Albumin and Transthyretin (TTR): Competitive binding of Neutral PFC-containing fractions from L. Ont. Herring gull Liver with 3,3',5-triiodo-L-thyronine (T_3) and S.S.S'.S'-tetra-iodothyronine or thyroxine (T_4); CPM, counts per minute -IT4 and ^{125}-IT$_3$.

Table 8. Arithmetic Mean Concentration of £PFCA (ng/g wet weight) in individual Herring gull livers collected from two colonies in the Great lakes in; Hamilton Harbour (HH) and Scotch Bonnet Island (SBI).

Site SBI	Average	SE	Min	Max
PFHxA	0	0	0	0
PFHpA	0	0	0	0
PFOA	0	0	0	0
PFNA	1.407	0.628	0	7.282
PFDA	6.763	1.081	0	12.048
PFUdA	11.414	2.193	0.032	24.745
PFDoA	0	0	0	0
PFTrDA	0.298	0.217	0	2.386
PFTeDA	0	0	0	0
PFPA	0	0	0	0
IPFCA	19.883	3.345	0.888	36.649
Site HH	Average	SE	Min	Max
PFHxA	0	0	0	0
PFHpA	0	0	0	0
PFOA	0.193	0.193	0	1.936
PFNA	3.775	1.283	0	12.429
PFDA	5.516	1.313	0	11.816
PFUdA	3.262	0.822	0	7.818
PFDoA	0	0	0	0
PFTrDA	0	0	0	0
PFTeDA	0	0	0	0
PFPA	0	0	0	0
IPFCA	12.748	3.260	0	30.202

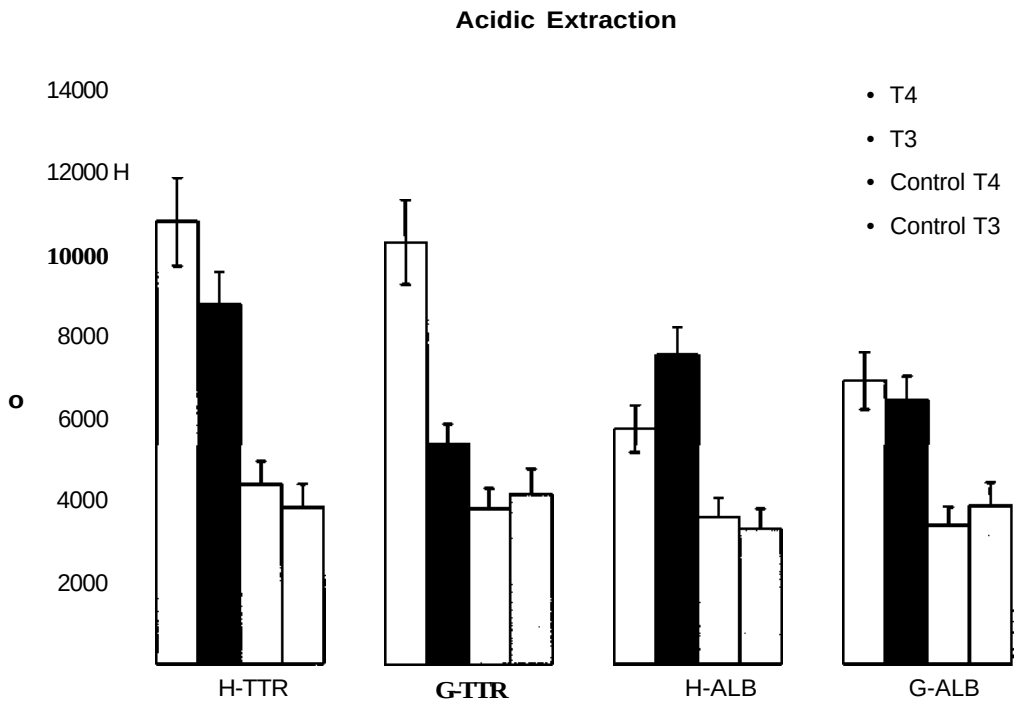

Figure 23. Human or Gull Albumin and Transthyretin (TTR): Competitive binding of Acidic fraction PFC-containing fractions from L. Ont. Herring gull Liver with 3,3',5-triiodo-L-thyronine (T_3) and S.S.S'.S'-tetra-iodothyronine or thyroxine (T4)); CPM, counts per minute -IT_4 and ^{125}-IT_3.

Albumin is one TH transport protein that is relatively non-selective for hormones in avian species and found at high concentrations in the blood (Baker, 2002), and serves as an important regulator of bioavailability, binding approximately 10% of circulating THs in humans (Benvenga et al, 2002) and contrary to humans is the major circulating TH transport protein in birds (75%), herbivorous marsupials and small eutherians (Richardson et al, 1994; 1996). Since PFCAs are structurally homologous to free fatty acids (Luebker et al 2002) and are expected to present affinity for for serum protein binding sites *in vivo* (Jones et al 2003). Jones et al, 2003 proposed that circulating serum albumin also tightly binds PFCs.

The complexity of fatty acids is a factor also, to be considered when the interaction of wide variety of PFCs are directly involve in the transportation of TH transport proteins such as TTR and albumin. For example, halogenated fatty acids which are one of the most interesting groups among the naturally occurring halogen compounds are not well known but several reviews on these compounds may be found. Halogenated fatty acids are found in different groups of organisms from microorganisms to the highest plants and animals. As halogen, they contain one or several atoms of fluor, chlorine, or bromine, and their interaction with TH transporters and their possible disruption by PFCs are not fully understood.

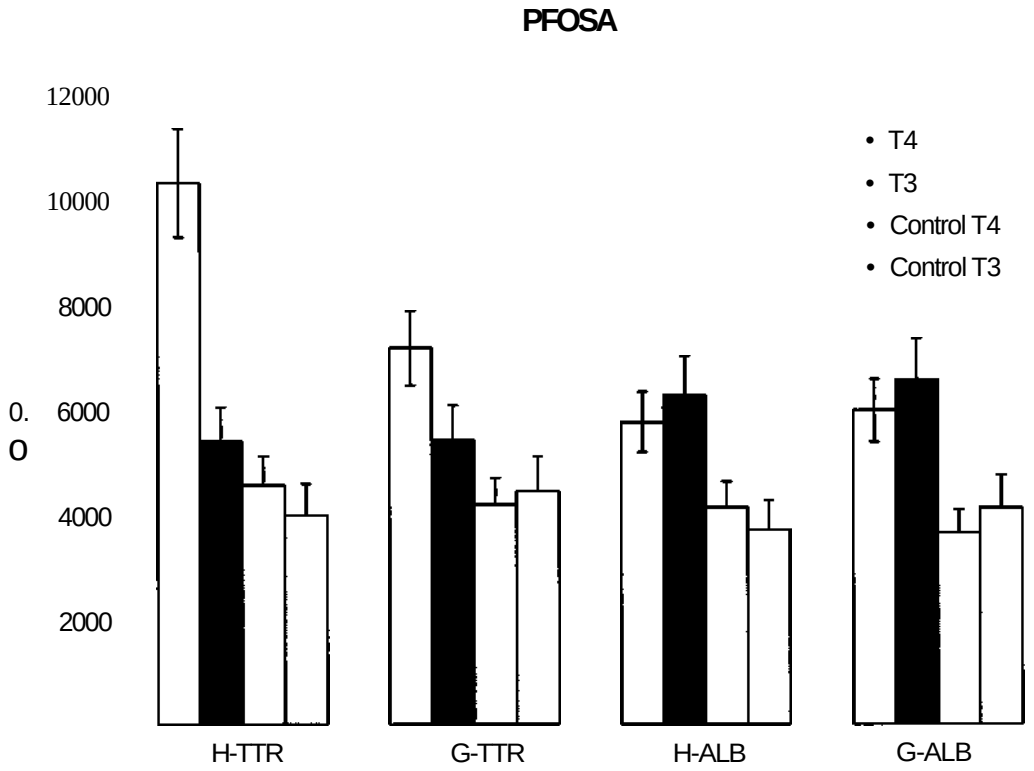

Figure 24. Human or Gull Albumin and Transthyretin (TTR): Competitive binding of Perfluorooctane sulfonamide (PFOSA) $CF_3(CF_2)7S02NH_2$ with 3,3',5-triiodo-L-thyronine (T_3) and 3,5,3',5'-tetra-iodothyronine or thyroxine (T_4)); CPM, counts per minute ^{n125}**-IT$_4$** and ^{125}-IT$_3$

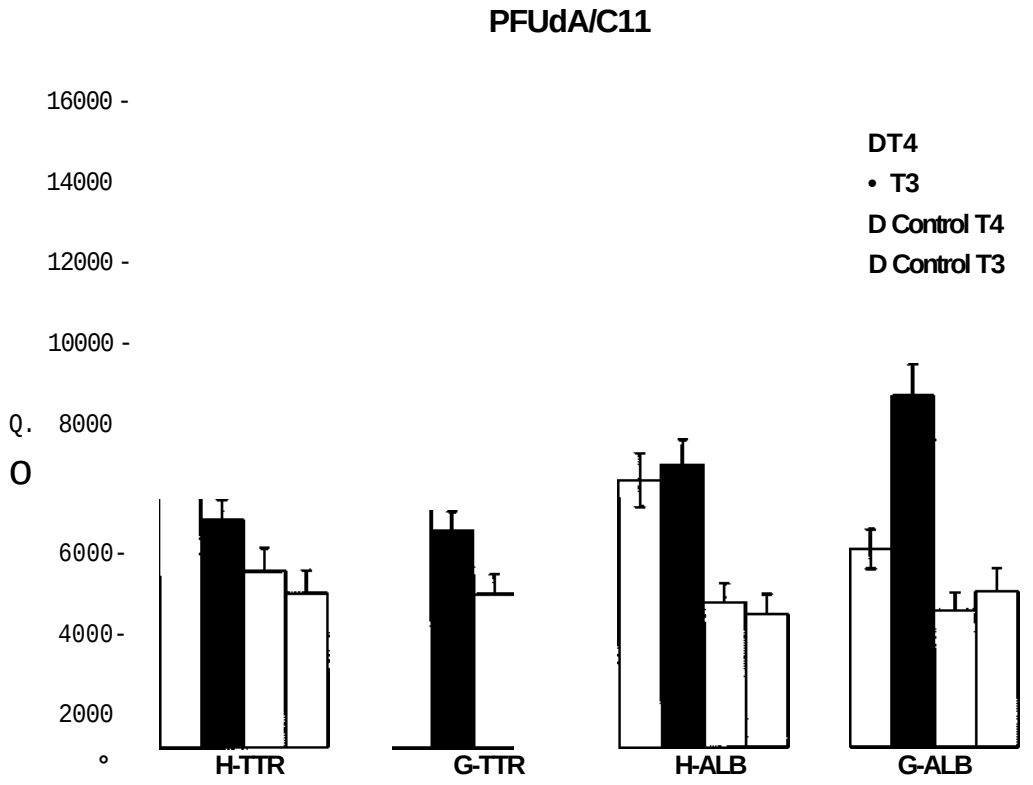

Figure 25. Human or Gull Albumin and Transthyretin (TTR): Competitive binding of Perfluoroundecanoic acid (PFUdA) $CF_3(CF_2)9C0_2H$ (Cn chain length) with 3,3',5-triiodo-L-thyronine (T_3) and S.S.S'.S'-tetra-iodothyronine or thyroxine (T_4)); CPM, counts per minute ^{1} -IT_4 and ^{125}-IT_3.

Even no statistically significant associations were detected, following *post hoc* power analyses raised the possibility for positive associations between FT4, PFDA and PFUdA, to be detected with moderate increases. In this study displaced $T_3$from binding herring gull albumin by PFUdA was observed. PFuDA (C11) had disrupting effects when albumin in gulls binds T_3 and T_4, while in human albumin no effect was observed, suggesting a more disrupting predisposition of albumin in birds than humans to PFCs.

Thyroid hormones play critical roles in human and avian neurodevelopment and adult neurocognitive function. POPs, such as PFCs tested in this competitive assays, may interfere with thyroid homeostasis and thus exposures to these compounds might represent risk factors for neurological and cognitive abnormalities. In a recent study, Bloom et al (2009) explored the potential associations between body burdens of six perfluorinated compounds, and their sum, with thyroid stimulating hormone (TSH) and FT4 among 31 licensed New York State anglers, using a cross-sectional study design, results of the study raise the possibility for associations for FT(4) with PFDA and PFUnDA, PFCs measured in low concentrations (Blooms et al 2009). Given the ubiquity of PFCs in the environment and the importance of thyroid function to neurodevelopment and neurocognitive endpoints.

In another study, Dellaire et al (2009) examined Adult Inuit (indigenous population north of Canada) plasma and found a negative association of PFOS concentrations with circulating TSH, tT_{3i} and TBG levels and a positive relationship with fT_4. Toxicological studies dedicated to investigating the thyroid-disrupting properties of PFOS are rare and focus mainly on maternal and fetal thyroid functions. In adult rats, PFOS was shown to compete with fT_4 for transport proteins (Chang et al 2008), leading to a transient increase in fT* and decrease in TSH concentrations. However, Dellaire et al (2009) also found a nontransient reduction in fT_4 and tT3, with the latter being less affected. These results in laboratory animals are consistent with perturbations observed on the TH homeostasis in the Inuit.

PFOS are widely distributed and persistent in the environment and in wildlife, and it has the potential for developmental toxicity; consequenty it is recommended to develop more studies studying the interaction of PFOs with THs and TH transport proteins. Since the molecular mechanisms that lead to these toxic effects are not well known, a new line of research is intending to address these unknown mechanisms. In a recent study, Xiongjie et al (2009) performed a proteomic analysis to investigate the proteins that are differentially expressed in zebrafish embryos exposed to 0.5 mg/l PFOS until 192 h post-fertilization. The analysis revealed that of 69 proteins used, all of them showed altered expression in the treatment group compared to control group with either increase or decrease in expression levels (more than two-fold differences). The study use proteins and

categorized them into diverse functional classes such as detoxification, energy metabolism, lipid transport/steroid metabolic process, cell structure, signal transduction, and apoptosis. Overall, proteomic analysis using zebrafish embryos serves as an *in vivo* model in environmental risk assessment and provides insight into the molecular events in PFOS-induced developmental toxicity. Since TH Transport proteins are strictly related to the supply of T_3 and T4 vital for intracellular process, the effect in the post-transcription of proteins could also be related to the increase, decrease or disruption of the transportation affinities of albumin and TTR.

6.3.3 Conclusions

Perfluorinated compounds (PFCs) analyzed in this study through their complex chemical fractions (neutral and acidic extractions) were able to disrupt the binding of T3 and T4 with TH transport Proteins TTR and albumin. Differences were observed in the response of TH transport proteins between humans and herring gulls (and between fractions. The acidic fraction was able to disrupt human and gull TTR binding T3 and T4. Neutral extraction have disruption effect in T4 binding human and gull albumin, and also TTR from human origin. Competitive assays with PFOSA show disruption of human TTR when binding T4. While PFUdA affected human and gull TTR when binding T4, but not for T3. This chapter contributes with novel data of PFCs concentration levels in livers of Herring gulls (Larus argentatus) from the Great Lakes, from two bird colonies (Hamilton Harbour and Scotch Bonnet Island) in Lake Ontario, Canada.

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CHAPTER SEVEN

Conclusions and Future Directions

7.1 Conclusions

The present gull TTR and albumin molecular characterization and competitive binding studies clearly showed that, although there are high amino acid sequence similarities among human and gull, and albumin and TTR, the human TH transport proteins cannot be used as a surrogate to assess the effects on circulating THs in wildlife, specifically birds such as the gull species *(Laridae).* Differences between human albumin and TTR for TH binding are several fold compared with herring gulls. The present study contributes with novel values, recording for the first time the binding affinities of gull albumin hormone transport protein binding T3 and T4, and observed through a set of environmentally relevant contaminants found in circulating plasma of gulls (organohalogens and perfluorinated compounds). It was observed in this research that OH-metabolites either chlorinated or brominated had higher binding affinity than THs to albumin and TTR. This indicates that the combination of the more TH-like brominated diphenyl ether backbone (relative to the chlorinated biphenyl backbone), and in combination of having an OH-group, results to more effective competitive ligand on gull hormone transport proteins relative to both T3 and T4. A more complex

ligand-binding parameter assessment (e.g. complex chemical mixtures) for TH transport protein in gull species, and in wildlife in general, are necessary to fully understanding the potential effects in reproductive, nutritional and physiological processes that could be influenced by changes in the circulating T4 and T3, and subsequently TH-dependent processes.

During this research investigation, molecular cloning, expression and purification of TTR and ALB was performed and was successful, and rec proteins could be used to assess two specific gull species, and then compared with human TH transport proteins (ALB and TTR) to observe binding affinities and potencies in the presence of various exogenous ligands and byproducts that theoretically and potentially could disrupt thyroid activities.

In this research, it was observed that PBDEs concentrations appears to follow the historical increase that has been previously reported for the Great Lakes Area, and thesis contributes with new data of brominated flame retardants concentrations found in analyzed livers and plasma of herring gulls. This is the first report of hydroxylated-PCBs/PBDEs (OH-PCBs and OH PBDEs), and Methoxylated brominated diphenyl ethers (£MeO-PBDEs) in circulating plasma and livers of Herring gulls from two selected colonies at Lake Ontario. To my knowledge, there were no previous reports on circulating OH-PCB, OH-PBDE, PBDE and MeO-PBDEs in herring gulls from the Laurentian Great Lakes. This is

the first report of concentrations of 6-OH-BDE47 and 4'-OH-BDE49 in plasma of herring gulls with values up to 1.1 ±0.62 ng/g (wet weight), which is ~0.002 nM.

Using an *in vitro* competitive binding analysis I used two extracted and selected phenolic and neutral fractions as complex chemical mixtures to observe a disruption in the binding of THs T3 or T_4 with TTR and albumin from gulls and humans. Differences were observed in the response of TH transport proteins among humans and herring gulls and among fractions, where phenolic fractions presented the higher affinity.

In contrast to OH-PBDEs, the mean concentrations of 4-OH-CB187 in the plasma of herring gull from Hamilton harbour was up to 37.5 ng/g ww (and 30.1 ng/g ww for Scotch Bonnet Island), which is ~0.09 nM and the the competitive potency (IC50) was ~5-10 nM for 4-OH-CB187 for T_3 and T_4 on gullTTR, and ~1-5 nM with gull albumin. The 4-OH-CB187 levels found in herring gulls in Lake Ontario during this study may be high enough to effect circulating T_3 or T4 levels, according to the observations during the competitive binding assays. Also, during this research, the patterns and levels of exposure to these OH-containing contaminants did not differ between the two colonies of birds (liver and plasma), therefore the potential effects on circulating T3 and T4 are the same for both areas. It is possible that levels of contaminants circulating in herring gull blood (and stored in liver) could disrupt TH homeostasis (to the fact that albumin had higher binding affinity than TTR), with consequently susceptibility of displacement

by competition by hydroxylated compounds such as OH-PCBs or OH-PBDEs, that had show higher binding affinity.

It is also possible, that an influence and/or change on the cellular TH levels and the ratio of T3 and T_4 could affect the TH homeostasis (Mendel, 1989), and the TH-dependent function and as consequence the health of exposed organisms. In birds and other wildlife, the competitive xenobiotic displacement of T4 from ALB or TTR would result in less T4 available to target tissues, and a subsequent diminish in the concentration of T4 substrate for deiodinase enzymes, including the 5' deiodinases Dl and DM. The lack of substrate T4 for deiodinases will have a direct effect in the T4 conversion to active T_3 and then a smaller concentration availability of T3 to thyroid receptors and targeted tissues (Brouwer et al., 1998; Verhoelst et al., 2005). In mammals for example, OH-PCB (4-hydroxy-2',3,3',4',5-pentachlorobipheny) binding affinity using rat liver cytosol and T2 suggested that the nature of the T2 sulfation inhibition is competitive (Schuur et al. 1998).

The present results also demonstrate that there are potential physiological consequences of the OH-containing organohalogens competitive binding to herring gull albumin and TTR. In birds the displacement of T4 from ALB or TTR would provide more T_4 to target tissues and would increase the amount of substrate for deiodinase enzymes, including the 5' deiodinases Dl and DM, which can convert T_4 to its active form T3 (Verhoelst et al., 2005). Like other bird species, gulls not only have TTR but also ALB, which also bind and are involved

in the transportation of thyroid hormones. However, relaying only in the context of overall thyroid hormone binding and transportation in birds, TTR may be of lesser importance than albumin. In birds, the proportion of circulating TH-binding transport proteins is low for TTR (McNabb et al, 1998). McNabb (2000) reported that in chicken the circulating T_4 is bound 75% to ALB, 17% to TTR and 7.5% to a a-globulin.

T_4 and particularly the primary metabolically active T_3 are considered the prime controllers for the regulation of metabolic functions and thermogenesis in mammals and birds (McNabb and Fox, 2003). T_4 and T3 are suspected to be involved in a partial dissipation of the mitochondrial proton electrochemical gradient that would uncouple phosphorylation from oxidation and hence produce heat. Therefore, high concentrations of contaminants in mammals and birds that alter circulating TH status could potentially affect temporally or permanently the basal metabolism and the capacity for adaptive thermogenesis.

Perfluorinated compounds on the other hand are a novel compounds, with just recent interest in the study of their capacity to disrupt or affect the Thyroid systems. In this perspective study of PFCs, differences were observed in the response of TH transport proteins among humans and herring gulls and among the selected complex mixtures. The acidic fraction in competitive binding was able to disrupt human and gull TTR binding T3 and T_4. Neutral extractions appear

to have effects mainly in T4 when binds albumin either from humans or gulls, however higher disruption was observed in human TTR.

PFOSA compound appears to have more disruption effects of human TTR when binding T_4. PFUdA compound affects and disrupt human and gull TTR in the binding of T4, but apparently does not have effect when is binding T_3. This investigation contributes with the first report of PFCs concentrations in livers of Herring gulls from the Great Lakes sampled in Lake Ontario two colonies (Hamilton Harbour and Scotch Bonnet Island).

7.2 Future directions

It is necessary to investigate tetra and pentachlorobiphenyl in liver cytosol in avians, due to the importance of the homeostasis in bird development, and reproduction. It has been observed in mammals, that OH-PCB (4-hydroxy-2',3,3',4',5-pentachlorobiphenyl, 4-OH-CB107) binding affinity using liver cytosol and T2 suggested that the nature of the T2 sulfation inhibition is competitive. In my suggestion, there are big gaps of knowledge in the sulfation and deiodination, and enzymatic reactions and relation in the process of the thyroid hormones to address.

PFCs in this preliminary screening of binding affinity capacity with thyroid hormones had higher disruption effects in human TTR than birds. Albumin is the

most important transporter in avian, but even accounts for the binding of approximately 10% of circulating thyroid hormone in humans, recent observations suggest that circulating serum albumin tightly binds PFCs. Recent findings suggest that oral dosing in rats with PFOS results in transiently increased tissue availability of the thyroid hormones and turnover of T4 with a resulting reduction in serum TT_4. Further investigations of PFCs compounds that are structurally homologous to free fatty acids are necessary (e.g. Cellular and membrane transportation). Questions need to be addressed about if they compete for serum protein binding sites *in vivo* and the description of the values of their binding affinity assessment *in vitro.*

New sets of ligands binding parameter assessments for TH transport protein in *Larus argentatus,* and in wildlife in general, are necessary to more fully understanding the potential effects in reproductive, nutritional, physiological and environmental (e.g., temperature) factors that can influence circulating T4 and T3 and subsequently on TH-depending processes. Ligands such as complex chemical extractions and the interactions of the two major TH transport proteins in birds, and in a-globulin. Further studies investigating the role of a-globulin are also necessary. Improving binding assays to a even more real environmental situations are necessary to observe interactions among thyroid hormones, ligands and TH transporters.

Investigations are also needed observing if there are changes in plasma thyroid hormone concentrations, mostly the depression of circulating T_4 concentrations due to exposure to OH-PCBs/OH-PBDEs *in vivo* and to deep investigate the consequence of several modes of action. First, to understand if exist a direct effect on the thyroid gland, both on thyroid gland morphology and on iodine transporters in the thyroid gland that can lead to a decreased synthesis of thyroid hormones by Hydroxylated compounds. Secondly, an altered metabolism of thyroid hormones, such as an increased biliary excretion of T_4, can decrease thyroid hormone concentrations. Thirdly, binding of OH-PCBs/PBDEs to the plasma thyroid hormone transport proteins can result in a displacement of the natural ligand of T4 *in vitro,* but we need to know the possible reaction *in vivo.* And, finally, interference of OH-PCBs/PBDEs with binding of thyroid hormones on their receptors has been described *in vitro,* but is necessary to include more elements such as direct observation in the iodine uptake at the thyroid gland, and their possible effect through changes in he sodium iodide symporter (NIS) which mediates iodide transport into cells.

Finally I will suggest further studies to observe effects e.g., *in-vitro* sub cellular and organohalogen compounds correlations at the *in vivo* (whole organism) level in free-ranging wildlife where TH-dependent effects are suggestive (for example in Arctic wildlife including birds) (Letcher et al., 2010). There is a lack of studies that have examined complex organohalogen exposures; and even fewer that have addressed competitive binding assays with THs and TH transport protein

interactions. More experiments are needed to address the differences on species and population specifics such as TH-related effects with TH transport proteins and confounding factors: (i) physiological status and timing (e.g., reproductively active); (ii) sensitivity as a function of other stressors (e.g., climate change), (iii) deleterious (chronic) effects and risks at organism or population levels.

7.3 References

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APPENDICES

Appendix I. Concentration levels of organohalogens and metabolites analyzed in plasma and liver of two herring gull colonies in Lake Ontario, Canada.

					percentage of samples positives			
ng/gr ww	**HH plasma**	**HH liver**	**SBI plasma**	**SBI liver**	**HH plasma**	**HH liver**	**SBI plasma**	**SBI liver**
1,2,4,5-Tetrachloroben...	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
1,2,3,4-Tetrachloroben...	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
Pentachlorobenzene	1.78	1.89	0.29	0.32	60	70	100	100
a-Hexachlorocyclohexane	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
Hexachlorobenzene	19.6	14.5	2.97	3.12	100	70	80	90
B-Hexachlorocyclohexane	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
Y-Hexachlorqc^clohexane	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
Sum of I	21.3	16.3	3.26	3.44				
Octachlorostyrene	**nd**	21.5	**nd**	nd	**0**	30	**0**	**0**
Heptachlor.epoxide	**nd**	3.35	**nd**	nd	**0**	30	**0**	**0**
Oxychlordane	**nd**	10.1	**nd**	2.56	**0**	40	**0**	30
t-Chlordane	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
c-Chlordane	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
t-Nonachlor	3.94	28.1	0.83	7.42	70	80	100	80
p,p'-DDE	296	2486	76	623	70	90	80	70
Dieldrin	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
p,p'-DDD	1.91	15.5	0.31	2.32	80	100	60	80
c-Nonachlor	4.25	51.1	0.78	10.6	70	80	100	90
p,p'-DDT	19.7	477	20.5	225	80	100	60	100
Photomirex	59.8	430	20.5	221	100	100	70	100
Mirex	128	1046	21.1	152	100	100	100	70
TCPM	**nd**	nd	**nd**	nd	**0**	**0**	**0**	**0**
Sum of *2*	513	4568	140	1243				
	HH plasma	**HH liver**	**SBI plasma**	**SBI liver**	**HH plasma**	**HH liver**	**SBI plasma**	**SBI liver**
p,p'-DDD	1.91	15.5	0.31	2.33	80	100	60	80
p,p'-DDE	296	2486	76	623	70	90	80	70
p,p'-DDT	19.7	477	20.5	225	80	100	60	100
Sum of *2*	317	2978	96.8	850				
Photomirex	59.8	430	20.5	221	100	100	70	100
Mirex	128	1046	21.1	152	100	100	100	70
Sum of I	187	1476	41.6	373				

Internal Std

13C- Tetrachlorobenzene
13C-Pentachlorobenzene
13C-Hexachlorobenzene
13C-p,p'-DDE

MLOQ < 0.10-0.25 ng/gr ww for blood and liver
nd= Not detected

ng/gr ww	HH plasma	HH liver	SBI plasma	SBI liver	percentage: of sartiples positives HH plasma	HH liver	SBI plasma	SBI liver
PCB-18	1.81	12.9	1.69	11.8	100	80	70	90
PCB-17	1.06	12.9	1.06	10.2	90	80	100	80
PCB-16/32	2.25	22.5	2.55	26.3	70	90	80	100
PCB-31	3.59	25.7	2.11	18.1	80	100	80	90
PCB-28	13.9	103	12.7	96.4	90	90	80	70
PCB-33/20	12.8	107	10.6	109	100	100	100	100
PCB-22	1.78	10.4	11.2	108	100	100	90	100
PCB-52	1.43	10.2	9.6	89.3	80	100	90	100
PCB-49	2.59	12.1	1.49	11.6	100	100	90	100
PCB-47/48	8.29	82.7	5.28	52.2	100	100	90	100
PCB-44	1.41	16.1	7.45	76.4	90	100	80	100
PCB-42	3.31	33.6	0.37	5.88	80	100	70	100
PCB-64/41	1.99	10.7	1.55	10.9	80	100	80	90
PCB-74	11.5	96.9	13.7	92.2	90	100	80	100
PCB-70/76	24.5	279	34.8	367	100	100	100	100
PCB-95	4.38	42.1	4.69	56.5	80	90	90	100
PCB-66	21.4	181	44.1	145	100	100	100	100
PCB-56/60	3.34	34.9	4.29	34.7	80	80	80	100
PCB-92	0.93	9.1	0.52	9.32	70	80	70	90
PCB-101/90	4.49	47.1	2.43	25.4	80	100	70	100
PCB-99	10.9	108	2.33	22.9	80	100	70	90
PCB-97	1.06	19.1	1.13	11.2	40	100	70	100
PCB-87	1.57	8.15	0.93	2.02	60	100	60	80
PCB-85	2.26	23.3	0.91	9.11	80	100	80	80
PCB-110	5.88	48.4	3.35	32.7	70	80	70	80
PCB-151	0.38	2.79	0.28	1.77	70	60	60	40
PCB-149	2.98	30.1	0.98	8.73	40	80	**60**	50
PCB-118	60.6	583	17.6	178	80	100	80	100
PCB-114	0.1	1.23	1.24	3.88	40	60	80	90
PCB-146	15.2	153	2.23	19.8	70	100	80	70
PCB-153	168	1619	29.1	325	100	100	100	100
PCB-105	14.7	116	9.25	81.3	80	80	60	100
PCB-179	0.1	0.1	0.24	3.88	50	40	60	80
PCB-141	4.85	47.9	2.58	23.2	40	80	50	30
PCB-130	4.56	38.1	0.41	3.24	40	70	60	60
PCB-176	0.22	2.41	0.37	3.8	30	70	60	60
PCB-137	3.99	26.9	6.79	15.3	10	40	40	80
PCB-138	162	1628	29.3	278	80	90	80	100
PCB-158	11.7	119	2.87	17.4	60	70	70	70
PCB-178	2.72	23.5	1.63	12.3	80	60	70	70

PCB-187	57.4	571	16.9	101	100	100	80	90
PCB-183	16.3	127	5.31	43.9	80	100	80	70
PCB-128	7.1	81.4	6.84	45.5	70	80	60	80
PCB-167	2.19	20.8	2.71	27.1	50	60	30	80
PCB-174	3.62	51.8	1.06	10.7	40	50	40	70
PCB-177	4.59	31.8	3.12	21.2	40	60	50	80
PCB-202	1.34	11.4	5.65	34.6	40	70	30	70
PCB-171	7.68	67.6	1.61	16.7	80	90	90	100
PCB-156	9.61	88.1	5.19	39.4	40	60	50	70
PCB-200	3.76	37.1	0.65	5.69	20	50	20	40
PCB-157	0.63	6.66	1.81	7.75	30	20	100	40
PCB-172	7.41	75.9	2.42	18.6	60	60	60	80
PCB-180	94.1	929	29.1	173	80	100	80	90
PCB-170/190	53.1	560	12.1	106	100	100	100	100
PCB-189	5.06	48.6	4.92	33.8	70	80	80	100
PCB-199	5.86	45.5	10.7	84.7	70	70	80	80
PCB-196/203	3.67	32.4	4.32	11.1	80	80	60	80
PCB-208	0.55	4.49	0.51	5.68	30	50	50	40
PCB-195	1.86	8.28	1.78	8.21	40	50	30	30
PCB-207	2.34	32.1	0.55	3.88	60	60	40	70
PCB-194	4.52	37.9	3.73	28.3	60	70	70	70
PCB-206	1.66	10.1	0.41	3.85	70	60	50	60
sum of *2*	**894**	**8626**	**407**	**3240**				

Internal Std
13C-PCB-28
13C-PCB-52
13C-PCB-118
13C-PCB-153
13C-PCB-180
13C-PCB-194

MLOQ < 0.10-0.25 ng/gr ww' for blood and liver
nd= Not detected

ng/gr ww					percentage of samples positives			
	HH plasma	HH liver	SBI plasma	SBI liver	HH plasma	HH liver	SBI plasma	SBI liver
BDE-17	nd	9.52	nd	3.56	0	20	0	60
BDE-28	2.12	17.2	1.24	8.54	60	60	50	70
BDE-49	1.54	19.5	0.33	2.56	50	50	30	60
BDE-47	20.1	268	28.1	312	80	90	80	100
BDE-66	nd	5.62	nd	2.53	0	20	0	30
BB-101	1.61	7.96	0.54	4.25	40	40	30	50
BDE-100	31.7	332	8.88	97.6	80	90	80	80
BDE-99	43.1	430	11.4	122	100	100	80	100
BDE-85	2.45	18.5	0.45	3.89	30	40	40	50
BDE-154/BB153	32.1	317	3.31	38.4	80	100	70	100
BDE-153	49.2	490	6.51	71.5	100	100	80	90
HBCDD	nd	nd	nd	nd	0	0	0	0
BDE-138	1.25	8.56	0.56	4.58	40	40	30	50
BDE-183	3.77	43.6	nd	13.1	60	70	0	50

BDE-190	2.53	18.52	1.14	9.56	60	70	30	**50**
BDE209	1.12	8.96	0.45	3.89	60	60	20	**40**
Sum of the Z	**192**	**1994**	**62.9**	**697**				

Internal Std
BDE-30

MLOQ < 0.10-0.25 ng/gr ww for blood and liver
nd= Not detected

ng/gr ww					percentage of samples positives			
	Plasma		**Liver**		**Plasma**		**Liver**	
	HH	**SBI**	**HH**	**SBI**	**HH**	**SBI**	**HH**	**SBI**
PCP	nd	nd	nd	nd	0	0	0	0
4-MeO-HpCS	nd	nd	nd	nd	0	0	0	0
4'MeO-CB79	nd	nd	nd	nd	0	0	0	0
4'MeO-CB120	nd	nd	nd	nd	0	0	0	0
2'MeO-CB114	nd	nd	nd	nd	0	0	0	0
3MeO-CB118	nd	nd	nd	nd	0	0	0	0
4MeO-CB107/4'MeO-CB108	nd	nd	nd	nd	0	0	0	0
4'MeO-CBlOl	nd	nd	nd	nd	0	0	0	0
4MeO-CB134	nd	nd	nd	nd	0	0	0	0
3'MeO-CB184	nd	nd	nd	nd	0	0	0	0
4MeO-CB146	nd	nd	nd	nd	0	0	0	0
4'MeO-CB127	nd	nd	nd	nd	0	0	0	0
3'MeO-CB138	nd	nd	nd	nd	0	0	0	0
4'MeO-CB130	nd	nd	nd	nd	0	0	0	0
4MeO-CB163	nd	nd	nd	nd	0	0	0	0
4MeO-CB178	nd	nd	nd	nd	0	0	0	0
3'MeO-CB182	nd	nd	nd	nd	0	0	0	0
3'MeO-CB183	nd	nd	nd	nd	0	0	0	0
4MeO-CB187	nd	nd	nd	nd	0	0	0	0
4MeO-CB97	nd	nd	nd	nd	0	0	0	0
4'MeO-CB159	nd	nd	nd	nd	0	0	0	0
4MeO-CB162	nd	nd	nd	nd	0	0	0	0
4'MeO-CB202	nd	nd	nd	nd	0	0	0	0
4'MeO-CB177	nd	nd	nd	nd	0	0	0	0
4'MeO-CB201	nd	nd	nd	nd	0	0	0	0
3'MeO-CB180	nd	nd	nd	nd	0	0	0	0
4'MeO-CB172	nd	nd	nd	nd	0	0	0	0
4MeO-CB193	nd	nd	nd	nd	0	0	0	0
3'MeO-CB203/4MeOCB198	nd	nd	nd	nd	0	0	0	0
4'MeO-CB199	nd	nd	nd	nd	0	0	0	0
4'MeO-CB200	nd	nd	nd	nd	0	0	0	0
4,4'diMeO-CB202	nd	nd	nd	nd	0	0	0	0

BJ3. s. a a *K K K*

0.10-0.25 ng

S:l&

ng/gr ww	HH plasma	HH liver	SBI plasma	SBI liver	percentage of samples positives HH plasma	HH liver	SBI plasma	SBI liver
OH-PCBs	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB79	0.19	0.52	3.69	2.83	70	**90**	100	90
4-OH-CB97	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB107/4	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB108	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
2-OH-CB114	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB120	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB162	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB163	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB177	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB178	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
3-OH-CB180	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
3-OH-CB182	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
3-OH-CB183	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB184	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB187	37.5	20.3	30.1	12.1	70	80	**90**	80
4-OH-CB193	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB198	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB199	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB200	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB201	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB202	0.18	1.03	2.31	1.19	80	80	90	100
4,4'-diOH-CB202	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
3-OH-CB203	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
4-OH-CB208	**nd**	**nd**	**nd**	**nd**	0	**0**	**0**	**0**
Sum Of *I*	37.8	21.8	36,1	16.1				

MLOQ< 0.02 ng/gr ww for blood and liver

	Plasma		Liver		percentage of samples positives Plasma		Liver	
OH-PBDEs	**HH**	**SBI**	**HH**	**SBI**	**HH**	**SBI**	**HH**	**SBI**
6-OH-BDE17	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
4-OH-BDE17	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
6-OH-CB107/4 OH CB108	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
2-OH-BDE68	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
6-OH-BDE47	0.31	0.15	0.23	0.21	70	90	100	100
3-OH-BDE47	0.1	**nd**	**nd**	nd	40	**0**	**0**	**0**
5-OH-BDE47	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
4-OH-BDE49	0.71	0.38	0.43	0.35	70	80	**90**	70
4-OH-BDE42	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**
6-OH-BDE90	**nd**	**nd**	**nd**	nd	**0**	**0**	**0**	**0**

6-OH-BDE99	0.06	nd	nd	nd	30	0	0	0
2-OH-BDE123	nd	nd	nd	nd	0	0	0	0
6-OH-BDE85	nd	nd	nd	nd	0	0	0	0
6-OH-BDE137	nd	nd	nd	nd	0	0	0	0
Sum of I	**1.18**	0.53	0.66	0.56				

Internal Std
13C-OH -PCB

MLOQ < 0.05 ng/gr ww for blood and liver
nd= Not detected