The effects of polychlorinated biphenyls 77 and 126 on the thyroidal status of a marine flatfish, the American plaice (*Hippoglossoides platessoides*).

by

Bruce A. Adams

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

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Department of Zoology

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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The Effects of Polychlorinated Biphenyls 77 and 126 on the Thyroidal Status of a Marine Flatfish, the American Plaice (*Hippoglossoides platessoides*)

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ABSTRACT

The effects of polychlorinated (PCB) congeners 77 (a tetrachlorobiphenyl) and 126 (a pentachlorobiphenyl) on the thyroid physiology of American plaice (*Hippoglossoides platessoides*) were investigated. The thyroid criteria examined were the activities of tissue thyroid hormone (TH) deiodinating enzymes, the plasma TH levels and the properties of the plasma TH-binding proteins. Deiodination pathways were examined in 7 major tissues. The tissues exhibiting the highest activity were liver for L-thyroxine (T4) outer-ring deiodination (T4ORD) which forms biologically active 3,5,3'-triiodothyronine (T3) and brain T3 inner-ring deiodination (T3IRD) which inactivates T3 to 3,3'-diiodothyronine. Characterization of liver and brain deiodination pathways showed the enzymes to be similar for most but not all criteria to deiodinases in other teleost fish.

Two *in vivo* experiments were performed. Injection of plaice with PCB 77 increased liver T4ORD activity, increased plasma T4 levels and decreased plasma T3 after one week, with no change in brain deiodinase activity. A second experiment with PCB 77 or PCB126 was performed. After one week, PCB 77 increased plasma T4 and PCB 126 increased liver IRD activities in some fish, but neither compound affected brain deiodinase activity. There is an up-regulation of the thyroid system in response to PCB 77 at one week indicated by effects of PCB 77 at both central (plasma T4 and T3) and peripheral levels (liver and brain deiodination) of the thyroid cascade and points to the liver as a key peripheral site.

In vitro studies involving incubation of plaice or rainbow trout liver microsomes with PCB 77, PCB 126 or three hydroxylated PCBs (OH-PCBs) showed no significant

effect by these compounds on deiodination. Using a dialysis technique with plaice and trout plasma, neither PCB 77 nor OH-PCBs were found to compete with T4 for binding to plasma proteins.

In an attempt to clone plaice deiodinase enzymes, ribonucleic acid (RNA) was isolated from liver and brain tissue of American plaice and Atlantic cod (*Gadus morhua*) and used in the reverse transcription polymerase chain reaction (RT-PCR). A 306-bp cDNA was isolated using cod liver RNA as a template. The cDNA has 79% homology with frog type III deiodinase, as well as high homology with other type III deiodinases.

The plaice is not highly responsive to injected PCBs, but does show some changes that parallel those in other poikilothermic vertebrates. PCBs have no demonstrated potential to directly impact TH deiodination or plasma binding of THs in plaice. Consequently the changes that do occur in liver deiodination and plasma TH levels may be due to indirect actions of PCBs or their metabolites on hepatic metabolism of TH.

Acknowledgements

I would like to thank my project supervisors Drs. D. G. Cyr and J. G. Eales for their interest, comments and support during this project. I would also like to thank my committee members, Drs. E. Huebner and L. Lockhart, as well as Dr. P. McKay for her participation as an external examiner. I want to extend my gratitude to Dr. R. McGowan for molecular biology support at the University of Manitoba.

This project is the result of the willingness of Drs. Cyr and Eales to collaborate on a project that would involve work in laboratories thousands of kilometres apart, the spirit of which is both commendable and important for science today. I appreciate their willingness and patience during this project. I have learned much about the modern process of science and consider this to have been a true growth experience.

This project involved more effort for coordination on the part of everyone involved, including laboratory support personnel. I would like to thank these people for their instruction, interest, comment and friendship that has made my project a rewarding experience on many levels. A l'institut Maurice Lamontage (Mont-Joli, Québec) merci à Paul Robichaud, Maria Gervais, Pierre Raymond, Jean Papillon, James Nagler, Nadine Coulomb, Catherine Hodges, and Christian Belanger. In Winnipeg, at the University of Manitoba, thanks to Audrey Waytiuk, Jennifer McLeese, Kathy Kohel, Glenn Fines, Ken Finnson and James Plohman.

Other people not already mentioned above have also profoundly influenced me during the project outside the laboratory. I want to thank them for their support, understanding and friendship, especially Ernie, Norm, Heather, Kate, my family, the cool zookids and my friends at the Winnipeg Rowing Club.

Special Note

This project originated in the laboratory of Dr. D. G. Cyr at the Maurice Lamontange Institute of the Department of Fisheries and Oceans in Mont-Joli, Quebec. Dr. Cyr provided considerable support, time, expertise and resources throughout the project with regards to the capture and housing of plaice, the *in vivo* experiments and particularly the attempts to clone plaice deiodinase. While the final written thesis has much less emphasis on the cloning aspect of the project, it is important to recognize the efforts and contributions made to this end by Dr. Cyr.

Dr. Cyr is currently at the Human Health Research Centre, INRS-Institut Armand Frappier, Université de Quebec in Pointe-Claire, Quebec.

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List of Abbreviations

AHH	Aryl hydrocarbon hydroxylase
ANOVA	Analysis of variance
ATG	Aurothioglucose
BAT	Brown adipose tissue
BHC	1,2,3,4,5,6-hexachlorocyclohexane
bm	Body Mass
C	Degrees Celsius
Ci	Curie
CYP450	Cytochrome P450-dependent monooxygenase
DDD	Tetrachlorodiphenylethane
DDE	Tetrachlorodiphenyldichloroethylene
DDT	Di-chlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetate
EROD	Ethoxyresorufin-*O*-deethylase
f-	Fempto-
FT3	Free T3
FT4	Free T4
g	Gram; X force of gravity
HBB	Hexabromobiphenyl

HPLC	High pressure liquid chromatography
hr	Hour
HSI	Hepato-somatic index
I^-	Free iodide
$*I^-$	I-125-radiolabeled free iodide
IAC	Iodoacetate
i.p.	Intraperitoneal
IRD/5D	Inner-Ring Deiodination
Km	Enzyme Affinity (Michaelis-Menten Constant)
L	Light; litre
LPO	Lipid peroxidation
lx	Lux
m	Milli-
MCR	Metabolic clearance rate
MMI	Methylmercaptoimidazole
MS222	Methane tricaine sulfonate
MW	Molecular weight
n	Nano-
OH-PCBs	Hydroxylated metabolites of polychlorinated biphenyls
ORD/5'D	Outer-Ring Deiodination
PBI	Protein-bound iodide
PCBs	Polychlorinated biphenyls
PCDF	Polychlorodibenzofuran

PCR	Polymerase chain reaction
PHAHs	Polyhalogenated aromatic hydrocarbons
ppm	parts per million
ppt	parts per thousand
PTU	6-*n*-propyl-2-thiouracil
p	Pico-
RAR	Retinoic acid receptor
RBC	Red blood cell
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rT3	3,3',5'-triiodothyronine (reverse T3)
*rT3	I-125-radiolabeled reverse T3
RT-PCR	Reverse transcription polymerase chain reaction
SA	Specific activity
SEM	Standard error of the mean
T0	Thyronine
T1	Monoiodothyronine
T2	Diiodothyronine
T3	3,5,3'-triiodo-L-thyronine
*T4	I-125-radiolabeled T3
T4	L-thyroxine, tetraiodothyronine
*T4	I-125-radiolabeled T4
TBG	Thyroxine (T4) binding globulin

TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TFA	Trifluoroacetic Acid
TG	Thyroglobulin
TH	Thyroid hormone
TETRAC	Tetraiodothyroacetic Acid
TR	Thyroid hormone receptor
TRIAC	Triiodothyroacetic Acid
tRNA	Transfer ribonucleic acid
TSH	Thyroid stimulating hormone
TT3	Total 3,5,3'-triiodo-L-thyronine
TT4	Total L-thyroxine
TTR	Transthyretin
u	Micro-
UGT	Uridine 5'diphosphoglucuronyl-transferase
wb	Whole body
WSF	Water soluble fraction

CHAPTER 1: General Introduction

In fish, the thyroid hormones (THs), L-thyroxine (T4) and its more active derivative 3,5,3'-triiodo-L-thyronine (T3), influence processes including growth, reproduction and development. The thyroid structure and physiology of fish is in many respects like that of other vertebrates. It is based on a complex cascade and feedback system of events, and TH action at the cellular level is believed to be similar to that of other vertebrates (Reviewed by: Eales and Brown, 1993; Leatherland, 1994; Cyr and Eales, 1996; Eales et al, 1999).

T4 is formed from iodinated tyrosine amino acids and stored in the colloid space formed by the follicular cells of the thyroid. THs may also be obtained from ingested prey (Eales, 1997). Regulated mainly by plasma TH levels, T4 is released into the circulation where it and its derivative, T3, are bound to the TH transport proteins, albumin and possibly transthyretin (TTR). Small amounts (<1%) of THs reside in a free form unbound to transport proteins in the plasma. Free T4 and free T3 are lipophilic molecules small enough to enter the cell by passive diffusion, although they may also penetrate the cell by transport processes. Once inside the cell, T4 and T3 are bound to cytosolic components including microsomes, cytoplasmic proteins, and mitochondria, and constitute the tissue pool of available T4 and T3. The receptors for THs are high-affinity nuclear receptors, part of the steroid and thyroid hormone receptor superfamily of DNA-binding receptors, and have a 10 times greater affinity for T3 than for T4. Binding of the receptor-hormone complex to the DNA initiates RNA synthesis, the first step in protein synthesis.

In fish, T4 undergoes extensive conversion in peripheral tissues where deiodination pathways are the primary regulators of thyroidal status by converting T4 to the more active T3. T3 may bind to receptors in the cell where it is formed or enter the plasma. Plasma T4 and T3 represent the circulating pool of THs that is available for exchange with the peripheral tissues. Changes in either the plasma pool of THs available for peripheral tissues or the activity of peripheral deiodination enzymes are therefore useful indicators of thyroidal status in fish.

Polychlorinated biphenyls (PCBs) are mixtures of chlorinated aromatic chemicals manufactured by chlorination of biphenyls. They are persistent chemicals used for industrial purposes including dielectric and heat-exchange fluids but now contaminate the entire planet (Eisler and Belisle, 1996). PCBs are notorious for their toxicity. While their production is now banned in most of the world, PCBs persist in our environment in all air, soil, water and biota including fish (Eisler and Belisle, 1996; Elskus et al, 1994).

PCB toxicity in animals is typically due to the acute effects or chronic and neurotoxic effects of a small number of more than 200 PCB congeners and due to the ability of a specific congener to induce biotransformation enzyme systems in animals (Brouwer, 1991; Kimbrough, 1995; Tilson and Kodavanti, 1998). Two congeners found to exhibit toxic effects at low concentrations are 3,4,3',4'-tetrachlorobiphenyl (PCB number 77; PCB 77) and 3,3',4,4',5-pentachlorobiphenyl (PCB number 126; PCB 126) (Walker and Peterson, 1991). These two congeners induce a variety of enzymes in mammals and birds including cytochrome P450 systems (or mixed-function oxidases) in many species (Dubois et al, 1996). In fish species, these PCBs induce similar enzyme pathways (Huuskonen et al, 1996; Otto et al, 1997; Sleiderink and Boon, 1996; Boon et

al, 1992; Palace et al, 1996), including aryl hydrocarbon hydroxylase (AHH) (Janz and Metcalfe, 1991) and conjugation pathways (Huuskonen et al, 1996).

While PCB congeners vary in their toxicity, they also have different mechanisms of toxic action, including direct and secondary effects on vitamin or endocrine systems. For example, PCBs are estrogenic in turtles (Crews et al, 1995) and PCB 77 is estrogenic in mice (Nesaretnam et al, 1996) and alters sex steroid hormone levels and ovarian development in fishes (Monosson et al, 1994). PCB 77 also affects tissue vitamin dynamics in brook trout and lake sturgeon (Ndayibagira et al, 1995), and alters cortisol dynamics in rainbow trout (Vijayan et al, 1997). PCB 126 affects vitamin levels in Lake Charr (Palace and Brown, 1994), immune function in catfish (Rice and Schlenk, 1995) and stress responsiveness of tilapia (Quabius et al, 1997).

PCBs and their hydroxylated metabolites (OH-PCBs) are sufficiently analogous in structure to THs that they may block TH receptors and other binding sites and alter TH metabolism (Brouwer, 1991; McKinney and Waller, 1994; Klasson-Wehler et al, 1998).

American plaice (*Hippoglossoides platessoides*) is a marine pleuronectid flatfish species found off the northeast coast of North America. Plaice may occupy sediments contaminated with industrial pollutants including PCBs, and are sensitive to these contaminants, as indicated by biotransformation enzyme induction (Nagler and Cyr, 1997). THs are known to play important roles in flatfish, including controlling metamorphosis (Inui and Miwa, 1985). Disruption of the normal thyroid physiology of plaice could be harmful to both individual plaice and to the population.

The main objective of this study was to determine whether specific PCB congeners alter two key criteria of thyroidal status, plasma TH levels and peripheral

tissue deiodinase activities in American plaice. This was achieved in a series of *in vivo* and *in vitro* experiments that intended to indicate whether there is potential for the thyroid system of plaice exposed to PCB-contaminated sediments to be disrupted. This will provide a framework for thyroid disruption studies, as well as expand our knowledge of thyroid function in pleuronectid fish species.

This thesis is organized as follows: Chapter 2 – Literature review of thyroid homeostasis and its disruption by xenobiotics; Chapter 3 – Characterization of deiodination pathways in tissues of American plaice; Chapter 4 – Determination of effects of PCBs injected *in vivo* on the thyroidal status of plaice; Chapter 5 – Determination of direct *in vitro* effects of PCBs on thyroidal status of plaice; Chapter 6 – An attempt to clone deiodinase genes in plaice to provide another measure of thyroidal status; Chapter 7 –General Discussion and Conclusions.

CHAPTER 2: Literature Review of thyroid homeostasis and its disruption by xenobiotics.

The Thyroid System.

Thyroid hormones (THs), L-thyroxine (T4) and 3, 5, 3'-triiodo-L-thyronine (T3) (Fig. 2-1), are involved in many physiological process in vertebrates. The main basis of TH action is regulation of cellular protein synthesis. THs are responsible for diverse actions in teleost fish. These include development, growth and aspects of reproduction in these and other fish taxa (Reddy et al, 1992; Eales et al, 1993; Cyr and Eales, 1996). The diversity of roles of THs in fish and other vertebrates likely stems from their permissive effects in optimizing the actions of other hormones (Eales and Brown, 1993; Leatherland, 1994; Cyr and Eales, 1996).

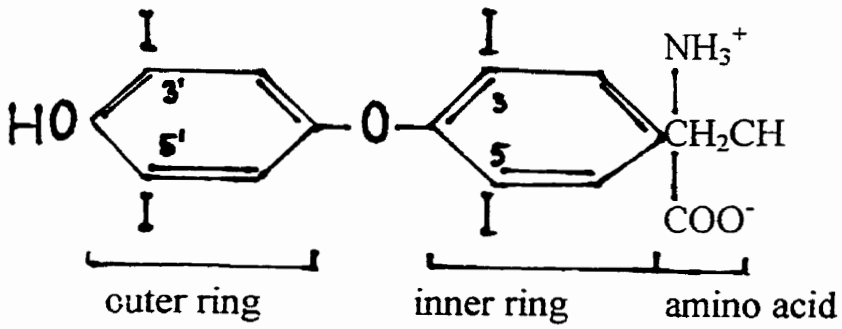
The thyroid system of fish is a complex cascade and feedback system of events. This cascade can be considered at three main levels (Fig. 2-2) (Eales et al, 1999): 1) Central control by the brain-pituitary-thyroid axis of the synthesis and secretion of T4 into the circulation from thyroid follicles, 2) Peripheral control of T3 formation from T4 and TH metabolism in tissues, and 3) Hormone action through T3 regulation of protein synthesis in the nucleus of target cells.

1) Central control by the brain-pituitary-thyroid axis

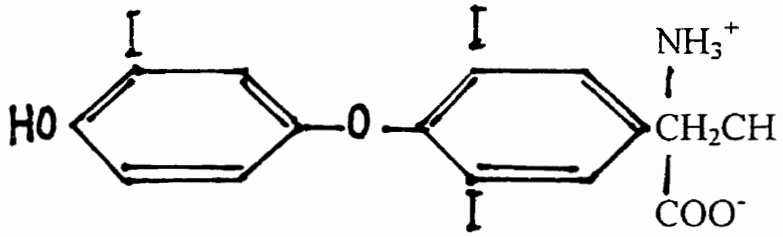
The primary TH produced and secreted by the thyroid tissue of teleost fish is the tetraiodothyronine, T4 (Chan and Eales, 1975; Eales and Brown, 1993). First, fish obtain iodide (I^-) mainly across the gills but also from dietary sources. I^- is transported into the lumen of the thyroid follicles that are commonly found in the basibranchial region of the fish. Some teleosts possess a plasma iodide-binding pre-albumin protein, which non-covalently and reversibly binds I^- (Leloup, 1970). This protein is not found in mammals.

Figure 2-1: Structure of L-thyroxine (T4), 3,5,3'-triiodo-L-thyronine (T3) and 3,3'5'-triiodo-L-thyronine (reverse T3).

THYROXINE = T4



3,5,3'-TRIIODOTHYRONINE = T3



3,3',5'-TRIIODOTHYRONINE (reverse T3) = rT3

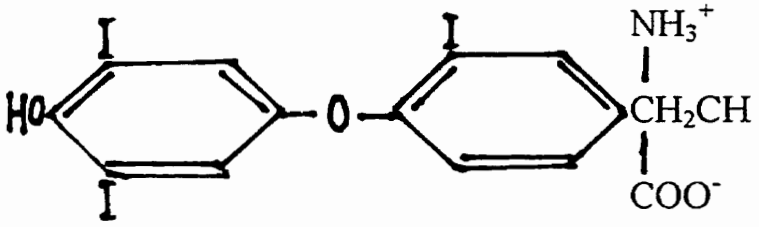
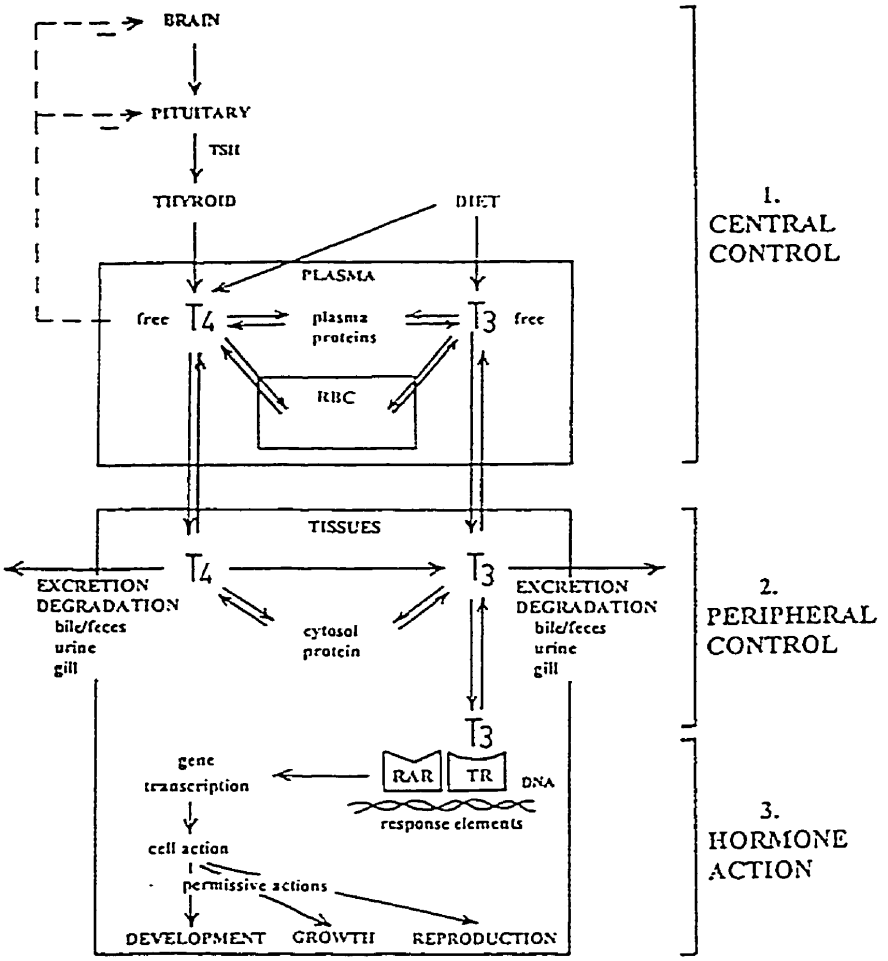


Figure 2-2: The fish thyroid cascade (from: Eales et al, 1999). RBC = Red blood cell, RAR = Retinoic acid receptor, TR = Thyroid hormone receptor.


BRAIN
PITUITARY
TSH
THYROID
DIET
PLASMA
free
T4
plasma proteins
T3
free
RBC
TISSUES
T4
T3
EXCRETION DEGRADATION
bile/feces
urine
gill
cytosol protein
EXCRETION DEGRADATION
bile/feces
urine
gill
T3
gene transcription
RAR
TR
DNA
response elements
cell action
permissive actions
DEVELOPMENT
GROWTH
REPRODUCTION
1. CENTRAL CONTROL
2. PERIPHERAL CONTROL
3. HORMONE ACTION

Within the thyroid follicle cells, iodoperoxidase oxidizes I^- that in turn replaces the hydrogen at the 3 and 5 positions of tyrosyls of the glycoprotein thyroglobulin (TG). It also couples adjacent diiodotyrosyls to form TG-incorporated tetraiodothyronine (T4) that is stored extracellularly in the colloid of the follicle. TG eventually undergoes endocytosis into the follicle cell, where the TG is digested by cathepsins, releasing T4 which then enters the plasma (McNabb, 1992). Plasma TH levels in fish are therefore derived from follicular T4, under control by pituitary-derived TSH, and, depending on diet, from food sources (Eales, 1997).

Once in the circulation T4 is bound rapidly and reversibly mostly to the TH transport protein albumin and maybe TTR, though some binding sites are found in erythrocytes (McLeese et al, 1998). Small amounts (< 1%) of T4 and T3 reside in a free form (FT4 and FT3) unbound to transport proteins in the plasma. The plasma level of FT4 exerts a negative feedback effect on the pituitary and hypothalamus to regulate TSH production and release and hence thyroid activity and ensure the plasma FT4 concentration. In contrast to mammals, T3, at or above physiological levels, does not appear to influence the activity of the fish hypothalamic-pituitary-thyroid axis (Fines et al, 1999).

Free T4 and free T3 are lipophilic molecules small enough (MWs 777 and 651 respectively) to enter target cells by passive diffusion, although they also penetrate the cell by facilitated diffusion transport processes (Ekins, 1986; Riley and Eales, 1993). Once inside the cell, T4 and T3 are bound to cytosolic components including microsomes, cytoplasmic proteins, and mitochondria. Free intracellular THs can

potentially undergo a variety of metabolic fates including deamination, decarboxylation, ether-link cleavage, conjugation and deiodination (Fig. 2-3).

2. Peripheral control of T3 formation and TH metabolism

Deiodination and conjugation are the main enzyme-regulated pathways for TH conversion. Conjugation by glucuronidation or sulfation of the TH 4'-hydroxyl group inactivates THs and increases their water solubility (Finnson and Eales, 1997; 1998). Though THs are more easily excreted when conjugated, in fish it appears they are also less prone to deiodination and sulfate conjugates may serve as a storage form for THs (Visser, 1990; McNabb, 1992; Finnson et al, 1999).

Deiodination is the removal of iodine from an iodothyronine, and is considered the key process for regulating TH in blood and peripheral tissues (McNabb, 1992; Eales et al, 1993; Kohrle, 1996; Visser, 1996). Outer-ring deiodination (ORD) of the T4 phenolic ring produces 3, 5, 3'-triiodothyronine (T3), the most active form of TH, and inner-ring deiodination (IRD) of the tyrosyl ring produces 3, 3', 5'-triiodothyronine (reverse T3, rT3), an inactive form of TH (Fig. 2-4). These iodothyronines can potentially undergo further deiodination by ORD and IRD to produce three diiodothyronines (T2s), two monoiodothyronines (T1s) and thyronine (T0). The IRD of T3 is particularly significant as it inactivates T3 to 3,3'-T2.

Deiodinases were first described in mammals. These enzymes have been characterized mainly on the basis of tissue type, enzyme-substrate affinity, TH analogue preference and action in the presence of inhibitors. Three different enzymes (types I, II and III) have been described in mammalians, mainly the rat (McNabb, 1992). These deiodinases are located usually in the microsomal fraction of the tissues and require a

Figure 2-3: The structure of thyroxine (T4) to indictate the potential metabolic pathways for iodothyronines: deiodination, conjugation, deamination, decarboxylation and ether-link cleavage.

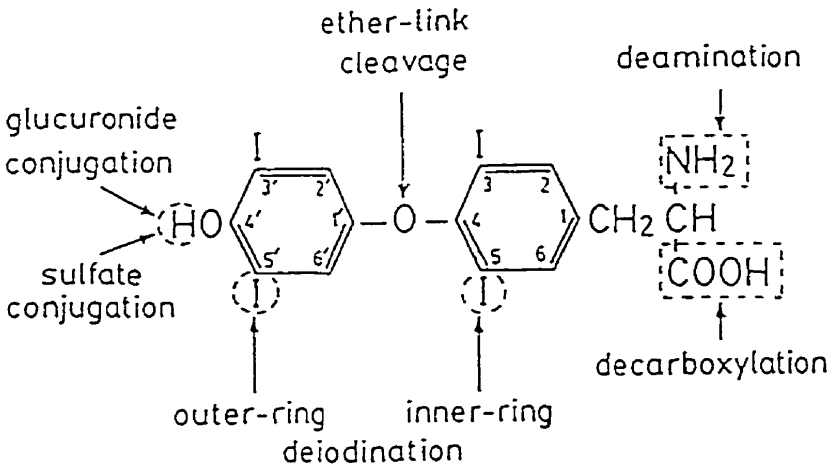
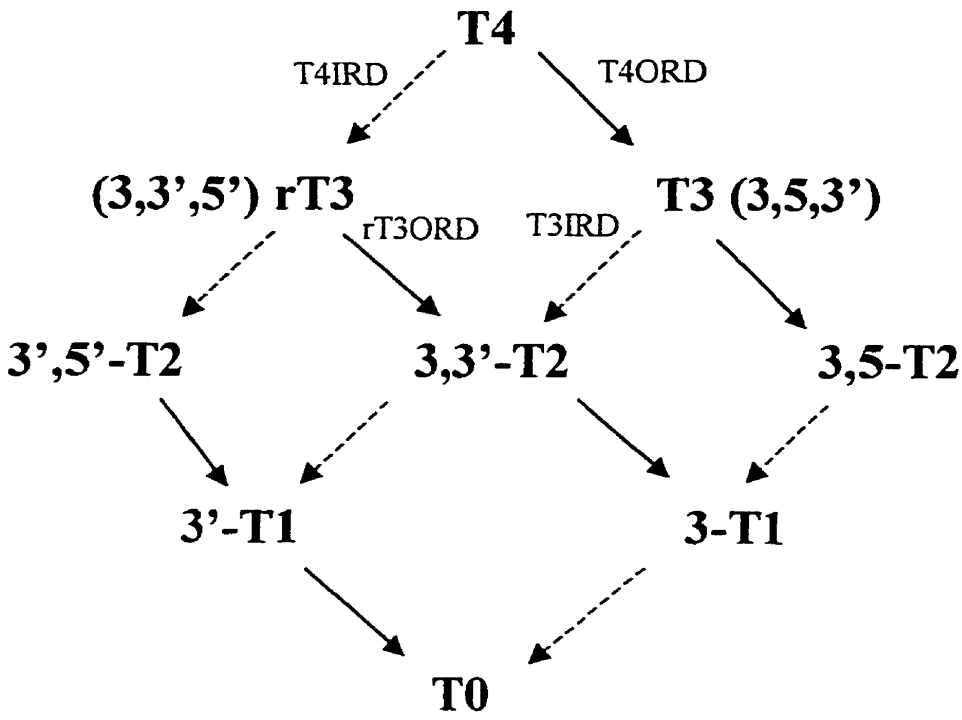

ether-link
cleavage
deamination
glucuronide
conjugation
sulfate
conjugation
HO
O
CH2 CH
NH2
COOH
decarboxylation
outer-ring
deiodination
inner-ring

Figure 2-4: Outer-ring deiodination (ORD = 5'D) and inner-ring deiodination (IRD = 5D) pathways for iodothyronines.


T4
T4IRD
T4ORD
(3,3',5') rT3
T3 (3,5,3')
rT3ORD
T3IRD
3',5'-T2
3,3'-T2
3,5-T2
3'-T1
3-T1
T0

thiol cofactor for activity. A sulfhydryl group of the deiodinase enzyme is oxidized to the sulfenyl state as a part of the deiodination reaction, then the sulfhydryl cofactor reduces the sulfenyl group on the enzyme to regenerate the deiodinase (McNabb, 1992).

Type I deiodinase is located mainly in the liver, kidney and thyroid of the rat. Type I catalyzes both ORD and IRD of iodothyronines, yet has a substrate preference of rT3>>T4>T3. This deiodinase has a high Km (~ 1uM), exhibits ping-pong kinetics with the thiol cofactor and is sensitive to inhibition by 6-*n*-propyl-2-thiouracil (PTU). The rat liver type I deiodinase enzyme is a major contributor to the circulating levels of plasma T3 by conversion of the prohormone T4.

Type II deiodinase is found in brain, pituitary and brown adipose tissue of the rat (Croteau et al, 1996). It catalyzes only ORD, likely regulating local (intracellular) T4 to T3 conversion, has a substrate preference of T4 > rT3 and does not significantly deiodinate T3. It is a low Km enzyme (~ 1 nM), exhibits sequential kinetics with the thiol cofactor and is mildly sensitive or insensitive to PTU.

Type III deiodinase is found in brain, skin and placenta of mammals. It catalyzes only IRD, has a substrate preference of T3 > T4 and is important in regulating local concentrations of T3 as well as the production of rT3 from T4. The types II and III enzymes also differ from type I by their low affinity for sulfated iodothyronine substrates.

In birds, three deiodinases have been described based on tissue and developmental stage. These include type I activity in chicken liver (Rudas, 1996) and intestines (Suvarna et al, 1993), and in the quail (Freeman and McNabb, 1991). Galton and Hiebert (1987) have described types I and III in chicken embryos, while Valverde et al (1993) have described types I, II and III activity in the chick embryo tissues.

Information on deiodination in reptiles is based on a few studies involving a lizard, snake and a turtle. Joos and John-Alder (1989) have described ORD activity in the liver of the lizard, *Sceloporous occidentalis*. In the striped racer snake, *Elaphe taeiniura*, ORD has been found in the liver, pancreas and kidney (Wong et al, 1993). Hugenberger and Licht (1999) have described two types of ORD pathways in turtle (*Trachemys scripta*) liver and kidney. One is an enzyme with a low T4 affinity (Km = 6.5 uM) similar to mammalian type I deiodinase and the other an enzyme with a high T4 affinity (Km = 1 nM), which is unlike either mammalian or avian high-T4-affinity (type II) enzymes.

The properties of deiodinases in amphibians have been described mainly by V. A. Galton and colleagues. There appear to be two types of deiodinases in amphibians. The first described in the bullfrog, *Rana catesbeiana*, is a high-affinity ORD in the skin, gut and tail tissue with physiological properties similar to mammalian type II deiodinase (Galton, 1988), but apparently differing considerably in structure (Leonard et al, 1999). A deiodinase similar in function to mammalian type III has been described in the liver of premetamorphic-climax frogs (Galton, 1988). Low ORD activity has also been described in the skin and gut of the salamander (*Ambystoma mexicanum*) (Galton, 1992). There has been no evidence to date for a deiodinase in amphibians with properties similar to a mammalian type I.

Deiodination pathways for TH have also been described in fish. Low Km-T4ORD, T4IRD and T3IRD pathways have been reported for several teleosts (Eales and Brown, 1993; Orozco et al, 1997; Mol et al, 1997, 1998). There is general agreement that low-Km T4ORD in fish is achieved by an enzyme that is closely related to the rat type II and that IRD of T4 and T3 is achieved by an enzyme (or enzymes) that resembles the rat

type III. A high-Km ORD activity has been reported in rainbow trout liver (Orozco et al, 1997; Finnson et al, 1999) and tilapia kidney (Mol et al, 1997), but it differs from the rat type I in several key properties (Finnson et al, 1999).

3) Hormone action through T3 regulation of protein synthesis

About 10% of the cell content of THs is present in the nucleus in most tissues (Sefkow et al, 1996). The fish receptors for THs are high-affinity nuclear receptors (Bres and Eales, 1986), and are part of the steroid and thyroid hormone receptor super-family of DNA-binding receptors. TH receptors have a 10 times greater affinity for T3 than T4. Binding of the receptor-hormone complex to the DNA initiates RNA synthesis, the first step in protein synthesis.

In summary, fish thyroid systems are similar in many respects to those of other vertebrates. However, in contrast to the system in mammals and birds, the fish thyroid system is not centrally-driven through the brain-pituitary-thyroid axis (Eales and Brown, 1993). In fish, T4 is secreted by the thyroid, and the plasma T4 level is regulated by the brain-pituitary-thyroid axis. However, the plasma level of T3, the active hormone, is controlled primarily by feedback and other regulatory mechanisms acting on the deiodination systems in the peripheral tissues.

Assays of thyroidal status.

There is currently a suite of assays for evaluating different aspects of the thyroid system at each of the three main levels of the thyroid cascade. Eales and colleagues (1999) have reviewed the validity and potential of these assays as indicators of thyroid disruption in fish.

Numerous assays have been developed to assess thyroidal status. Early evaluation relied mainly on thyroid histological examination of epithelial cell height, follicle size, and colloid content. Methods were then developed for measuring the uptake of radioiodide by the thyroid. Introduction in the 1970s of radioimmunoassay (RIA) technology has provided measures of levels of total T4 or T3, or FT4 and FT3 when used in conjunction with separatory techniques such as dialysis. Injected I-125 labeled T4 (*T4) or I-125 labeled T3 (*T3) can be used to estimate clearance rates of T4 and T3. Deiodinase assays measure the affinities and enzyme activity level for converting iodothyronines to more active or less active forms through specific iodine removal. The affinity and number of receptors for TH can now be determined. Also, it is now possible with the cloning of TH-receptors (Yamano and Inui, 1995) to determine their location in tissues by *in-situ* hybridization (Yamano and Miwa, 1998).

In summary, three main levels of the thyroid cascade have been discussed: 1) plasma T4 levels and properties of plasma T4-binding proteins give a measure of the regulation of central control by the brain-pituitary-thyroid axis responsible for T4 secretion; 2) plasma T3, properties of plasma T3-binding proteins and peripheral deiodination activities give information about the status of peripheral control of T3 production and availability; and 3) regulation of protein synthesis by T3 binding to DNA to increase mRNA transcripts. In this thesis, the first two of these three levels are measured, and an attempt is made to develop a tool to measure mRNA synthesis, the first step in protein synthesis.

Effects of xenobiotics on the thyroid system.

Due to the production and release of a vast number of chemicals by human industrial practices, some animals and even populations of animals are believed threatened. The thyroid system, as other endocrine systems, operates through chemical messengers and may be affected by these xenobiotics. Some xenobiotics may interfere directly with the thyroid system, or interact indirectly with another enzyme or hormone system that influences the thyroid system. Without direct study of the impact of these compounds on the thyroid system, it is difficult to determine whether hormonal dysfunction is the result of direct toxicity to the thyroid system or to a related but separate enzyme or hormone system (Rattner et al, 1984).

The purpose of this review is to determine the extent of vertebrate thyroid system disruption by xenobiotics. It is based on laboratory and field reports of the effects of contaminants on the thyroid system in the main vertebrate classes (Table 2-1). Effects are grouped in each section according to contaminant class, including such classes as halogenated hydrocarbons, aromatic hydrocarbons (eg. petroleum) and metals.

Halogenated hydrocarbons include aromatic compounds that have been halogenated mainly with chlorine (organochlorines or polyhalogenated aromatic hydrocarbons (PHAHs)), yet bromination is also common. Organochlorines include chemicals such as o,p'-DDD, Mirex, chlorinated dioxins, chlorinated furans and PCBs. They have been used for many purposes, but most commonly as insecticides and industrial compounds and are the by-products of some industrial processes.

Organochlorines, including PCBs, are fat-soluble and highly persistent in the environment. Consequently, they often accumulate in vertebrate tissues.

PCBs are double benzene ring structures with various degrees of chlorination (Fig. 2-5). The degree and position of chlorine substitution defines a specific congener, of which there are 209. Each congener varies in its toxicity, as some congeners more readily bind to the aryl hydrocarbon receptor that can induce various harmful effects on organisms. When available commercially for industrial processes, PCBs were commonly sold as a mixture of congeners under such names as Aroclor 1254. PCBs are often found as by-products of paper bleaching process or as residual material in incinerators. In this review, for the purposes of clarity, PCBs will be referred to only as specific congeners (eg. PCB 77) or as a mixture of PCBs.

Exposure of animals to aromatic hydrocarbons, which are non-halogenated, is often the result of an event such as an oil spill. These compounds tend to have a shorter half-life than chlorinated hydrocarbons yet there is evidence for both direct and indirect toxic effects on the thyroid system (Peakall et al, 1981; Stephens et al, 1997a,b).

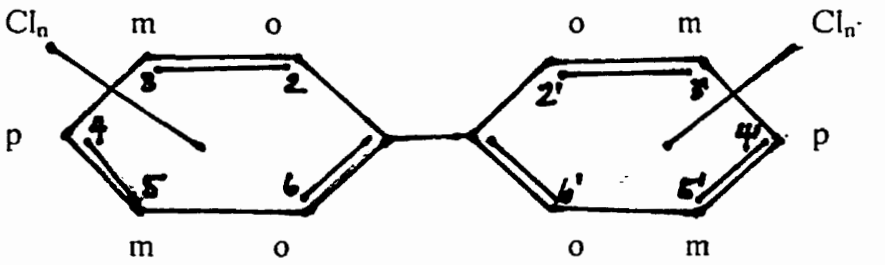
Metals in the environment above normal background levels are often industrial by-products from mining or manufacturing. Fish and bird (particularly waterfowl and fish-eating) species are those most often at risk of contamination and thyroid disruption by heavy metals (Chaurasia et al, 1996; Gupta and Kar, 1999).

Mammals

There is evidence that mammalian thyroid function can be disrupted by exposure to halogenated organic compounds or metals such as cadmium, lead, mercury and zinc (Table 2-1). Most studies have used the rat model. However other reports involve species

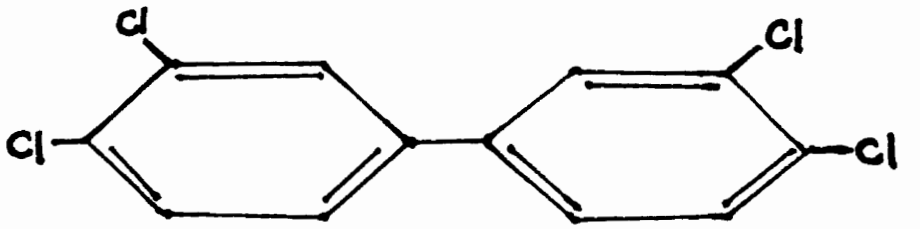
Figure 2-5: A) The general structure of PCBs; B) PCB 77 and C) PCB 126.

A.



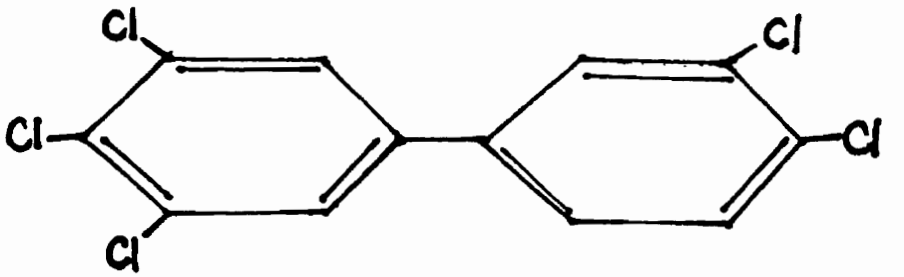
General Structure of PCBs
(o, m, p denote ortho, meta and para positions, respectively)

B.



3,3',4,4'-Tetrachlorobiphenyl
(PCB 77)

C.



3,3',4,4',5-Pentachlorobiphenyl
(PCB 126)

such as mice, mink, squirrels or seals. Capen (1997) has reviewed the action of many compounds, mainly drugs used for treating human disease, on the thyroid system of mammals.

Exposure to halogenated organic compounds typically decreases plasma TH levels in mammals. At least one dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), can decrease plasma T4 (Bastomsky, 1977; Raasmaja et al, 1996), but this is not always the case (Birnbaum et al, 1998). Coinciding with this decrease in T4 may be a decrease in plasma levels of T3 (Bastomsky, 1977), however, this is not always found (Raasmaja et al, 1996). Similar effects on plasma TH levels have been reported for mammals exposed to other halogenated aromatics. Rats exposed to the PCB mixture Aroclor 1254 have experienced many common responses: reductions in T4 and moderate decreases in T3 (Goldey et al, 1995); decreases in T4 and no change in levels of T3 (Morse et al, 1996); or a decrease in T4 (Gray et al, 1993). Exposure of rats, mice and seals to specific PCB congeners has also reduced plasma T4 levels and has sometimes increased T3 (Byrne et al, 1987; Morse et al, 1993; Darnerud et al, 1996). These studies often use different routes of exposure and dosages, which may account for some of the differences found.

Changes in plasma TH levels may be the result of changes in activities of enzymes that metabolize THs. Deiodination, the major pathway of TH metabolism can be affected by contaminants. Decreases in T4 may be accounted for by the increase in ORD (Type II deiodinase) (Raasmaja et al, 1996; Morse et al, 1993; 1996; Byrne et al, 1987; Darnerud et al, 1996), the enzyme responsible for converting T4 to T3. To maintain plasma T3 levels after a reduction in T4, the thyroid axis may respond with an increase in

activity of Type I deiodinase (Raasmaja et al, 1996), an enzyme that can convert T4 to T3.

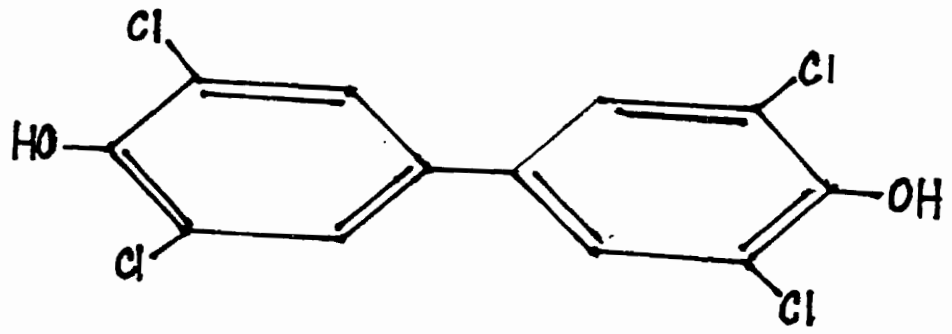
PCB metabolites also have the potential to alter normal endocrine function. PCBs appear to be able to induce both phase I and phase II enzyme reactions. Phase I reactions are the predominant biotransformation pathway and includes the cytochrome P450 or mixed function oxidase system the primary role of which is hydroxylation of compounds such as steroids and xenobiotics (Sipes and Gandolfi, 1991). Phase II enzyme reactions are energy consuming and include glucuronosyltransferases, sulfotransferases and methylation. Variables that can affect xenobiotic metabolism include species, strain, age, sex, time of day, enzyme induction, enzyme inhibition, nutrition and disease states (Sipes and Gandolfi, 1991).

TCDD-induced cytochrome P450 1A activity does not always decrease THs (Besselink et al, 1997). However, CYP450 by-products can affect thyroidal status. Hydroxylated-PCBs (OH-PCBs) (Fig. 2-6), metabolites of PCBs produced by the hydroxylation action of CYP450 isozymes, can lower T4 levels in fetal mice (Sinjari and Darnerud, 1998) as well as decrease sulfotransferase activity in rat cytosol (Gerlienke Schurr et al, 1998). Sulfotransferase is an important process in the normal thyroid function of mammals (Visser, 1996). Also, OH-PCBs have been found to be estrogenic or anti-estrogenic in human cell lines (Kramer et al, 1997; Connor et al, 1997).

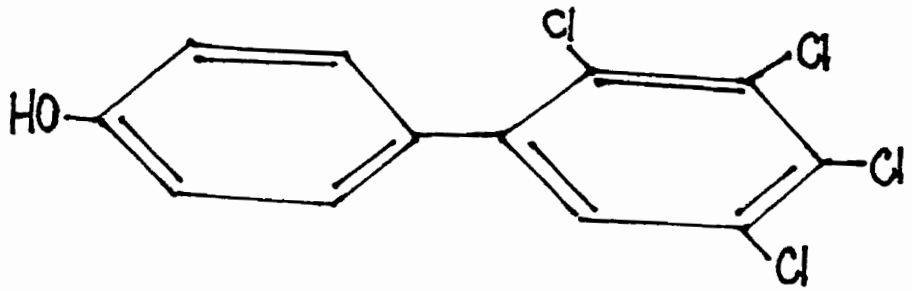
Brouwer and colleagues (1990a) have offered another explanation for the action of PCBs in mammals. OH-PCB metabolites of PCB 77 can competitively inhibit T4 binding to the transport protein TTR, although PCB 77 alone caused no such inhibition. Another similar compound, although not a PCB, o,p'-DDD, competes with T4 for

Figure 2-6: The structure of 3 hydroxylated PCBs: A) 3,3',5,5'-tetrachloro-4,4'-biphenylol (OH-PCB-A); B) 2',3',4',5'-tetrachloro-4-biphenylol (OH-PCB-B); and C) 3,4',5-trichloro-4-biphenylol (OH-PCB-C).

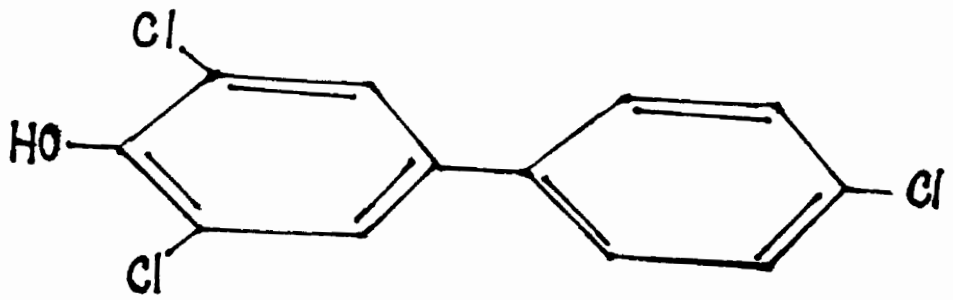
A.



B.



C.



binding sites on the TH transport protein, thyroxine-binding globulin (TBG) (Marshall and Tompkins, 1968). This could result in a decrease in the normal circulation of T4 as well as an increase the proportion of T4 that is free in the plasma and available for cell uptake or excretion.

Pathways for TH metabolism other than deiodination may also be influenced by contaminants, with a subsequent effect on thyroidal status. A decrease in T4 may be caused by in an increase in hepatic T4-glucuronidation which increases body clearance of T4 (Hotz et al, 1997; Birnbaum et al, 1998). Spear and colleagues (1990, 1994) have found that exposure of fetal or neonate Wistar rats to hexabromobiphenyl (HBB) can reduce plasma T4 and increase uridine 5'diphosphoglucuronyl-transferase activity (UGT), suggesting an increase in metabolic clearance rate (MCR) of T4 through T4 glucuronidation.

Another group of PCB metabolites, methylsulfonyl PCBs, is the third most abundant in Canadian biota (Bergman et al, 1994). For example, methylsulfonyl PCBs compete for binding to the human glucocorticoid receptor (Johansson et al, 1998) and decrease both T4 and T3 in mink (Lund et al, 1999).

There is good evidence for disruption of the thyroid system by contaminants other than halogenated hydrocarbons as well. Heavy metals such as mercury, cadmium, and lead show a strong affinity for sulfhydryl groups and inhibit a large number of enzymes having functional sulfhydryl groups (Paier et al, 1993;Watanabe et al, 1999). Thiol groups play an important role in the enzymatic deiodination of T4 to T3 (Visser et al, 1976). Cadmium decreased ORD activity in rats (Yoshida et al, 1987; Paier et al, 1993; Gonzalez-Pondal et al, 1995; Chaurasia et al, 1996b), and also decreased circulating

levels of T4 and T3 (Paier et al, 1993; Yoshida et al, 1987). Zinc also decreased ORD activity in rats. However, this occurred with no change in levels of T4 or T3 (Gonzalez-Pondal et al, 1995). Lead caused a similar effect to cadmium by decreasing rat hepatic ORD activity as well as levels of T4 and T3 (Chaurasia et al 1996b). Metals may affect other components of the thyroid cascade. Lead nitrate decreased I-131 uptake and decreased the colloid content of follicles in the ground squirrel (*Funambulus pennanti*) (Shrivastava et al, 1987). While it is unlikely that metals act by a similar mechanism to organic compounds to alter thyroid physiology, this information may be useful for interpreting general responses of thyroid systems to contaminants, particularly if indirect effects are predominant.

Birds

The structure and function of the thyroid gland in birds closely resemble those of mammals. In birds, THs influence a variety of physiological processes including reproduction, growth, metabolism, temperature regulation, molting, and a variety of behaviours such as migration (Norris, 1997). There is evidence in birds that contaminants affect these processes by altering thyroidal status (Jeffries, 1975; McArthur et al , 1983; Barron et al, 1995).

The compounds DDE, dieldrin and DDT increased the mass of the pigeon (*Columba livia*) thyroid and decreased the colloid content (Jefferies and French 1969, 1971, 1972). In the quail (*Coturnix coturnix japonica*) DTT decreased T4 levels, while it increased T3 levels (Grassle and Biessmann, 1982), suggesting an increase in T4 to T3 conversion.

Responses to TCDD seem more variable in bird species than in mammals. There was no effect of this compound on plasma levels of T4 or T3 in *Gallus gallus*, *Columba livia*, or *Ardeo heodias* exposed *in ovo*, perinatally (Janz and Bellward, 1996). Great Blue herons (*Ardea herodias*) exposed to TCDD had increased plasma levels of T4 although there was no change in T3 (Janz and Bellward, 1997). PCB 77 decreased plasma T4 and T3 in ring doves and changed thyroid histology (Spear and Moon, 1985). PCB mixtures have caused alterations in the thyroid histology of birds, including increase in thyroid mass and reduction in colloid content (Jefferies and Parslow, 1976), as well as reduced levels of plasma T4 and T3 (Grassle and Biessmann, 1982).

The common tern (*Sterna hirundo*) experienced no significant changes in levels of T4, T3 or in T4-UGT activity upon exposure to PHAHs (Murk et al, 1994), but three species of seabirds had increased plasma levels of T4 after exposure to crude oil (Peakall et al, 1981).

While there is little information on the effects of metals on the thyroid physiology of birds, cadmium can decrease plasma T3 levels in the Indian Rock pigeon (Prakash et al, 1997) as well as plasma T3 level and hepatic ORD in chicken (Gupta and Kar, 1999).

Black ducks reared on acidified wetlands had reduced plasma levels of T3 (Rattner et al, 1987).

Amphibians and Reptiles

The physiological actions of TH are perhaps most dramatic in certain amphibians. THs influence many processes in both anurans and urodeles, including reproduction metabolism, growth, and molting (Norris, 1997). However, they are also responsible for metamorphosis, the conversion of the tadpole to an adult frog. Much of our current

understanding of the thyroid system actions and mechanisms is derived from comparative studies involving the frog species *Xenopus laevis* and *Rana catesbeiana*. In reptiles, the thyroid system is important in tissue oxygen consumption, reproduction, molting, and growth (Norris, 1997). There is surprisingly little information available on thyroidal status of reptiles or amphibians exposed to xenobiotics. Generally, amphibians are considered more sensitive to aquatic contaminants than other aquatic vertebrates (Boyer and Grue, 1995).

The planar chlorinated hydrocarbon TCDD caused no alteration in the time to metamorphosis by three species of frogs (Jung and Walker, 1997). Exposure of *Xenopus* frogs to methoxychlor results in a failure of these frogs to undergo metamorphosis from a tadpole to an adult (Morrell et al, 1998). These results suggest that methoxychlor may be interfering with some aspect of the thyroid system thereby inhibiting metamorphosis, as metamorphic tail resorption in frogs is dependent on TH-induced apoptosis. Using tail resorption as a measure for metamorphosis, Fort and colleagues (1998) found that pentachlorophenol decreased tail resorption in *Xenopus*, while nonylphenol increased tail resorption. Atrazine, a herbicide, increases levels of T4 in larval tiger salamanders (Larson et al, 1998). Physical and reproductive abnormalities found in alligators from contaminated lakes in Florida are related in part to endocrine disrupting chemicals (Guillette et al, 1994). While there has been little focus on the thyroid physiology of these animals, male alligators from these lakes were found to have high levels of T4 (Crain et al, 1998).

Fish

TH play an important 'permissive' role in fish species by optimize the function of other hormones systems (Norris, 1997), such as those responsible for reproduction (Cyr and Eales, 1996). They also promote growth and development in most species, including metamorphosis in flatfish species (Inui and Miwa, 1985), although the actions may be different for some more ancient species, such as lampreys (Manzon et al, 1998).

There are more reports of disruption of the thyroid system in fish species than in other vertebrate classes. In fish, the greatest range of species, measuring parameters (histology to deiodinase activity), and contaminants have been described. More than half the reports of thyroid disruption occur in salmonids (salmon, trout and charr).

Thyroid disruption in fish was reported first in the late 1970's. Leatherland and colleagues reported changes in thyroid histology and plasma TH levels in coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) collected in the Great Lakes or exposed to Great Lakes water, and in coho salmon and rainbow trout (*Oncorhynchus mykiss*) exposed to PCB mixtures or Mirex (Leatherland and Sonstegard, 1978, 1979, 1980a; Muller, 1983). Flounder (*Platichthys flesus*) exposed to a PCB mixture experienced increases in both T4 and T3 in plasma (Besselink et al, 1996). However, exposure to the closely related TCDD caused no alterations in plasma TH levels in the same species (Besselink et al, 1997).

Exposure to contaminant mixtures has also been reported. Bleached kraft mill effluent, which typically contains various dioxins and PCB compounds, increased plasma T4 in white suckers (*Catastomus commersoni*) (Munkittrick et al, 1991). Near industrial centres in St. Lawrence Estuary, water and sediments contains a variety of industrial

effluents as well as agricultural run-off and can cause reductions in plasma T4 with increases in plasma T3 in perch (*Perca flavescens*) (Hontela et al, 1995).

The impact of non-PCB-compounds such as pesticides on the fish thyroid has been studied using the murrel (*Channa punctatus*) and catfish species (*Claris batrachus* and *Heteropneustes fossilis*). Malathion exposure both increased and decreased plasma T3 of catfish species (Sinha et al, 1991; Yadav and Singh, 1986), depending on sexual maturity (Sinha et al, 1992). Hexadrin and cythion decreased plasma TSH levels, determined using a conversion ratio and I-131 uptake values in relation to pituitary gland extract and blood serum, and inhibited I-131 uptake (Singh and Singh, 1980). Carburan decreased plasma T4 and increased plasma T3 (Sinha et al, 1991) while fenitrothion and carburan decreased thyroid follicular diameters (Saxena and Mani, 1988). Endosulphan increased plasma T4 levels and decreased plasma T3 in the catfish (Sinha et al, 1991), and caused hyperplasia and a reduced colloid content in tilapia (Bhattacharya, 1995).

There are also reports of thyroid disruption in fish exposed to metals. A combination of low pH and aluminum decreased hepatic T4ORD activity in mature rainbow trout, with no changes in plasma T4 or T3 levels (Brown et al, 1990). This same treatment also reduced tissue levels of T3 (Fok et al, 1990). Immature rainbow trout exposed to only a low pH had increased levels of plasma T4 and decreased plasma T3 (Brown et al 1984), while brown trout (*Salmo trutta*) increased their plasma levels of T4 upon exposure to low pH alone (Brown et al, 1989) or a combination of low pH and aluminum (Whitehead and Brown, 1989). Brown trout exposed only to aluminum increased levels of both THs and liver 5'D (Waring and Brown, 1997). Lead and mercury-based compounds have caused hypertrophy, reduction in colloid content and

inhibited uptake of I-131 in the catfish (*Claris batrachus*) (Katti and Sathyanesan, 1987; Kirubagaran and Joy, 1989), while lead alone decreased plasma THs and ORD activity (Chaurasia et al, 1996a or b). Rainbow trout exposed to mercurial compounds experienced increases in both T4 and T3 (Bleau et al, 1996). Acute (hours) cadmium exposure increased plasma T4 levels in rainbow trout, while a subacute (days) exposure decreased plasma T4 and neither dosage changed plasma T3 (Hontela et al, 1996).

Folmar and others (1982) found increased plasma T4 and T3 levels of coho salmon exposed to fuel oil. While in more recent studies exposure of turbot (*Scophthalmus maximus*) to oil fractions caused whole body increases in T4 but no change in T3 (Stephens et al, 1997a, 1997b). Cyanide compounds decreased levels of plasma T4 (Ruby et al, 1993; Lanno and Dixon, 1996a) and T3 (Ruby et al, 1993). Histological changes including hyperplasia and reduction in colloid content have also been reported (Lanno and Dixon, 1996a, 1996b).

The results for fish suggest some differences in the response of the thyroid system to a contaminant stress when compared to birds and mammals. In fish, T4 generally seems to increase in the presence of a xenobiotic challenge, but typically there are no changes in plasma levels of T3. This suggests a change in the central control of the fish thyroid hormone system responsible for T4 production. There is a small set of evidence to suggest that peripheral control of TH can be influenced by aluminum in rainbow trout (Fok et al, 1990). In lake trout, PCB 126 caused changes in thyroid epithelial cell height, plasma T4 kinetics and T4-glucuronidation, without changes in ORD activity, T3 glucuronidation or plasma TH levels (Brown et al, 1997).

There is no evidence indicating whether any class of contaminants might alter receptor and post-receptor actions of TH. Thus, the trend in fish is for an increase in T4 in response to a contaminant challenge. This is similar to the response seen in reptiles and amphibians suggesting thyroid homeostasis may be comparably disrupted in the less recently evolved species. This may be related to the evolution of the thyroid system in these classes of vertebrates or to the differences in uptake and exposure of animals in their particular environments.

General Conclusions

Of all the xenobiotics studied, there seems to be greatest potential for TCDD and TCDD-like compounds to disrupt the thyroid system in vertebrates. There are a large number of gaps in our understanding. To what extent do xenobiotics alter the peripheral metabolic systems in vertebrates? How effective are metals in disrupting the thyroid systems of vertebrates? There are few reports that deal with the impact of the metabolites of contaminants on the thyroid system. However, what reports there are suggest this is an area of concern. There has been little study of the direct response of the thyroid systems of birds, reptiles, amphibians and fish to a contaminant challenge. For all vertebrate classes, a greater range of thyroidal parameters, including at least one from each level of control of the thyroid system (central control of T4 production, peripheral control of T3 formation and hormone action through T3 regulation of protein synthesis), would give a better indication of the impact of a xenobiotic on thyroidal status.

Most studies report a single parameter of the thyroid system making it difficult to fully estimate or assess the impact on the thyroidal status of the organism, as multiple parameters are required to make this evaluation. Newly developed tools, such as the

cDNAs for deiodinases and TH receptors should be used to assess potential contaminant action at the pretranslational level, giving clearer indications of alteration in TH action. A better understanding of the influence of TH on vertebrate behaviour may help to determine if contaminant influences on the thyroid system of an organism would also affect that aspect.

Table 2-1: Effects of xenobiotics on thyroid parameters in vertebrates.

Species	Parameter	Contaminant	Effect	Reference
MAMMALIAN				
Rat (Long-Evans) (*Rattus norvegicus*)	ORD (liver), ORD (BAT) T4, T3	TCDD	-ORD (liver),+ORD (BAT) -T4, ncT3	(Raasmaja et al, 1996)
	T4,T3,TSH	PCBs (Araclor 1254)	-T4, -T3 (moderate)	(Goldey et al, 1995)
Rat (Sprague -Dawley)	T4 binding (to TTR)	OH-PCBs	-T4 binding	(Brouwer et al, 1990)
	T3, T4 Thyroid response to TSH	PCB, PBB	-T4, -T3	(Byrne et al, 1987)
	TT4, FT4 T4-UGT	TCDD TCDD-like	ncTT4, FT4, +T4-UGT - TT4, - FT4, + T4-UGT	(Birnbaum et al, 1998)
	T4, T3, UGT	PCBs (Araclor 1254)	-T4, -T3, +UGT	(Barter and Klaassen, 1992)
	TSH, T4, T3, rT3, T4-UGT	Thiazopyr	+TSH, -T4, +T3, +rT3 +T4-UGT	(Hotz et al, 1997)
Male hooded rats	T3, T4, TSH	TCDD	-T4, +T3	(Bastomsky, 1977)
Rat (Wistar) (fetal, neonates)	TT4, FT4 TT3, FT3 ORD activity	PCBs (HCB, TCB)	-T4, ORD activity	(Morse et al, 1993)
	T4, T3 5'DII activity	PCBs (Araclor 1254)	-T4, ncT3, +5'DII activity	(Morse et al, 1996)
	T4	Hexabromobiphenyl	-T4 (26%)	(Spear et al, 1994)

	T4, T3	Hexabromobiphenyl	-T4, ncT3, incr. MBC T4, +UGT	(Spear et al, 1990)
Species	**Parameter**	**Contaminant**	**Effect**	**Reference**
Rat (Wistar) – Male	T4, T3, ORD	Cadmium	-T4, -T3,-ORD	(Paier et al, 1993)
Rat (Wistar) – Male (*in vitro* and *in vivo*)	T4, T3, ORD	Zinc Cadmium	nc T4, nc T3, -ORD -T4, -T3, -ORD	(Gonzalez-Pondal et al, 1995)
Rat (Wistar) – Female	T4, T3, ORD	Cadmium (CdCl2)	ncT4, -T3,-ORD	(Yoshida et al, 1987)
Rat	selenium binding protein (SBP)	PCB 126	induction of SBP	(Ishii et al, 1996)
Rat (Wistar) – cytosol	iodothyronine sulfo-transferase activity	OH-PCBs	-sulfotransferase activity	(Gerlienke Schuur et al, 1998)
Rat (Wistar) – liv. homog.	T4, T3, ORD, LPO	Cadmium (Cd), Lead (Pb)	-T4, -T3, -ORD, +LPO	(Chaurasia et al, 1996b)
Rat (Fischer 344)	T4	PCBs (Araclor 1254)	-T4, incr. liver mass	(Gray et al, 1993)
Mouse (*Mus domesticus*)	T4 T4 binding	PCB 77	-FT4, -TT4	(Darnerud et al, 1996)
Mouse (mother and fetal)	T4	OH-PCBs	-T4	(Sinjari and Darnerud, 1998)
Mouse (fetal)	T4, T3, ORD, IRD	Methylmercury	ncT4, ncT3, ncORD, -IRD	(Watanabe et al, 1999)
Mink (*Mustela vison*)	TT3, TT4	mix. MeSu-PCBs, DDE	-TT3, -TT4	(Lund et al, 1999)
Common seal (*Phoca vitulina*)	TT4, FT4 TT3	PCB-contam. Fish	-TT4,-FT4,-TT3	(Brouwer et al, 1989)
Indian ground squirrel (*Funambulus pennanti*)	I-131 uptake, hist., PBI	Lead nitrate	-I-131uptk., -colloid,-PBI	(Shrivastava et al, 1987)

Species	Parameter	Contaminant	Effect	Reference
AVIAN				
Pigeon (*Columba livia*)	Thyroid mass Colloid content	pp'-DDE, dieldrin	+thyroid mass -colloid content	(Jefferies and French, 1972)
	Thyroid mass Colloid content	pp'-DDT	+thyroid mass -colloid content	(Jefferies and French, 1969,1971)
Guillemots (*Uria aalge*)	Thyroid mass Colloid content	PCB (Araclor 1254)	+thyroid mass -colloid content	(Jefferies and Parslow, 1976)
Indian rock pigeon	T4, T3	Metal-Cadmium	-T3 (sugg -T4 to T3)	(Prakash et al, 1997)
Gallus gallus, *Columbia livia*, *Ardeo heodias* (in ovo - perinatal)	T4, T3	TCDD	No changes	(Janz andBellward,1996)
Ring doves (*Streptopelia risoria*)	Histology TT4, TT3	PCB77	hyperplasia reversal -TT4, -TT3	(Spear and Moon, 1985)
Great Blue herons (*Ardea herodias*)	TT4	TCDD	+T4, ncT3, nc T3/T4	(Janz and Bellward,1997)
Common tern (*Sterna hirundo*)	TT4, fT4, TT3 T4-UGT activity	PHAHs	No significant changes	(Murk et al, 1994)
Japanese quail (*Coturnix coturnix japonica*)	Histology, T4, T3	DTT PCBs	-T4, +T3 -T4, -T3	(Grassle and Biessmann, 1982)
Herring gulls (*Larus. argentatus*)	T4	Crude oil	+T4	(Peakall et al, 1981)

Species	Parameter	Contaminant	Effect	Reference
Black guillemots (*Cepphus grylle*)				
Leach petrels (*Oceanodroma leucorhoa*)				
Black ducks (*Anas rubripes*)	T3	low pH	-T3	(Rattner et al, 1987)
Chicken (*Gallus domesticus*)	T4, T3, ORD (liver)	cadmium	-T3, nc T4, -ORD	(Gupta and Kar, 1999)
REPTILES				
Alligators (*Alligator mississippiensis*)	T4, T3	contam. lakes	+T4m	(Crain et al, 1998)
AMPHIBIANS				
Xenopus laevis	Tadpole metamorphosis	methoxychlor	fail to metamorphose	(Morrell et al, 1998)
Xenopus laevis	Tail resorption	pentachlorophenol nonylphenol	- tail resorption +tail resorption	(Fort et al, 1998)
Larval tiger salamander (*Ambystoma tigrinum*)	T4, metamorphosis	Atrazine	+T4	(Larson et al, 1998)
FISH				
Catfish (*Claris batrachus*)	Histology I-131 uptake	Lead nitrate	Hypert., +cell height, vacul, -coll. cont., Uptake inhib.	(Katti and Sathyanesan, 1987)
Catfish (*Claris batrachus*)	Histology I-131 uptake	Mercurials	Hypert. epith,-coll.cont -I-131 uptake, -PBI	(Kirubagaran & Joy,1989)

Species	Parameter	Contaminant	Effect	Reference
Catfish (*Clarias batrachus*)	T4,T3,T3/T4	Endosulfan Malathion Carbaryl	+T4, -T3, -T3/T4 ncT4, -T3, -T3/T4 -T4, +T3, +T3/T4	(Sinha et al, 1991)
Catfish (*Clarias batrachus*)	T4, T3, peroxidase T4toT3 conversion	Malathion	changes depending on fish phase	(Sinha et al, 1992)
Catfish (*Clarias batrachus*)	T4, T3, ORD, LPO	Lead (Pb)	-T4, -T3, -ORD, +LPO	(Chaurasia et al, 1996)
Catfish (*Heteropneustes fossilis*)	TSH content I-131 uptake	Pesticides: cythion hexadrin	-pit., serumTSH -I-131 uptake	(Singh&Singh, 1980)
Catfish (*Heteropneustes fossilis*)	T4	Malathion, BHC	+/-T4, -T3(BHC), +T3(Malathion)	(Yadav and Singh, 1986)
Flounder (*Platichthys flesus*)	TT4, fT4 TT3, fT3	PCBs (Clophen A50)	+T4, +T3 (at one time point)	(Besselink et al, 1996)
Flounder (*Platichthys flesus*)	TT4, fT4 TT3, fT3	TCDD	ncT4, ncT3	(Besselink et al, 1997)
Turbot (*Scophthalmus maximus*)	wb T4,T3	WSF oil	+whole body T4, ncT3	(Stephens et al, 1997a)
Turbot (larval) (*Scophthalmus maximus*)	wb T4, T3	WSF oil	+whole body T4, nc T3	(Stephens et al, 1997b)
Coho salmon (*Oncorhynchus kisutch*)	T4, T3	PCB, Mirex Mirex	PCB ncT4, -T3 (high dose) -T4, -T3, -bw	(Leatherland & Sonstegard, 1978)
Coho salmon (*Oncorhynchus kisutch*)	T4, T3	PCBs (Araclor 1254) No. 2 fuel oil	+T4, +T3 (after duration)	(Folmar et al, 1982)

Species	Parameter	Contaminant	Effect	Reference
Coho and Chinook salmon (*O. kisutch, O. tshawytscha*)	Histology	Great Lakes	differences between lakes	(Moccia et al, 1981)
Murrel (*Channa punctatus*)	Histology	Fenitrothion carburan	-follicle diameters	(Saxena and Mani, 1988)
Murrel (*Channa punctatus*)	Histology	NH4SO3 fertilizer	Hypertrophy, hyperplasia, reduction in colloid content	(Narayan Ram&Sathyanesan,1987)
Pink salmon (*Oncorhynchus gorbuscha*)	Histology, T4, T3	Great Lakes	Hyperplasia, -T4, ncT3	(Noltie and Leatherland, 1988)
Pink salmon (*O. gorbuscha*)	Histology	Great Lakes	Hyperplasia	(Noltie, 1988)
Perch (*Perca flavescens*)	T4, T3	various	-T4, nc or +T3	(Hontela et al, 1995)
White sucker (*Catastomus commersoni*)	T4, T3	BK mill effluent	+T4, ncT3	(Munkittrick et al, 1991)
Tilapia *(Oreochromis mossambicus)*	Histology	Endosulphan	Hyperplasia, foll. Atrophy -colloid content	(Bhattacharya, 1995)
Brown trout (*Salmo trutta*)	T4, T3, liver ORD	Aluminum	+T4, +T3, +liver ORD	(Waring and Brown,1997)
Brown trout (*Salmo trutta*)	T4	pH + Aluminum	+T4	(Whitehead and Brown, 1989)
Brown trout (*Salmo trutta*)	T4, T3	pH	+T4, ncT3	(Brown et al, 1989)
Lake trout (*Salvelinus namaycush*)	Hist., T4, T3, ORD, T4- and T3-gluc.	PCB 126	+cell height, ncT4, ncT3, ncORD +T4-gluc.	(Brown et al, 1997)

Species	Parameter	Contaminant	Effect	Reference
Rainbow (RB) trout (*O. mykiss*)	T3, T4	mercury chloride methylmercury cl	+ T4, trend +T3	(Bleau et al, 1996)
RB trout (immature) (*O. mykiss*)	Histology T4, T3	Acidification	+T4,-T3 (depend. on expt)	(Brown et al, 1984)
RB trout (*O. mykiss*)	Tissue T3 levels	low pH + Aluminum	-Tissue T3 pool)	(Fok et al, 1990)
RB trout (*O. mykiss*)	T4, T3, ORD	low pH + Aluminum	ncT4, ncT3, -ORD	(Brown et al, 1990)
RB trout (*O. mykiss*)	T4, T3	cadmium (acute, sub)	acute + T4 nc T3 subacute -T4, nc T3	(Hontela et al, 1996)
RB trout (*O. mykiss*)	T4, T3	HCN	-T3, trend -T4	(Ruby et al, 1993)
RB trout	Histology T4, T3	PCBs, Mirex	PCB nc hist.,ncT4, ncT3 Mirex nc hist., -T4, -T3	(Leatherland &Sonstegard. 1979)
RB trout	T4, T3	PCBs, Mirex Mirex -T3, +T4/T3	PCB nc histology, -T4, -T3 (at highest dose)	(Leatherland and Sontegard, 1980b)
RB trout	T4, T3	PCDF	ncT4, ncT3	(Brown et al, 1998)
RB trout	Histology, T4	Thiocyanate	hyperplasia, -coll cont, -T4	(Lanno and Dixon, 1996a)
	Histology, T4	Thiocyanate	-coll. content	(Lanno and Dixon, 1996b)

CHAPTER 3: Characterization of deiodination pathways in tissues of the American plaice, *Hippoglossoides platessoides*.

Introduction

Several deiodination pathways have been described in vertebrates using T4, T3, and rT3 as substrates (Visser, 1996). Measurement of deiodinase activity has been proposed as a key component of a suite of assays than can be used to assess thyroidal status in fish (Eales et al, 1999). In fish, the main pathways are T4ORD to form the more biologically active T3, and T4IRD and T3IRD pathways that degrade T4 and T3 to inactive forms (Eales and Brown, 1993). In order to use deiodination as an indicator of thyroidal status in American plaice (*Hippoglossoides platessoides*), it is necessary to determine in which tissues these pathways occur and to optimize the conditions necessary for *in vitro* assays of their activity.

There is little information on the thyroid physiology of plaice or other pleuronectid fish. Osborn and Simpson (1969, 1978) described the histology of the thyroid and examined aspects of thyroid hormone metabolism in the European plaice (*Pleuronectes platessa* L.). Eales and Fletcher (1982) described seasonal TH cycles in the winter flounder (*Pseudopleuronectes americanus* Walbaum). THs play a major role in regulating flatfish metamorphosis (Inui and Miwa, 1985). Deiodination pathways have been characterized recently in the turbot (*Scophthalmus maximus*) (Mol et al, 1998). There have been no studies to date investigating deiodination pathways in any plaice species.

Information on deiodinase pathways in plaice is essential to better understand the role of TH in these species and, for purposes of this project, to determine how contaminants may influence plaice thyroid physiology. The objectives here are to

determine the tissues of American plaice in which deiodination occurs, and the properties of the enzymes involved. Optimal assay conditions for deiodinating systems for this species will also be determined for later investigation of polychlorinated biphenyl (PCB) effects.

Materials and Methods

Animal collection and maintenance: American plaice were captured in Spring 1998 by trawl on the St. Lawrence Estuary near Matane, Quebec, Canada. The fish were maintained for at least 2 months prior to sampling in 3.6 X 1.2 X 0.45-m fiberglass tanks supplied with aerated, filtered, flowing seawater, under conditions of ambient temperature (5-9°C) and natural photoperiod. Fish were fed chopped capelin twice weekly and every sixth feeding fed in-house prepared capelin-based food pellets supplemented with vitamins (Nagler and Cyr, 1997; Scott and Scott, 1988).

Chemicals. Supplies of [125-I] T4 (*T4; specific activity (SA) = 1250 uCi/ug, [125-I] T3 (*T3; SA 3300 uCi/ug) and [125-I] rT3 (rT3*; s. a. = 730 uCi/ug) were obtained from New England Nuclear (NEN) Life Science Products. Unlabelled T4, T3, rT3, 3,5-diiodo-L-thyronine (3,5-T2), tetraiodothyroacetic acid (TETRAC), diothiothreitol (DTT) and 3,5,3'-triiodothyroacetic acid (TRIAC) were obtained from Sigma (St. Louis, MO, USA).

Blood and tissue sampling. After anesthetization by immersion in methane tricaine sulfonate (MS222) (1.0 g/litre), fish were sampled by bleeding from caudal blood vessels into heparinized syringes. Fish were then killed by a blow to the head. Brain, liver, gill, kidney, muscle, heart and a distal section of the intestine were collected, frozen in liquid nitrogen and subsequently stored at –80°C. Tissues were packed on dry ice and sent to the University of Manitoba where they were stored at – 76°C.

Preparation of microsomal fractions: Microsomes were prepared based on the methods of Shields and Eales (1986). Partially thawed tissues were added to 4 volumes (weight/volume; w/v) of buffer (0.1 M KH2PO4/Na2HPO4, 0.25 M sucrose, 1 mM EDTA, 20 mM DTT (except for DTT optimum assay), pH 7.4) in 55-ml Wheaton homogenizer chambers. Tissues were homogenized in a Polytron (Brinkman Instruments) at setting 5 for 10 s followed by two strokes with a motorized pestle (Tri-R Instruments Inc, New York). The resulting crude homogenate was filtered through two layers of cheesecloth. This and subsequent steps were performed on ice or at 4°C Aliquots of homogenate were transferred into centrifuge tubes and centrifuged for 20 min at 730 g in a Ti-50 fixed-angle rotor (Beckman Instruments, Palo Alto, California). The supernatant from this was centrifuged at 25 200 g for 20 min resulting in a crude mitochondrial/lysosomal pellet. The supernatant was again centrifuged this time for 1 hour at 110 000 g in order to obtain the final microsomal pellet. Pelleted fractions (resuspended in 1-2 ml of buffer) were stored at –70°C (Shields and Eales, 1986).

Deiodinase assays: The deiodinase assay followed Shields and Eales (1986), as modified by MacLatchy and Eales (1992) and Johnston and Eales (1995). Tissue distributions are the mean from at least six different fish of triplicate measures of the tissues of individual fish. All other assays were performed using a pool of microsomes prepared from at least three different fish. Tissue microsomal fractions were thawed on ice and diluted with buffer (pH 7.2) containing 20 mM DTT and 1 mM EDTA to a final protein concentration of 0.2 to 0.9 mg/ml, determined by the method of Bradford (1976). A volume of 0.5 ml-1.0 ml of diluted microsomal fraction was added to siliconized test tubes and equilibrated in darkness in a water bath for 30 minutes (9°C; 140 rpm). Adding a mixture of cold and

I-125 labeled iodothyronine (T4, T3 or rT3) started the reaction. After either 60 or 90 minutes the reaction was stopped with 0.5 ml of methylmercaptomethizmadol-methanol (MMI-methanol), vortexed and centrifuged at 15 600 g for 4 to 5 minutes. A 420-ul aliquot of the supernatant was transferred to 700-ul amber vials for HPLC analysis. Optima were determined for a parameter over a range: DTT (0-100 mM); pH (5-9); temperature (0-35°C); ATG (0.1-1000 uM); IAC and PTU (10-1000 mM); TETRAC, T4, T3, rT3, TRIAC and T2 (1-100 nM).

HPLC analyses: HPLC analyses were, in the manner of Sweeting and Eales (1992), conducted using Gilson-IBM binary gradient HPLC using a C-18 column (5 u, 16 X 146 mm, at 30°C) and an acetonitrile (0.1 % TFA)/ H2O (0.1% TFA) solvent system at a flow rate of 1 ml/min. For T3 and its metabolites the % acetonitrile was 40 or 42 % run isocratically. For T4 and rT3 and their metabolites the % acetonitrile was 40 or 42 % for 0-10 min and then increasing linearly to 54% (10-18 min) and then decreasing linearly to 40 or 42 % (18-26 min). The identity of peaks was established from numerous previous UV analyses of authentic iodothyronines. The proportion of substrate converted is determined from the area under the curve. All solvents used were HPLC grade and degassed with helium before use.

Statistics: Statistical parameters and analyses were completed using Microsoft Excel 97 or SigmaStat 8.0. Specific tests are designated in the Results section or in figure legends.

Results

Representative HPLC profiles for T4, T3 and rT3 deiodinase assay products are found in Figs. 3-1, 3-2 and 3-3, respectively. T4 ORD activity under standard conditions (0.49 nM T4, 20 mM DTT, 12 C, pH 7.2) was highest in plaice liver with an activity

Figure 3-1: Representative HPLC profiles of an extract of (A) labeled T4 substrate incubated with liver microsomes, and (B) labeled T4 substrate in the absence of microsomes (blank). The retention times of authentic I⁻, T3, rT3 and T4 are shown. C-18 column (5 u, 16 X 146 mm, at 30°C). Solvent flow = 1 ml min –1, acetonitrile/water (0.1% TFA) with acetonitrile gradient (40 or 42% for 0-10 min and then increasingly linearly to 54% and then decreasing linearly to 40 or 42% (18-26 min)). Radioactivity was measured by an on-line flow through gamma detector which records radioactivity in arbitrary units.

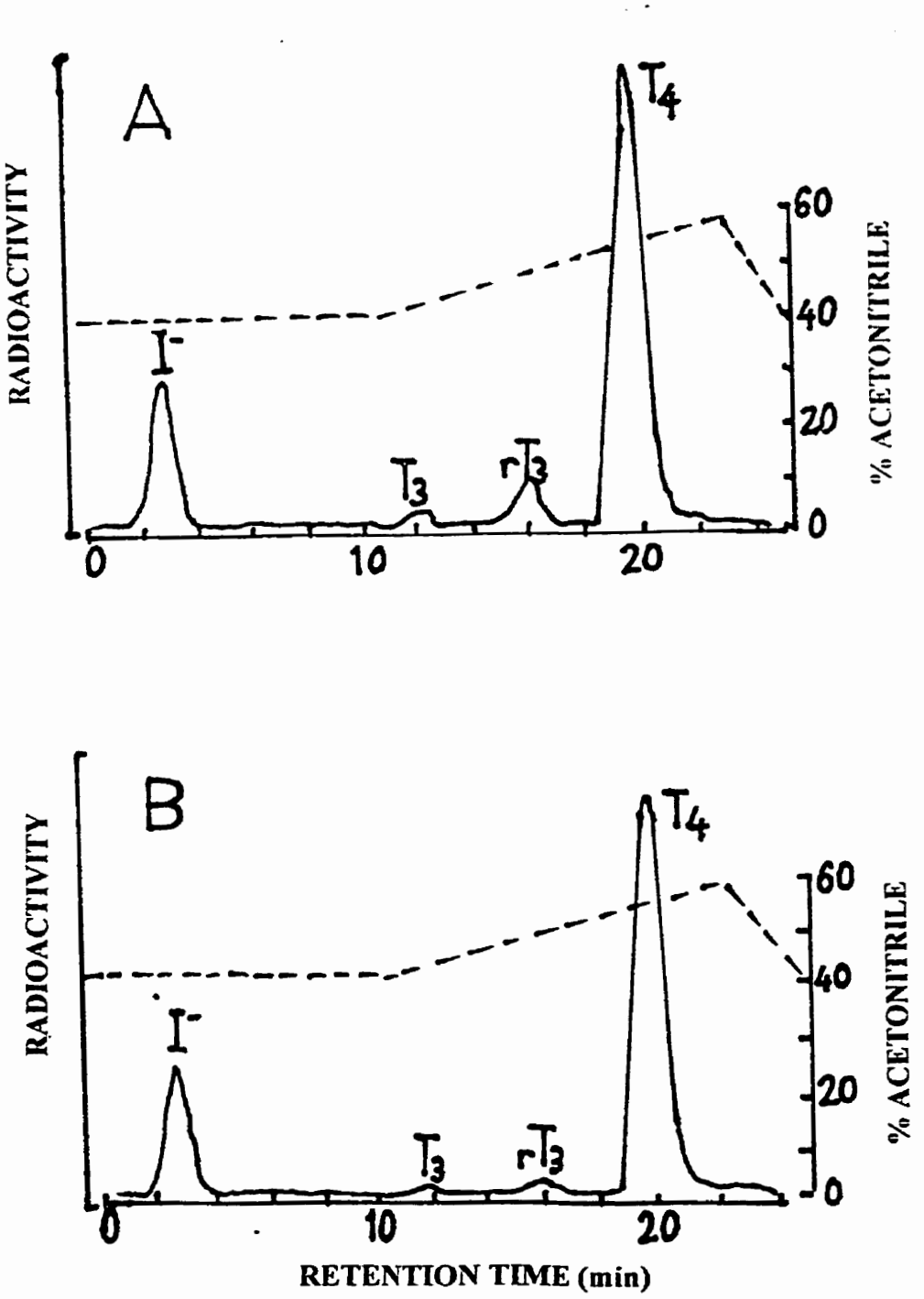

A
B
RADIOACTIVITY
% ACETONITRILE
I⁻
T3
rT3
T4
0
10
20
40
60
RETENTION TIME (min)

Figure 3-2: Representative HPLC profiles of an extract of (A) labeled T3 substrate incubated with liver microsomes, and (B) labeled T3 substrate in the absence of microsomes (blank). The retention times of authentic I^-, T2 and T3 are shown. C-18 column (5 u, 16 X 146 mm, at 30°C). Solvent flow = 1 ml min –1, acetonitrile/water (0.1% TFA) with acetonitrile gradient (40 or 42 % run isocratically). Radioactivity was measured by an on-line flow through gamma detector which records radioactivity in arbitrary units.

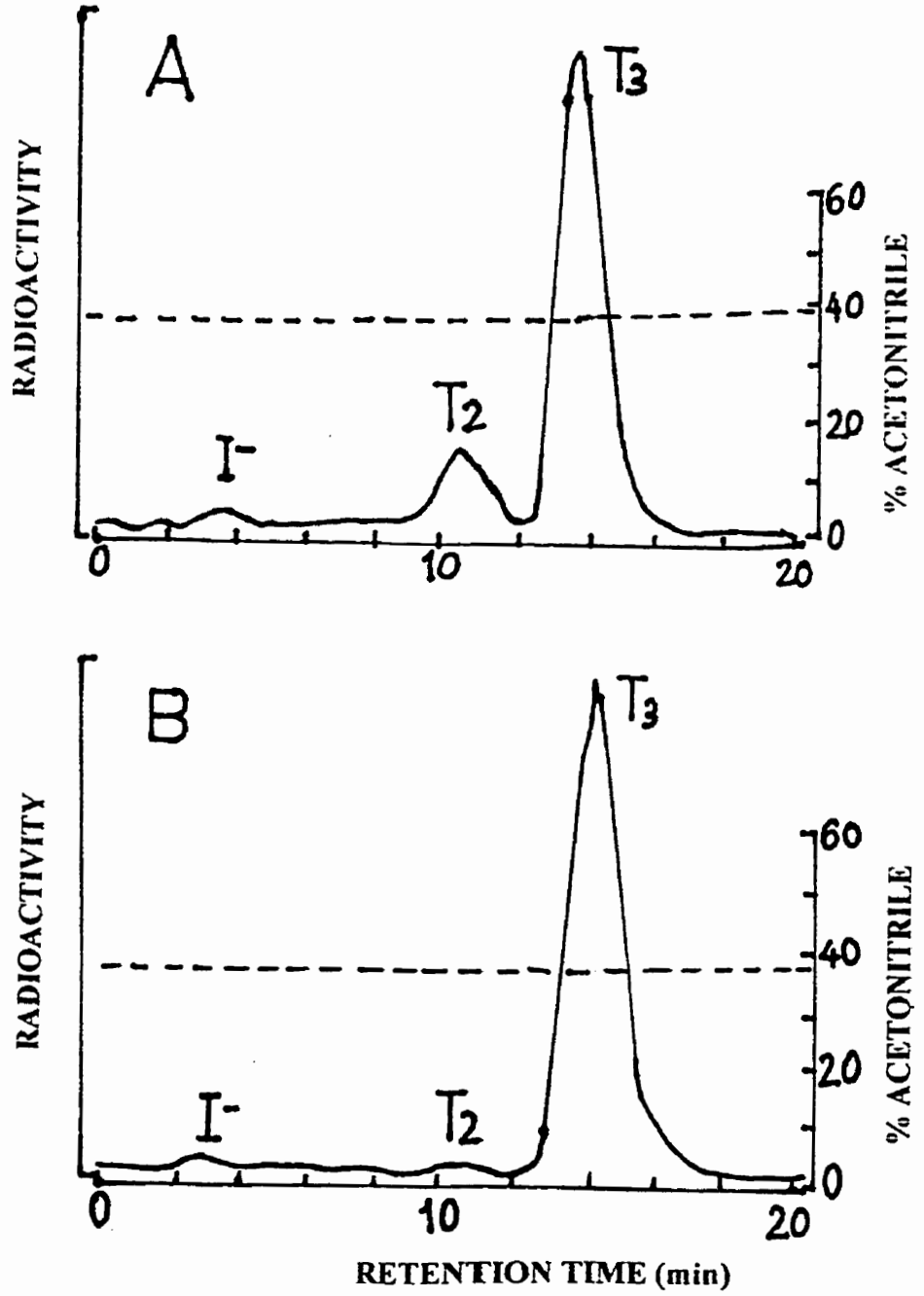
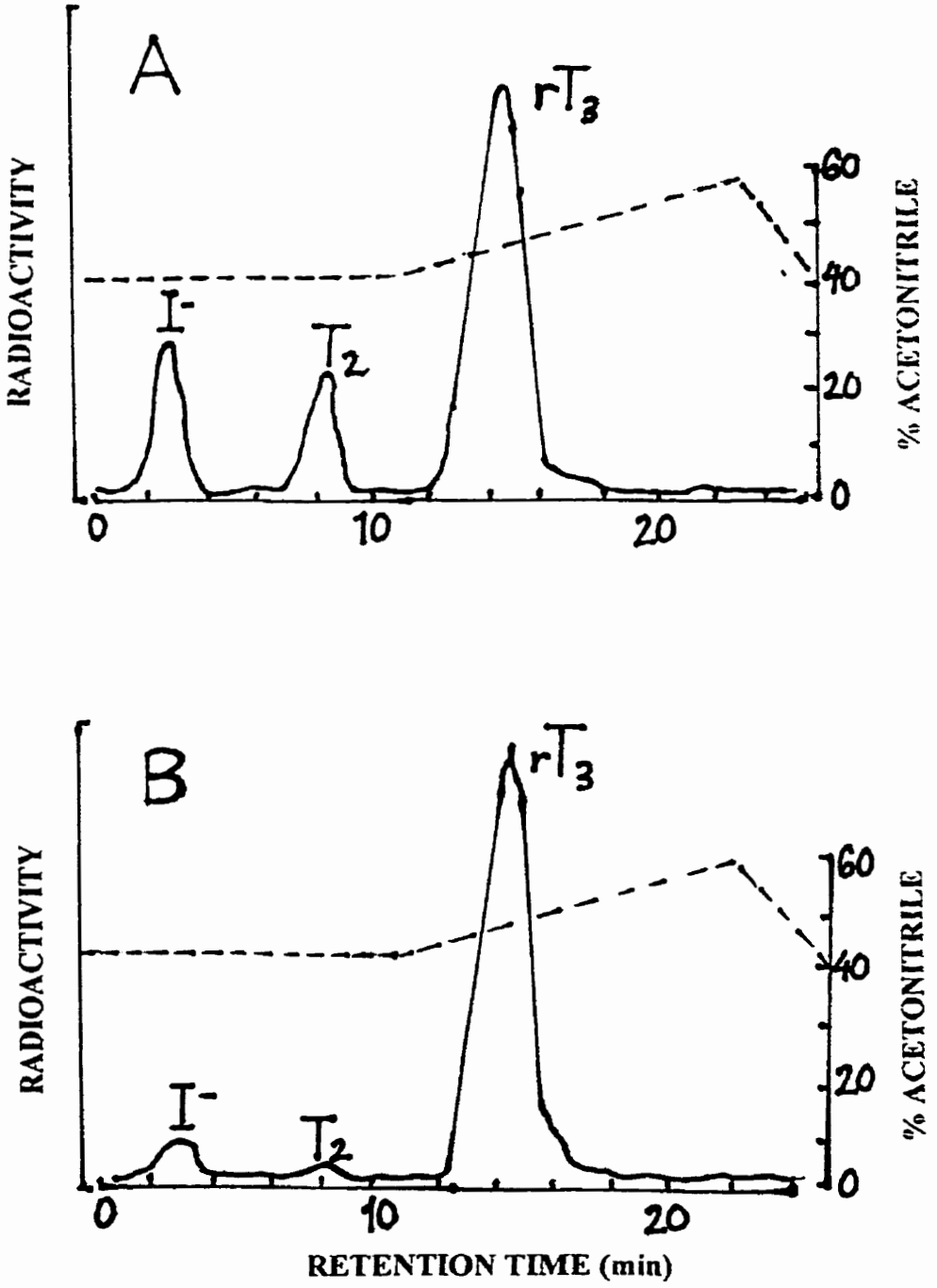

A
T3
T2
I-
RADIOACTIVITY
% ACETONITRILE
60
40
20
0
0
10
20
B
T3
T2
I-
RADIOACTIVITY
% ACETONITRILE
60
40
20
0
0
10
20
RETENTION TIME (min)

Figure 3-3: Representative HPLC profiles of an extract of (A) labeled rT3 substrate incubated with liver microsomes, and (B) labeled rT3 substrate in the absence of microsomes (blank). The retention times of authentic Γ, T2 and rT3 are shown. C-18 column (5 u, 16 X 146 mm, at 30°C). Solvent flow = 1 ml min –1, acetonitrile/water (0.1% TFA) with acetonitrile gradient (40 or 42% for 0-10 min and then increasingly linearly to 54% and then decreasing linearly to 40 or 42% (18-26 min)). Radioactivity was measured by an on-line flow through gamma detector which records radioactivity in arbitrary units.


A
rT3
I-
T2
RADIOACTIVITY
% ACETONITRILE
60
40
20
0
0
10
20
B
rT3
I-
T2
RADIOACTIVITY
% ACETONITRILE
60
40
20
0
0
10
20
RETENTION TIME (min)

level of 0.12 pmol (120 fmol) T4 converted/hr/mg protein (Fig. 3-4A). Some activity was detected in intestine and low or no activity was detected in gill, kidney, heart, brain or muscle. T4 IRD activity (measured simultaneously in the same assay as T4ORD activity under similar conditions) was detected in all tissues and was highest in brain (Fig. 3-4B). rT3ORD activity was highest in liver (Fig. 3-4C), although minor activity was detected in all tissues examined. No rT3 IRD activity was detected in any of the tissues studied. Based on these activities, subsequent enzyme characterizations of T4ORD activity and T4IRD (or T3IRD) activity were conducted using liver or brain tissues respectively.

The optimum level for the thiol cofactor (DTT) was 25 to 50 mM for liver T4ORD (Fig. 3-5A), while 12.5 to 25 nM was optimum for liver T3IRD (Fig. 3-5B). The low levels of liver T4IRD and brain T3IRD activities did not change over the concentration range used (with at least 12.5 nM). A common DTT cofactor level of 20 nM was selected for use in later assays.

The optimal pH for liver and brain T4ORD and brain T3IRD was between pH 7 and 8 (Fig. 3-6). A pH of 7.2 was used for the remainder of the characterization experiments, based both on the above results, as well as the pH optimum of 7.2 determined in other fish species (Frith and Eales, 1996; Mol et al, 1998).

Brain T4IRD and T3IRD and liver T4ORD activities were highest at an incubation temperature of 25°C (Fig. 3-7). Activity at 37°C was similar to values from 0 to 10°C for both brain and liver tissues. The remaining characterization assays were conducted at 12°C in order to compare with activities of assays in other fish species performed at 12°C (MacLatchy and Eales, 1992). Deiodinase assays for PCB-treated

Figure 3-4: Deiodinating activity in tissues of American plaice: T4ORD (pmol/hr/mg protein) (0.49 nM T4 substrate level), T4IRD (pmol/hr/mg protein) (0.49 nM T4 substrate level) and rT3ORD (fmol/hr/mg protein) (0.48 nM rT3 substrate level). Values represent the mean $\pm$ SEM of measures of 7 different plaice that were each measured in triplicate.

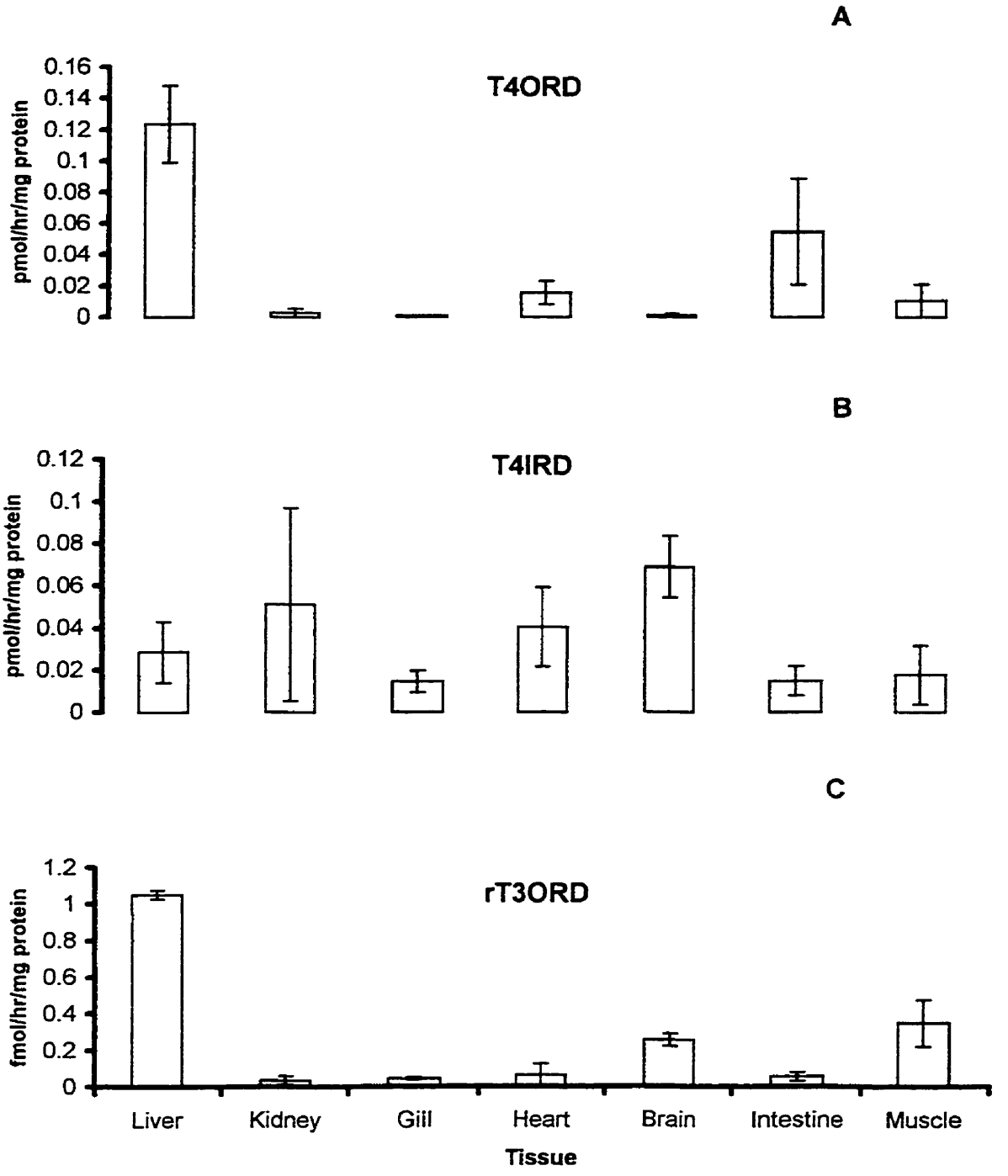

A
T4ORD
pmol/hr/mg protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
B
T4IRD
pmol/hr/mg protein
0.12
0.1
0.08
0.06
0.04
0.02
0
C
rT3ORD
fmol/hr/mg protein
1.2
1
0.8
0.6
0.4
0.2
0
Liver
Kidney
Gill
Heart
Brain
Intestine
Muscle
Tissue

Figure 3-5: The effect of DTT on plaice liver T4ORD, liver T4IRD, brain T3IRD and liver T3IRD activities in fmol/hr/mg protein. Values represent the mean of 3 measures on a pool of plaice microsomes.

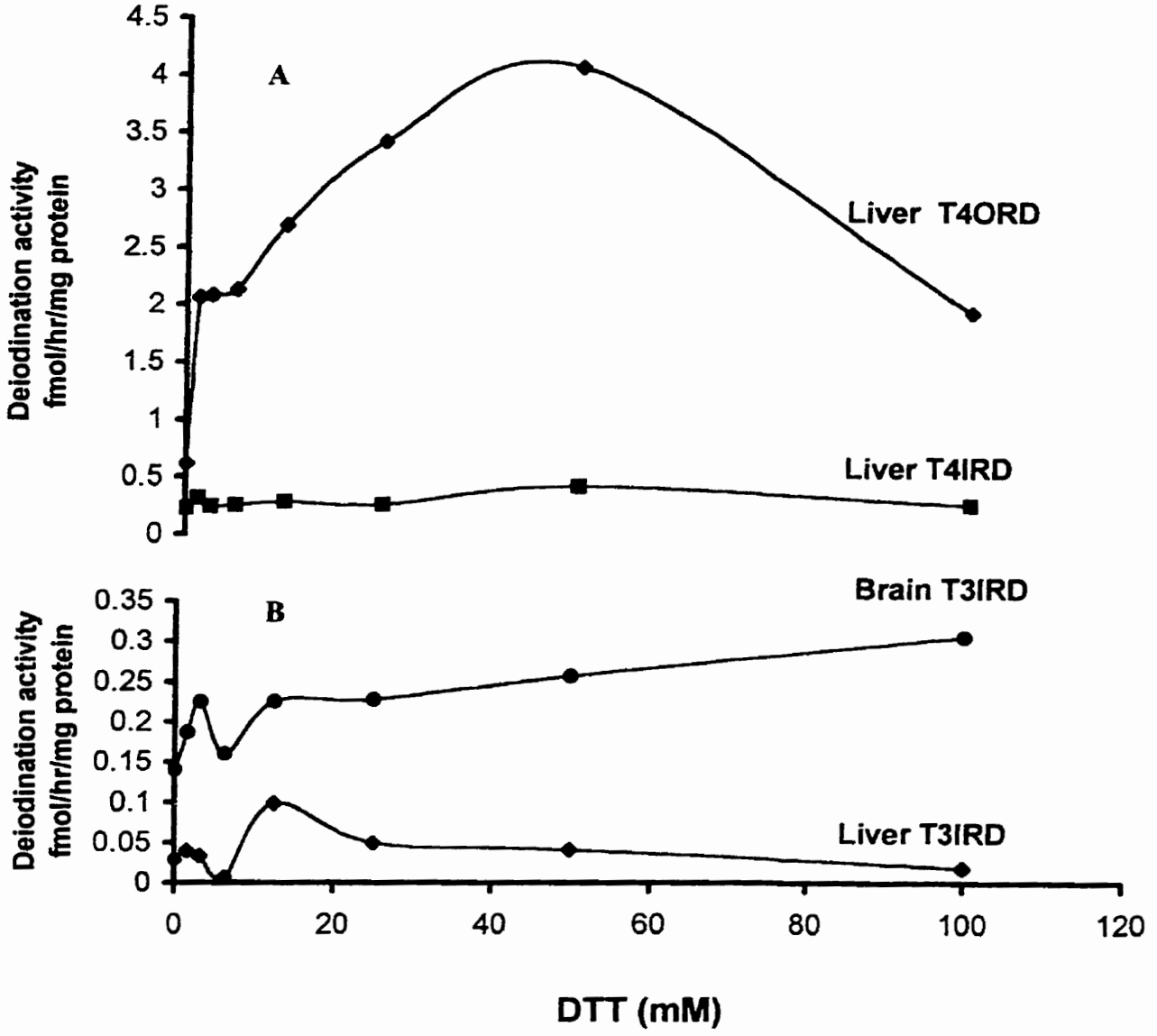

A
Deiodination activity
fmol/hr/mg protein
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Liver T4ORD
Liver T4IRD
B
Deiodination activity
fmol/hr/mg protein
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Brain T3IRD
Liver T3IRD
0
20
40
60
80
100
120
DTT (mM)

Figure 3-6: The effect of pH on plaice liver T4ORD, brain T3IRD and brain T4ORD activities in fmol/hr/mg protein. Values represent the mean of triplicate samples from a pool of plaice microsomes.

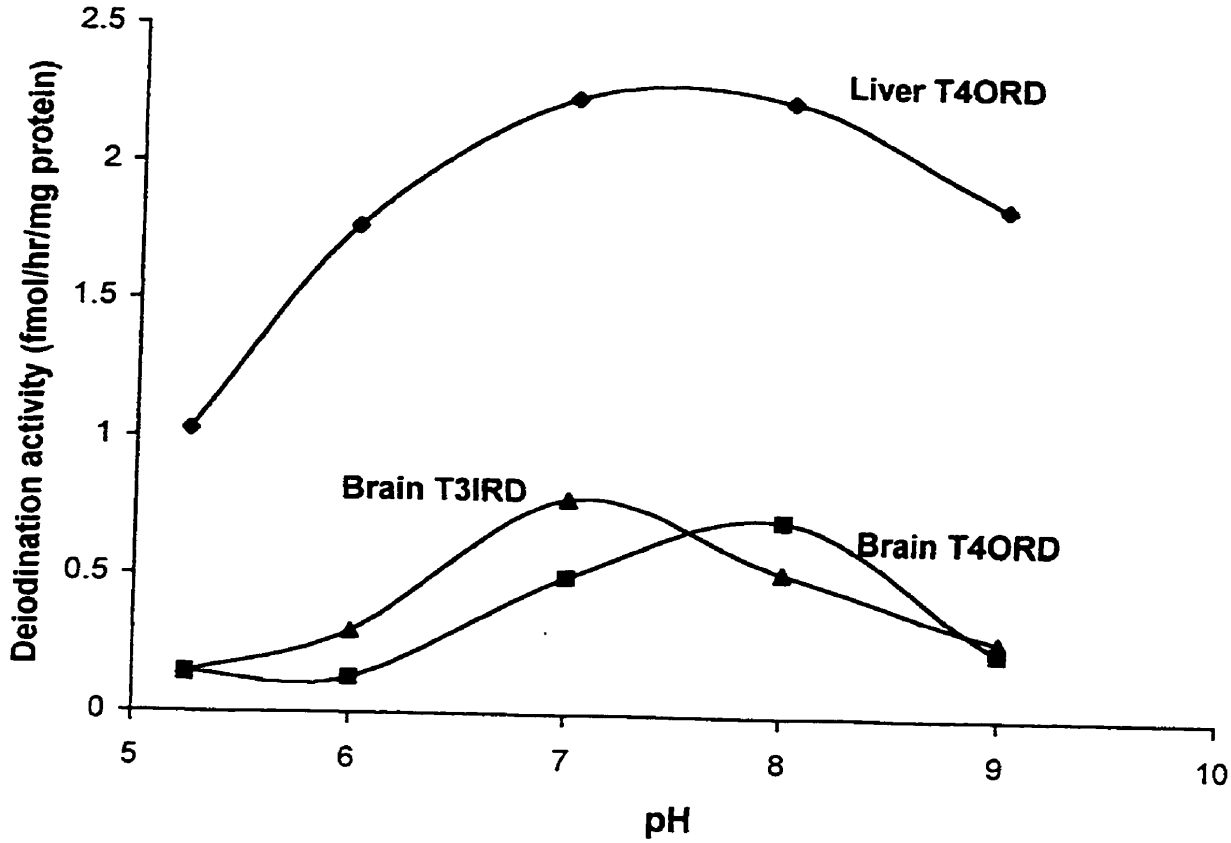
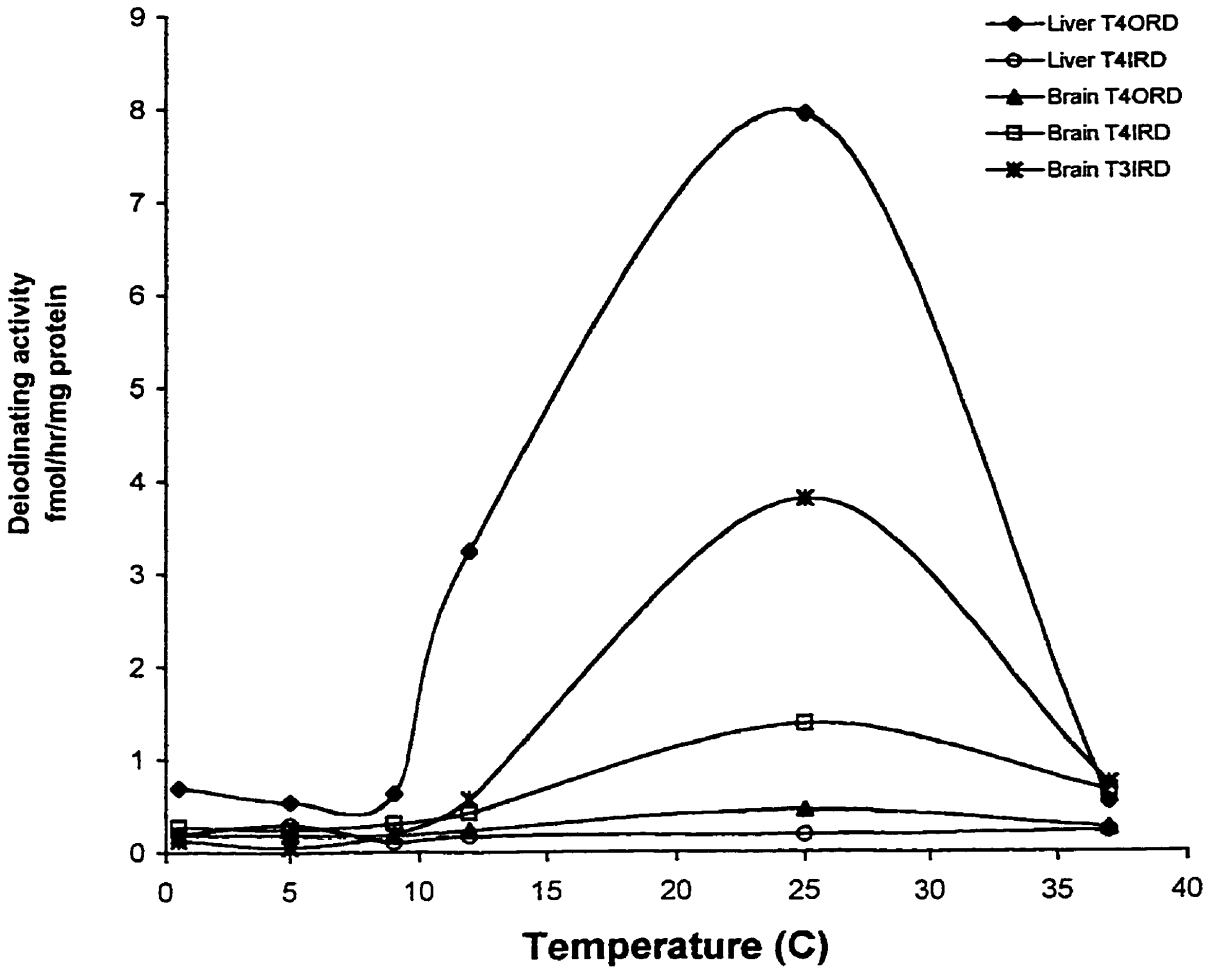

Deiodination activity (fmol/hr/mg protein)
2.5
2
1.5
1
0.5
0
5
6
7
8
9
10
pH
Liver T4ORD
Brain T3IRD
Brain T4ORD

Figure 3-7: The effect of temperature on deiodinating activity in plaice liver and brain in fmol/hr/mg protein. Values represent the mean of triplicate samples from a pool of plaice microsomes.


Liver T4ORD
Liver T4IRD
Brain T4ORD
Brain T4IRD
Brain T3IRD
Deiodinating activity
fmol/hr/mg protein
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25
30
35
40
Temperature (C)

plaice were conducted at 9°C, a temperature close to that at which the fish were held in the experiment.

Liver T4ORD was insensitive to iodoacetate (IAC) and 6-*n*-propyl-2-thiouracil (PTU) inhibition. However, it was inhibited by aurothioglucose (ATG) at concentrations greater than 1 uM (Fig. 3-8A). Brain T4IRD was sensitive to ATG and IAC, but not PTU (Fig. 3-8B). However, brain T3IRD was sensitive only to ATG, but not to IAC or PTU (Fig. 3-8C).

Liver T4ORD had an affinity for T4>TETRAC>T3=rT3=TRIAC>T2 in contrast to liver T4IRD (rT3>T2>T3>TETRAC=T4=TRIAC) and liver T3IRD (T3>TRIAC>TETRAC>T4>T2>rT3) (Fig. 3-9). Brain T4IRD had an affinity profile very similar to liver T3IRD (T3>TETRAC=TRIAC>T4>T2>rT3), and somewhat similar to brain T3IRD (TETRAC>T2=T3>T4=TRIAC=rT3=T2) (Fig. 3-9).

Lineweaver-Burk double-reciprocal plots indicated an apparent Km of 0.6 nM for plaice liver T4ORD (Fig. 3-10), 1.8 nM for liver T4IRD, 1.6 nM for liver T3IRD (Fig. 3-11), 3.6 nM for brain T3IRD (Fig. 3-11) and 0.6 nM for brain T4IRD.

Discussion

This is the first study of the deiodination pathways in American plaice. The only other study of deiodination pathways in a pleuronectid species was undertaken for turbot (Mol et al, 1998). This information is required for selection of tissues and assay conditions for the study of PCB effects on deiodination in plaice. It also provides useful comparative data.

The survey of seven tissues in plaice indicates that the highest T4ORD activity is in the liver. This resembles the situation in other teleost fish species, including rainbow

Figure 3-8: The effect of the potential inhibitors ATG (0.1 – 1000 uM), IAC (10 – 1000 uM) and PTU (10 – 1000 uM) on plaice liver T4ORD and brain T4IRD and T3IRD activities. Values represent the mean of triplicate samples from a pool of plaice microsomes.

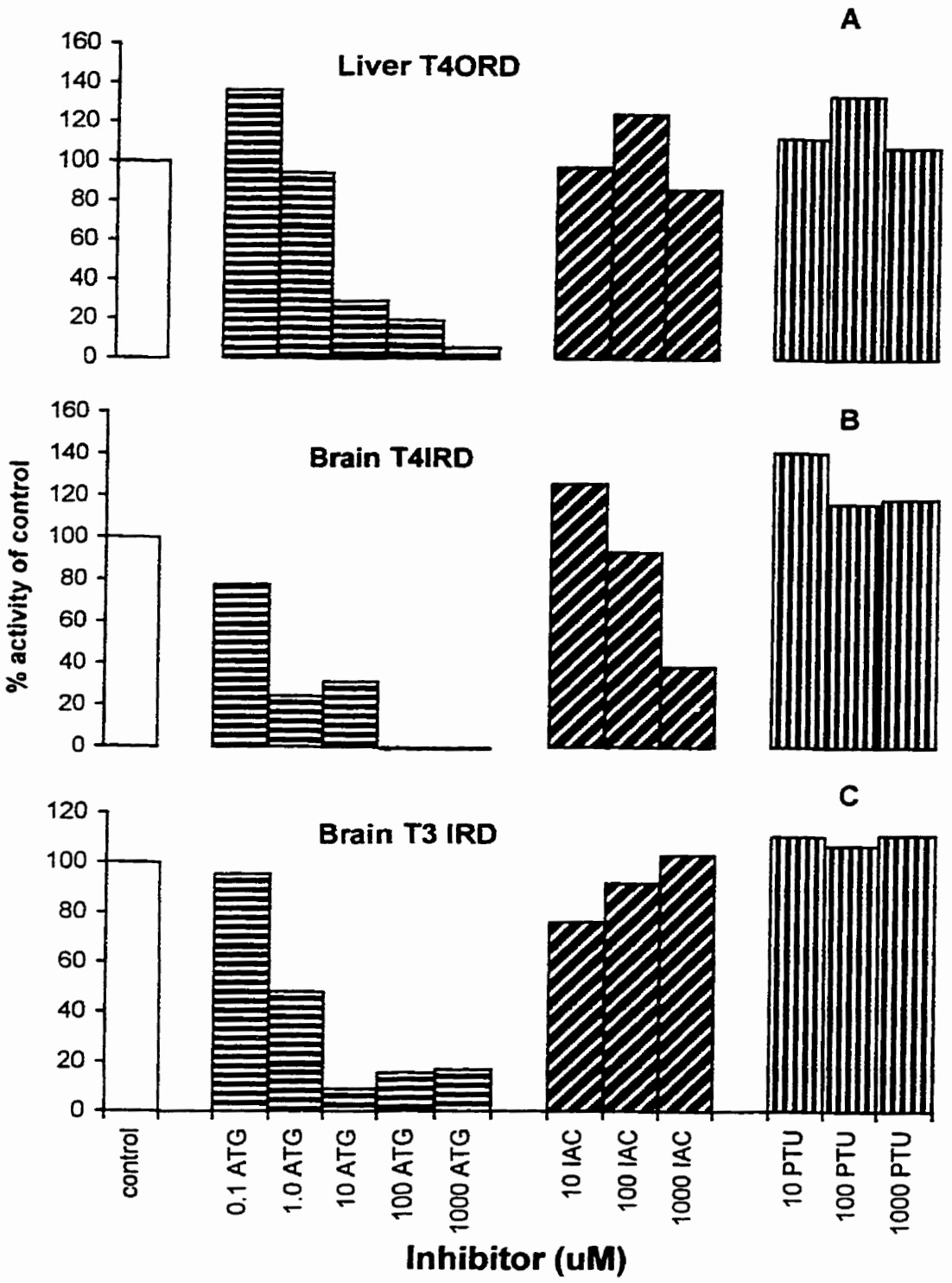

A
Liver T4ORD
160
140
120
100
80
60
40
20
0
B
Brain T4IRD
C
Brain T3 IRD
% activity of control
control
0.1 ATG
1.0 ATG
10 ATG
100 ATG
1000 ATG
10 IAC
100 IAC
1000 IAC
10 PTU
100 PTU
1000 PTU
Inhibitor (uM)

Figure 3-9: The effect of thyroid hormone analogues (1 – 100 nM) on liver and brain deiodinating activity. Values represent the mean of triplicate samples from a pool of plaice microsomes.

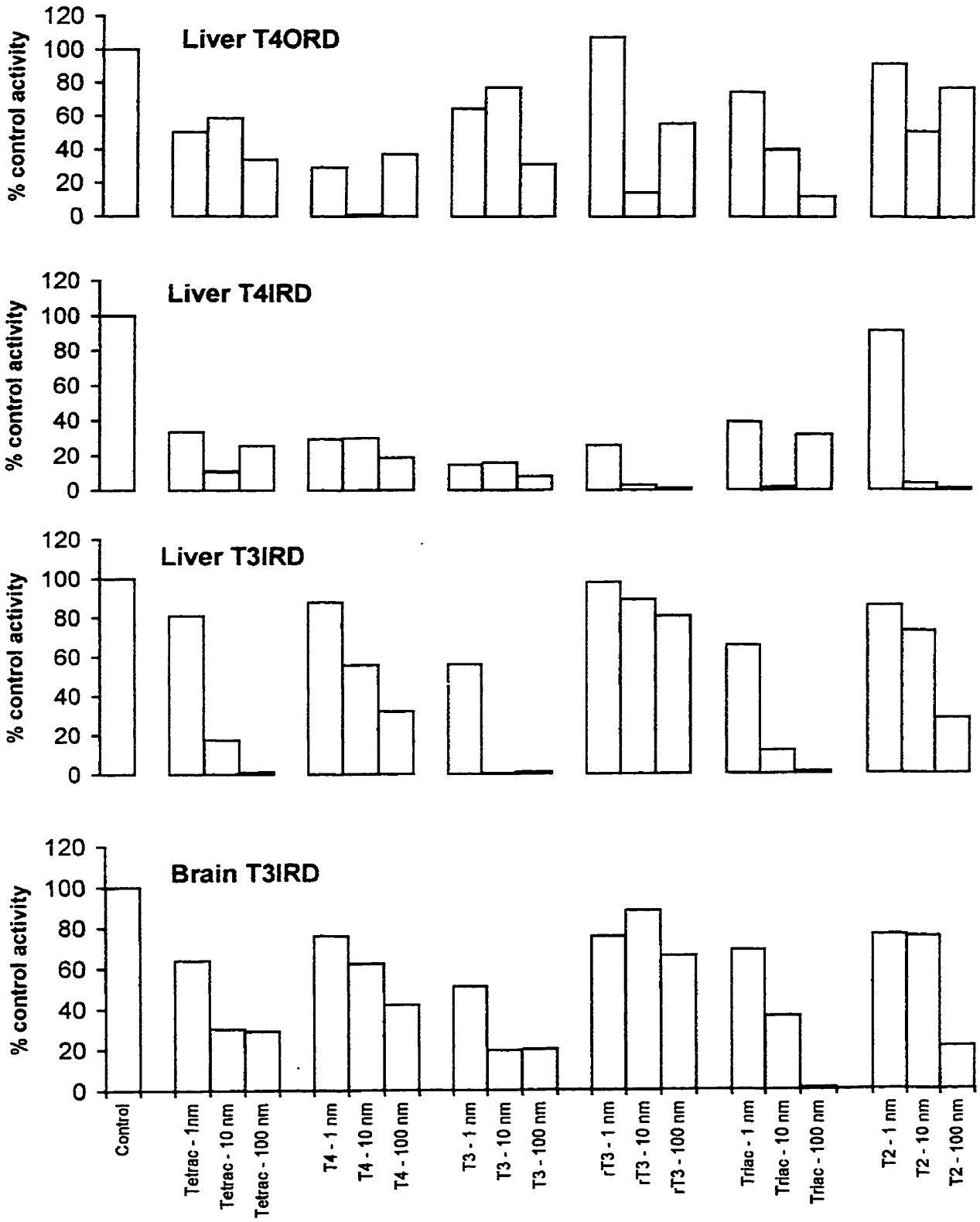

Liver T4ORD
Liver T4IRD
Liver T3IRD
Brain T3IRD
% control activity
120
100
80
60
40
20
0
Control
Tetrac - 1nm
Tetrac - 10 nm
Tetrac - 100 nm
T4 - 1 nm
T4 - 10 nm
T4 - 100 nm
T3 - 1 nm
T3 - 10 nm
T3 - 100 nm
rT3 - 1 nm
rT3 - 10 nm
rT3 - 100 nm
Triac - 1 nm
Triac - 10 nm
Triac - 100 nm
T2 - 1 nm
T2 - 10 nm
T2 - 100 nm

Figure 3-10: Lineweaver-Burk of plaice liver T4ORD plotted as the inverse of the substrate concentration (1/S (1/nM)) versus the inverse of the enzyme activity (1/V (1/pmol T4 converted/hr/mg protein)). The Vmax is 0.0002 pmol T4 converted/hr/mg protein. Values represent the mean of triplicate samples from a pool of plaice microsomes.

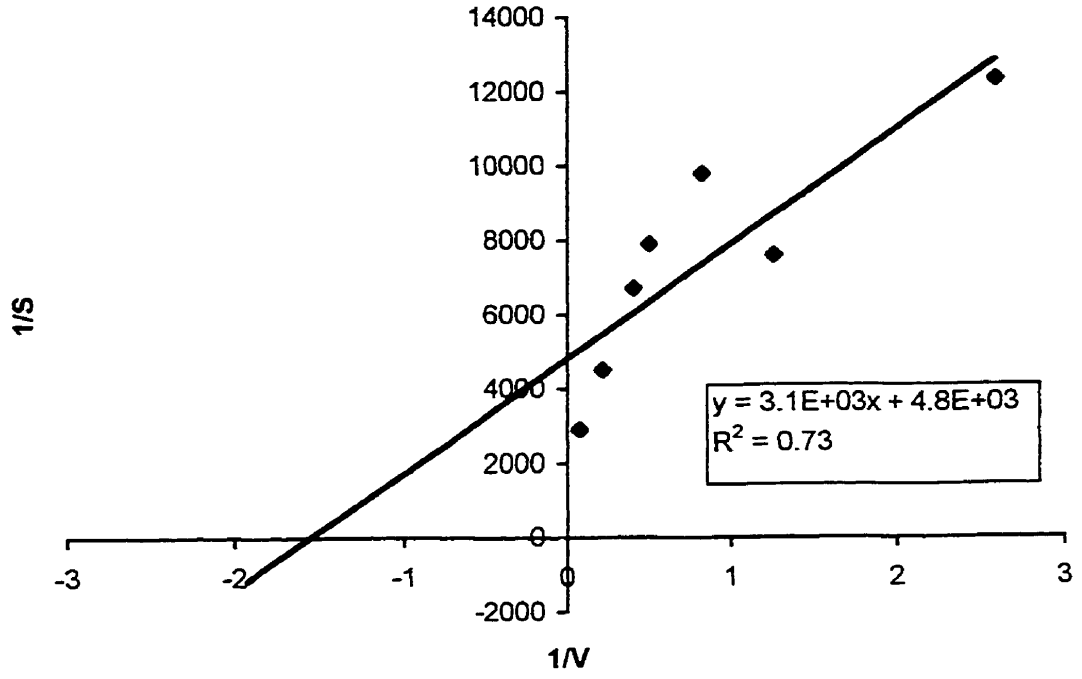
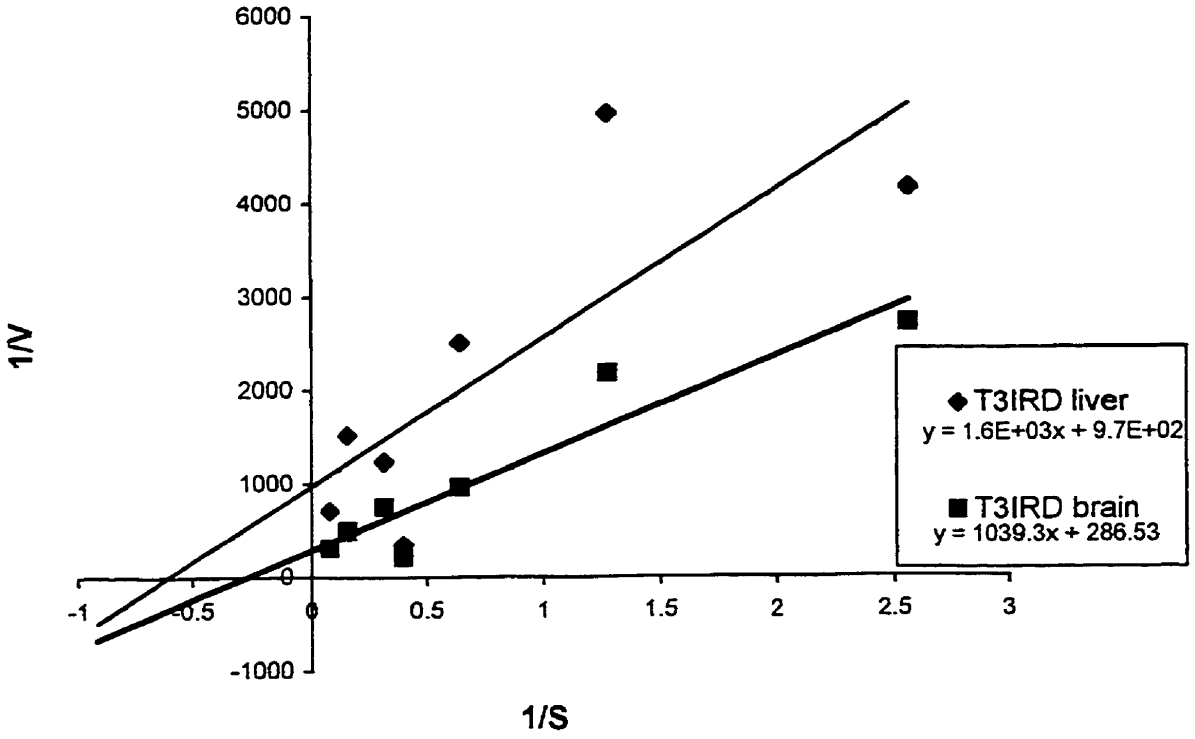

14000
12000
10000
8000
6000
4000
2000
0
-2000
-3
-2
-1
0
1
2
3
1/S
1/V
y = 3.1E+03x + 4.8E+03
R2 = 0.73

Figure 3-11: Lineweaver-Burk graph of plaice liver and brain T3IRD plotted as the inverse of the substrate concentration (1/S (1/nM)) versus the inverse of the enzyme activity (1/V (1/pmol T4 converted/hr/mg protein)). The Vmax values are 0.001 and 0.003 pmoles T3 converted/hr/mg protein for liver and brain, respectively. Values represent the mean of triplicate samples from a pool of plaice microsomes.


6000
5000
4000
3000
2000
1000
0
-1000
-1
-0.5
0
0.5
1
1.5
2
2.5
3
1/V
1/S
T3IRD liver
y = 1.6E+03x + 9.7E+02
T3IRD brain
y = 1039.3x + 286.53

trout (MacLatchy and Eales, 1992), Atlantic cod (Cyr et al, 1998), tilapia, African catfish and the turbot (Mol et al, 1998). There was significant activity in the intestine, the tissue with highest activity in the sea lamprey (Eales et al, 1997). Low, but detectable, T4ORD activity occurred in heart and muscle. Negligible T4ORD activity was detected in brain and gill tissues, while no activity was detected in the kidney. Because it had the highest T4ORD activity, plaice liver tissue was selected for examining susceptibility to PCBs.

IRD activity, based on T4IRD, was highest in brain tissue of plaice. This agrees with findings in rainbow trout, Atlantic cod and turbot (Frith and Eales, 1996; Cyr et al, 1998; Mol et al, 1998). However, unlike turbot, IRD activity was found at some levels in all the tissues examined; kidney, heart and liver showing considerable activity, with lower levels in muscle, intestine and gill.

rT3ORD had a tissue distribution profile similar to T4ORD. This high deiodination of rT3 by the liver is similar to the turbot (Mol et al, 1998). However, there was little deiodination of rT3 by the plaice kidney or heart that are high in the turbot. The tissue profile for rT3ORD activity in plaice is most similar to that of the catfish, with the exception of catfish brain that lacks activity (Mol et al, 1998). The catfish has considerable liver activity, as well as low muscle activity.

A thiol cofactor (DTT) concentration of 20 mM was chosen for the remainder of experiments, based on the considerable activity measured in all deiodination pathways at this level. This level was generally similar for that used in other teleosts (Cyr et al, 1998; Mol et al, 1998).

Profiles for the effect of pH suggest liver ORD activity to be less variable at high pH than IRD activities. A pH of 7.2 was selected as that which would ensure

considerable activity for all the pathways that will be analyzed further in liver and brain. This selected pH is slightly higher than the optimum pH found for turbot, tilapia, catfish (Mol et al, 1998) and cod (Cyr et al, 1998), yet similar to the pH optimum used for deiodinase enzymes in sea lamprey (Eales et al, 1997) and trout (MacLatchy and Eales, 1992).

A temperature of 12°C was used in most assays in order to reflect the temperature at which plaice were held and to directly compare activity levels in other species such as trout which have been routinely run at 12°C (MacLatchy and Eales, 1992). However, the assays indicate a temperature optimum in plaice of around 25°C for all major deiodinase pathways, which include liver T4ORD, brain T3IRD and brain T4IRD. This temperature optimum for plaice liver ORD is similar to that found in the liver of other species such as trout and turbot (Mol et al, 1998). However, the activity of plaice brain IRD contrasts with the 37°C optimum found in brain of other fish species to date. In turbot, the only other pleuronectid studied, the brain IRD optimum was 30 to 37°C (Mol et al, 1998).

While the full geographical range of American plaice is not known, they occur on both sides of the North Atlantic ocean and extend from Frobisher Bay, Baffin Island to Rhode Island, USA. It is believed to have one of the widest depth ranges (36-713 m) of any Scotian Shelf species studied (Scott and Scott, 1988). Plaice have a preference for temperatures from just below 0°C to 1.5°C. They have been found on the Scotian Shelf to occupy water at temperatures of 0-13°C (Scott and Scott, 1988), though this range may depend on sex (Swain et al, 1998). Plaice of the southern Gulf of St. Lawrence occupy habitats of –0.1 to 0.3°C in September and 5.2 to 5.4°C in January (Swain et al, 1998). The fish in our studies were caught in the St. Lawrence River near Matane, QC, which is

likely warmer than Gulf of St. Lawrence waters. The fish in the present study were housed in natural temperature St. Lawrence seawater ranging from 5 to 9°C during most of the summer.

Habitat selection by poikilotherms such as plaice may be used to help manipulate enzyme activities during certain times of the year. Temperatures influence various aspects of the thyroid system in trout, including T4 production and iodide uptake, the proportion of FT4 and deiodinase systems (Eales et al, 1982; Eales and Shostak, 1986; Johnston and Eales, 1995). However, while temperature was found to influence T4ORD activity in cod, T4ORD activity was not different in cod acclimated at 2-4°C or 6-10°C (Cyr et al, 1998). The role and regulation of deiodinase function and habitat would require further study in plaice.

Potential inhibitors are used to help characterize deiodinase enzymes, as some deiodinases are not affected by certain compounds, while others are. Certain inhibitors were effective on plaice deiodinase activity. ATG inhibited liver T4ORD, but not brain T4ORD, suggesting a different enzyme is responsible for ORD in the two tissues. Brain T4IRD was inhibited by ATG and high concentrations (1000 uM) of IAC. The lack of effect of IAC in liver and on brain T3IRD contrasts with that found in the tissues of other species which experience inhibition by IAC (Cyr et al, 1998; Mol et al, 1998). PTU did not affect deiodinase activity in plaice, however, PTU can be used to induce hypothyroidism in coho salmon (*O. kisutch*) (Ebbesson et al, 1998).

The preference for certain TH analogues as substrates is also used to help characterize deiodinase enzymes. Substrate analogues had different effects depending on the pathway studied. The substrate inhibition profile of liver T4ORD was similar to that

found in catfish and turbot livers with regards to T4, rT3 and T3 (Mol et al, 1998) and cod liver (Cyr et al, 1998). The substrate preferences for liver T3IRD and T4IRD pathways differed, particularly in response to rT3 and T2, which suggest there may be a separate enzyme in American plaice for IRD of T4 and T3. The brain IRD pathways for T4 and T3 showed similar profiles of substrate preference, consistent with a single enzyme for this pathway. The profiles for these pathways were more similar to liver T3IRD than to liver T4IRD, suggesting a deiodinase in brain is similar to a deiodinase in the liver of plaice. These brain IRD and liver T4IRD pathways are generally similar in inhibition profiles to those described in tilapia and turbot brains for the substrates T4, T3 and rT3 (Mol et al, 1998).

The deiodinases studied here had low Km values in the nM range. This suggests a strong affinity by these enzymes for T4 and T3. The only high Km (~1 uM) deiodinases in fish that have been recorded use rT3 as a substrate (Orozco et al, 1997; Sanders et al, 1997; Finnson et al, 1999). A high substrate rT3 pathway (uM range) was not investigated in this study.

This characterization of deiodinase enzymes in American plaice suggests a strong similarity in deiodination pathways to those of other fish. Mainly, liver is responsible for T4ORD, likely to produce the more biologically active T3, while brain has high T3IRD activity, likely responsible for degrading T3 to protect the brain from an excess of TH.

While there is predominantly ORD in the liver of fish, birds and mammals, the ORD in birds and mammals is achieved by a type I deiodinase. The fish liver deiodinase has characteristics more similar to a type II deiodinase, which is what was found in this study for plaice liver deiodinase. It is believed the regulation of TH levels in peripheral

tissues is in large part a coordination of efforts of deiodinases responsible for both ORD and IRD of THs (Eales and Brown, 1993; Darras et al, 1998).

The optima determined in this study for pH, DTT and temperature can be used in establishing a protocol using deiodination as an indicator or thyroidal status for investigating contaminant effects on plaice thyroid systems. Information on the deiodination of TH in American plaice extends understanding of metabolism in fish and vertebrates. American plaice exhibit a high degree of individual variability in enzyme activity, as shown by the high SEM values and the variation in means between assays that represent different pools of plaice microsomes. This individual variability may be an important consideration in further studies with this animal. This may also be related to individual variability shown by this species in other parameters such as reproduction (Nagler et al, 1999).

Chapter 4: Injection of American plaice with PCB 77 or PCB 126.

Introduction

An increasing number of environmental contaminating chemicals or their metabolites have been identified as endocrine disrupters. These chemicals impact on normal hormone physiology and have the potential to interfere with the processes these hormones control, such as growth, reproduction, energy production and osmoregulation. In fish, reproduction and links with endocrine disrupters have come into recent focus (Arcand-Hoy and Benson, 1998).

Assessment of thyroid function in fish can best be achieved using a suite of measures currently available, including histological examination of thyroid tissue, plasma TH levels and deiodinase assays (Eales et al, 1999). The objective in this set of experiments is to determine if intraperitoneal (i.p.) injection of PCB 77 or PCB 126 alters the thyroidal status of American plaice (*Hippoglossoides platessoides*) after one or four weeks, as measured by plasma TH levels and deiodination activities in liver and brain. These parameters provide an indication of the systemic availability of plasma T4 (presumed prohormone) and T3 (active hormone) as well as the activities of the enzymes responsible for the systemic regulation of T3 (liver) and local regulation of T3 (brain).

Materials and Methods.

Fish collection: American plaice were captured by trawl on the St. Lawrence Estuary near Matane, Quebec, Canada, in 1995 and 1997. The fish were maintained for at least two months prior to the experiment in 3.6 X 1.2 X 0.45 –m fiberglass tanks supplied with aerated filtered flowing seawater, under conditions of ambient temperature (5-9°C) and natural photoperiod. Fish were fed chopped capelin twice weekly and every sixth feeding

fed in-house prepared capelin-based food pellets supplemented with vitamins (Nagler and Cyr, 1997). Fish were not fed one week before they were killed and sampled.

Chemicals. PCB 77 (3,3',4,4'-tetrachlorobiphenyl) and PCB 126 (3,3',4,4',5-pentachlorobiphenyl) were obtained from UltraScientific (Kingston, RI). These PCBs are tested and considered >99% pure based on the manufacturer's specifications. The PCBs were dissolved in an acetone-peanut oil (1:99, v/v) carrier. Antibodies for T4 and T3 were from Calchemical Lab Supplies, Ltd., Calgary, or Sigma Chemical (St. Louis, MI). Other chemicals used were as described in Chapter 3.

Experiment 1 (November 1996): Male American plaice (78 g average) were randomly selected from a stock tank of plaice, anaesthetized with MS222 (1.0 g/litre) and given an i.p. injection of PCB 77 in carrier medium containing 0 (carrier only), 5 or 25 ng PCB congener/g bm. Injected plaice were grouped in 3.6 X 1.2 X 0.45 –m fiberglass tanks based on their treatment and then sampled after one or four weeks. Fish were not fed one week before they were killed and sampled.

Experiment 2: In June, 1997, male or immature female plaice (121 g average) were randomly selected from a stock tank of fish, anesthetized, and given an i.p. injection of PCB 77 or 126 at a dose of 0, 5, 50 or 500 ng PCB congener/g bm. Injected plaice were grouped in one of seven 3.6 X 1.2 X 0.45 –m fiberglass tanks according to treatment and sampled after one week. Fish were not fed after injection.

Blood and tissue sampling. Fish were weighed and bled by the caudal blood vessels. Vials containing heparanized blood were placed on ice, and were later centrifuged for five minutes at 15 600 g and the upper plasma layer was transferred to a clean vial and stored on ice and later stored at –80°C. Fish were anesthetized, killed and brains and

livers were collected, frozen in liquid nitrogen and subsequently stored at –80°C. The hepato-somatic index (HSI) was calculated as liver mass X 100/(fish mass – liver mass).

Plasma analyses. Radioimmunoassays (RIAs) for T3 and T4 followed established procedures (Brown and Eales, 1977; Omeljaniuk et al, 1984), with the following modifications. Hormones were extracted three times with methanolic ammonia (99:1 v/v) using a 1:1 (plasma: extractant) ratio. Spiking plasma with a known amount of T4 or *T4 indicated a T4 recovery of T4 of 93.5 $\pm$ 2.7%. Columns used in the assay were 5-ml Quik-Sep columns containing 0.3 g (dry weight) of G-25 fine Sephadex (Pharmacia). Barbital buffer (pH 8.6) was used for the T4 RIA, and phosphate buffer (100 mM Na2HPO4, 3 mM Na2EDTA, pH 7.4) was used instead of barbital buffer for the T3 RIA.

Preparation of microsomal fractions: Brain and liver microsomes were prepared following the methods described in Chapter 3.

Deiodinase assays: The brain and liver deiodinase assays were performed as described in Chapter 3, except that all assays were performed for 90 min at 9°C.

HPLC Analysis: Products were analyzed by HPLC as described in Chapter 3.

Statistics. Differences in HSI, plasma TH levels and deiodinase activity were determined using one-way ANOVA or the non-parametric Kruskal-Wallis test for data that did not conform to normality. The Levene statistic was used for testing equality of variance. All analyses were done using SigmaStat or SPSS 7.5 for Windows. Unless otherwise stated, the significance level was $p \leq 0.05$.

Results:

Experiment 1 (PCB 77 treatment, sampling at 1 and 4 weeks). There were no statistically significant differences in HSI or body mass between PCB-treated plaice and controls at

either one or four weeks (Table 4-1). Relative to controls, there were no significant differences in plasma T4 levels due to PCB treatment at either one or four weeks. However, after one and four weeks, fish injected with 5 ng PCB 77/g bm had significantly lower T4levels than fish injected with 25 ng PCB 77/g bm (Fig. 4-1). After one week, plasma T3 levels were significantly lower than control levels for both the 5 and 25 ng/g bm dosages. There were no differences in plasma levels of T3 for any of the groups at four weeks (Fig. 4-1).

Hepatic T4ORD activity was increased for both the low-dose and high-dose groups at one week. This was only significant for the high dose yet there was a trend for a dose-dependent increase in T4ORD activity (Fig. 4-2). At four weeks, fish injected with the low dose of PCB had lower liver T4ORD activities than those injected with the high dose, though neither group was significantly different from their respective controls (Fig. 4-2). In plaice treated with PCB 77 there were no changes in liver T4IRD activity at either one or four weeks (Fig. 4-2). There were no significant ($p \leq 0.05$) changes in brain T4ORD or IRD activities (Fig. 4-3). However, after four weeks T4ORD activity was lower ($p < 0.10$) in PCB 77-treated plaice.

Experiment 2 (PCB 77 or 126 treatment, sampling at one week). There were no differences in the HSI or body mass between control and either PCB 77- or PCB 126-treated plaice (Table 4-1). There were more females in the low dose PCB 77-treated group than in the other PCB 77-treated groups or in the controls.

There were no significant differences in plasma T4 due to PCB 126-treatment (Fig. 4-4). Plasma T4 of PCB 77-treated plaice did not differ significantly from that of controls, but plasma T4 levels at 500 ng PCB 77/g bm were significantly greater than

Table 4-1: The number of fish, mean mass, hepatosomatic indices (HSI) and sex distribution of plaice used in experiments 1 and 2.

Treatment	N	Mean fish mass (range), g	HSI (SEM)	Males	Females
Experiment 1					
1-week controls	8	74.6 (41-116)	1.10 (0.163)	8	0
1-week 5 ng/g	12	75.5 (42-122)	1.12 (0.142)	12	0
1-week 25 ng/g	14	97.1 (46-134)	1.49 (0.183)	14	0
4-week controls	10	74.1 (49-125)	1.58 (0.240)	10	0
4-week 5 ng/g	10	68.1 (42-103)	1.42 (0.228)	10	0
4-week 25 ng/g	10	77.9 (43-138)	1.67 (0.205)	10	0
Experiment 2					
Controls	13	135 (69-244)	1.05 (0.082)	5	8
5 ng/g PCB 126	11	131 (95-222)	0.99 (0.073)	6	5
50 ng/g PCB 126	9	111 (58-237)	0.91 (0.090)	5	4
500 ng/g PCB 126	12	133 (56-282)	0.88 (0.086)	7	5
5 ng/g PCB 77	13	127 (52-249)	1.01 (0.079)	3	10
50 ng/g PCB 77	13	99 (36-153)	1.02 (0.088)	10	3
500 ng/g PCB 77	12	113 (47-232)	0.96 (0.078)	7	5

Figure 4-1: Plasma levels of T4 (top) and T3 (bottom) (ng T4 or T3/ml plasma) in plaice injected with 0 (n=8 for T4, n=7 for T3), 5 (n=12 for T4, n=9 for T3) or 25 (n=12) ng PCB 77/g bm and sampled at one week and 0 (n=10), 5 (n=9) or 25 (n=10) ng PCB 77/g bm after four weeks. Differences among means at any one sampling time are indicated by lowercase superscripts ($p<0.05$) or uppercase superscripts ($p<0.10$).

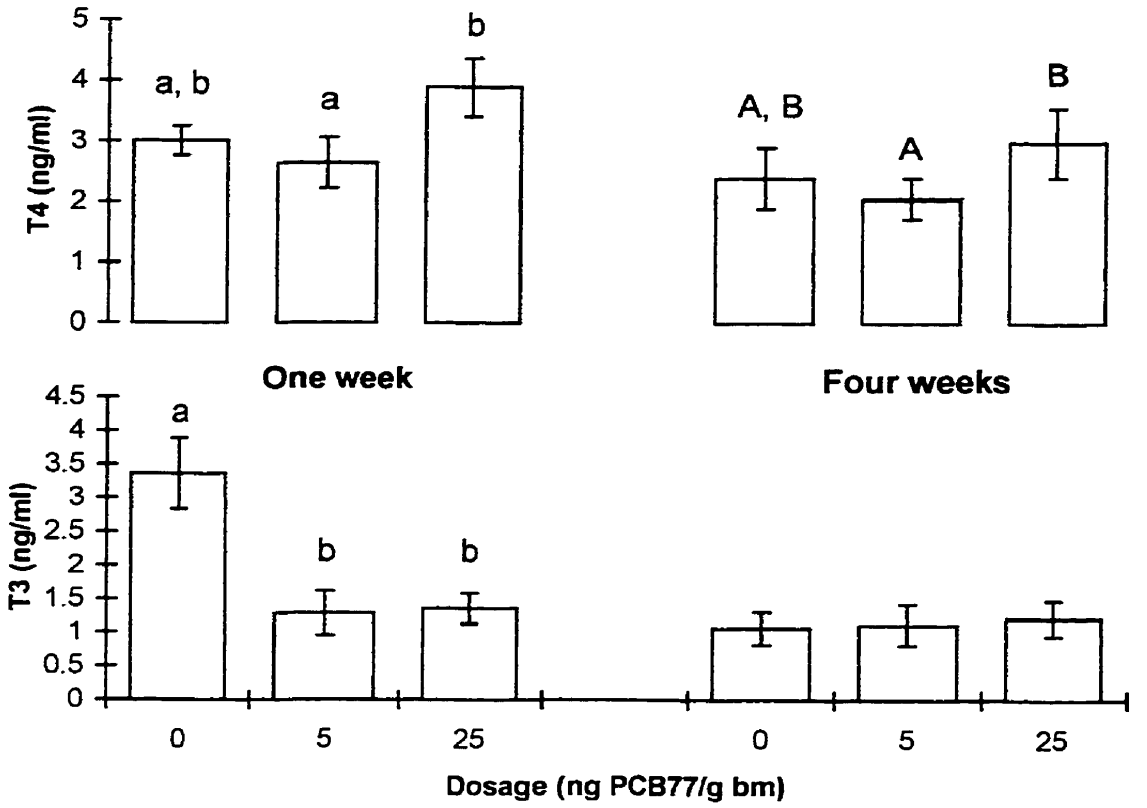

T4 (ng/ml)
5
4
3
2
1
0
a, b
a
b
A, B
A
B
One week
Four weeks
T3 (ng/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
b
0
5
25
0
5
25
Dosage (ng PCB77/g bm)

Figure 4-2: Liver T4ORD (top) and T4IRD (bottom) activities in plaice injected with 0 (n=8), 5 (n=12 for T4ORD , n=11 for T4IRD) or 25 (n=12) ng PCB 77/g bm and sampled at one week, and 0 (n=10), 5 (n=10) or 25 (n=10) ng PCB 77/g bm after four weeks. Differences among means at any one sampling time are indicated by lowercase superscripts ($p<0.05$).

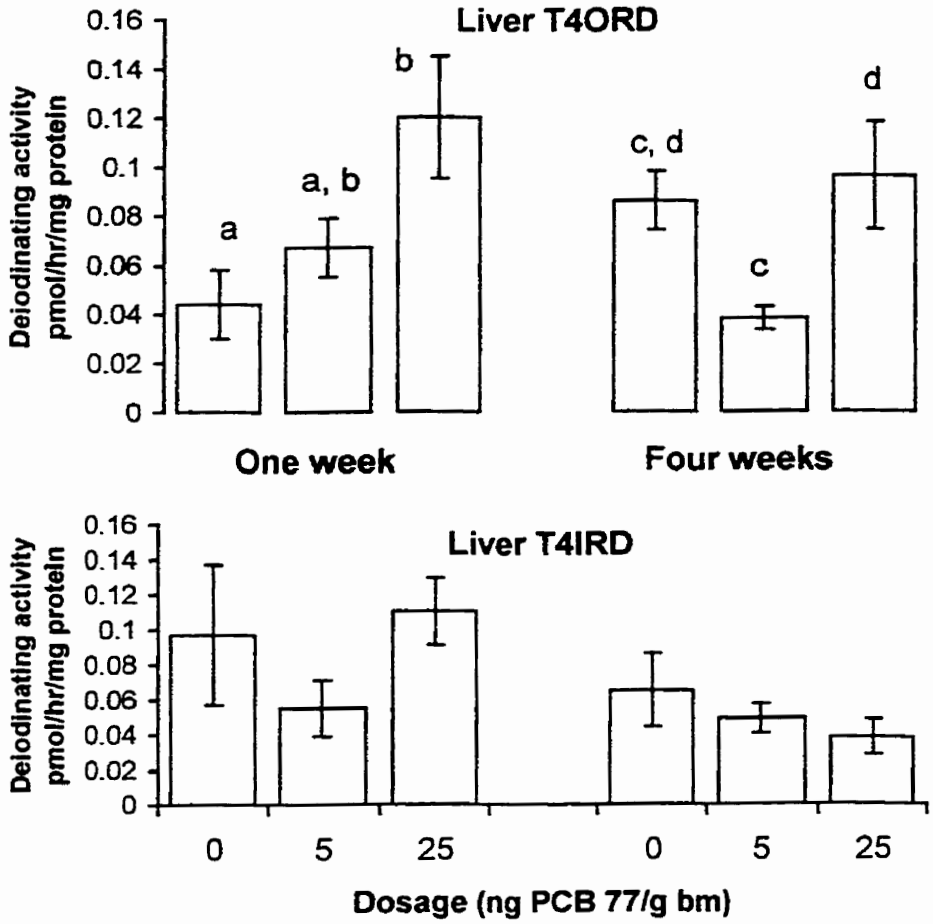
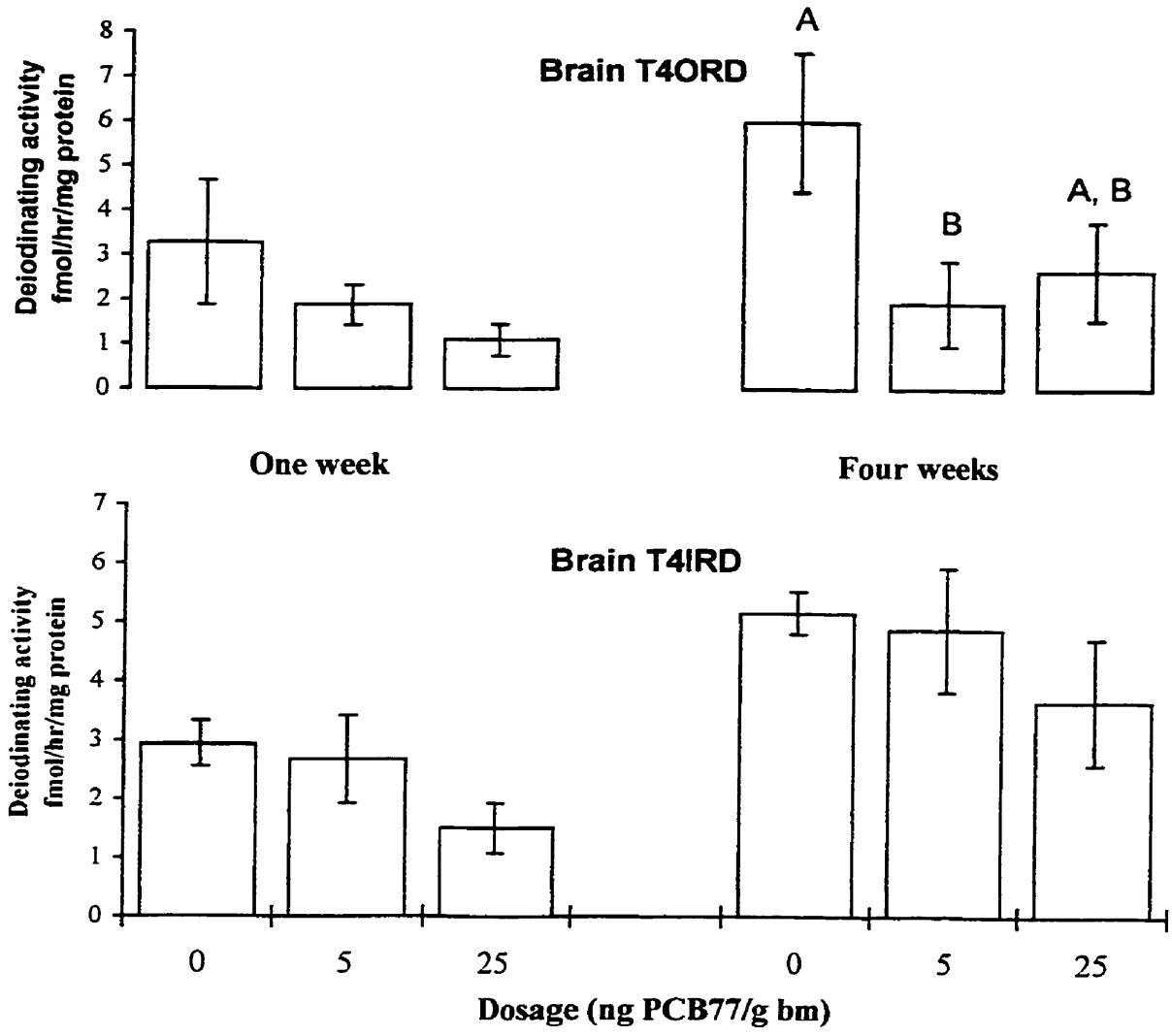

Liver T4ORD
Deiodinating activity
pmol/hr/mg protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a, b
b
c, d
c
d
One week
Four weeks
Liver T4IRD
Deiodinating activity
pmol/hr/mg protein
0
5
25
0
5
25
Dosage (ng PCB 77/g bm)

Figure 4-3: Brain T4ORD (top) and T4IRD (bottom) activities in plaice injected with 0 (n=7), 5 (n=8 for T4ORD, n=9 for T4IRD) or 25 (n=11) ng PCB 77/g bm and sampled at one week, and 0 (n=8), 5 (n=9) or 25 (n=9) ng PCB 77/g bm after four weeks. Differences ($p \leq 0.10$) among groups are indicated by different superscripts.


Brain T4ORD
Deiodinating activity
fmol/hr/mg protein
8
7
6
5
4
3
2
1
0
A
B
A, B
One week
Four weeks
Brain T4IRD
Deiodinating activity
fmol/hr/mg protein
7
6
5
4
3
2
1
0
0
5
25
0
5
25
Dosage (ng PCB77/g bm)

those at 5 ng PCB 77/g bm (Fig. 4-4). No differences were found in plasma T3 for either PCB treatment at any dosage (Fig. 4-4).

Liver T4ORD activity increased with increasing PCB 77 concentration, although only the 500 ng PCB 77/g bm dosage was significantly increased (Fig. 4-5). High doses of PCB 126 tended to decrease liver T4ORD activity, though not significantly. Hepatic T4IRD activity of plaice treated with PCB 126 did not differ significantly from that of controls. However, some PCB 126-treated fish had very high T4IRD amd T3IRD activity and regression analysis revealed a significant dose-dependent increase in T4IRD activity. There were no statistically significant changes in brain deiodinase activity for T4ORD or T4IRD (Fig. 4-6). While not affected by PCB 77 ($p \leq 0.05$), the 50 ng PCB 126/g bm treatment caused a decrease in brain T3IRD activity ($p \leq 0.10$). Also, there was a tendency (not statistically significant; $p \leq 0.10$) for brain T4ORD and T4IRD activity to be lower than control values in PCB 126-injected plaice.

Discussion:

The objective in this set of experiments was to determine if i.p. injection of PCB 77 or PCB 126 alters the thyroidal status of American plaice. This was assessed by measuring plasma TH levels and deiodination activities in liver and brain. These are parameters which indicate the systemic availability of plasma T4 (presumed prohormone) and T3 (active hormone) as well as the activities of the enzymes responsible for the systemic regulation of T3 (liver) and local regulation of T3 (brain).

The fish used in the two experiments differed in a number of ways. Experiment 1 used only males, while, due to fish availability, Experiment 2 used both males and immature females. Also, the average mass of the fish in Experiment 1 was smaller than

Figure 4-4: Plasma levels of T4 (top) and T3 (bottom) (ng T4 or T3/ml plasma) in plaice injected with 0 (n=12), 5 (n=10), 50 (n=7) or 500 (n=11) ng of PCB 126 or 5 (n=10), 50 (n=12) or 500 (n=11) ng of PCB 77 per g bm and sampled after one week. Differences ($p \leq 0.05$) among means are indicated with different lowercase superscripts.

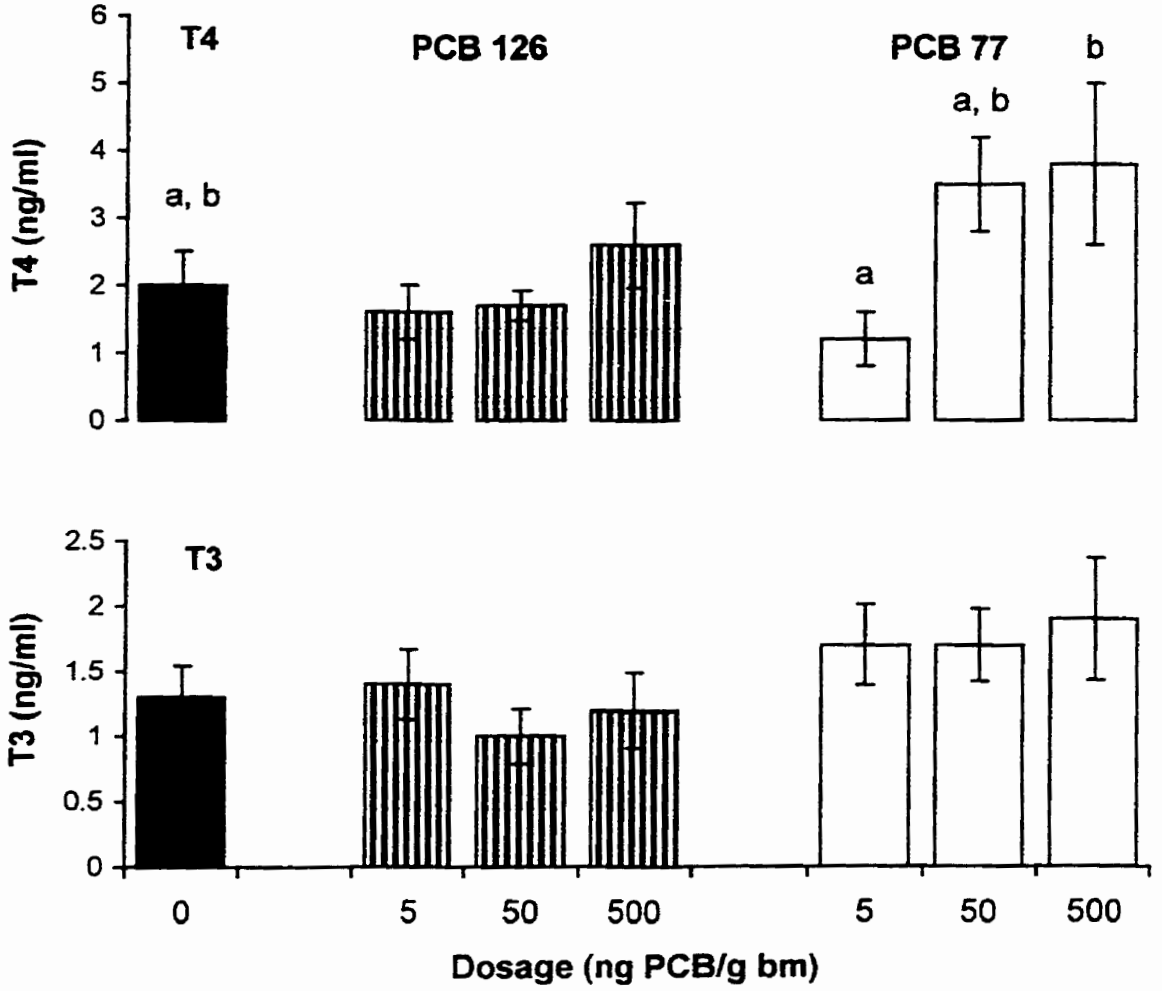

T4
PCB 126
PCB 77
T4 (ng/ml)
a, b
a
a, b
b
T3
T3 (ng/ml)
0
5
50
500
5
50
500
Dosage (ng PCB/g bm)

Figure 4-5: Liver T4ORD, T4IRD or T3IRD activities in plaice injected with 0 (n=11), 5 (n=10), 50 (n=7) or 500 (n=11) ng of PCB 126 or 5 (n=11), 50 (n=10) or 500 (n=8) ng of PCB 77 per g b.w. and sampled after one week. Differences ($p \leq 0.10$) among means for each PCB are indicated with different uppercase superscripts.

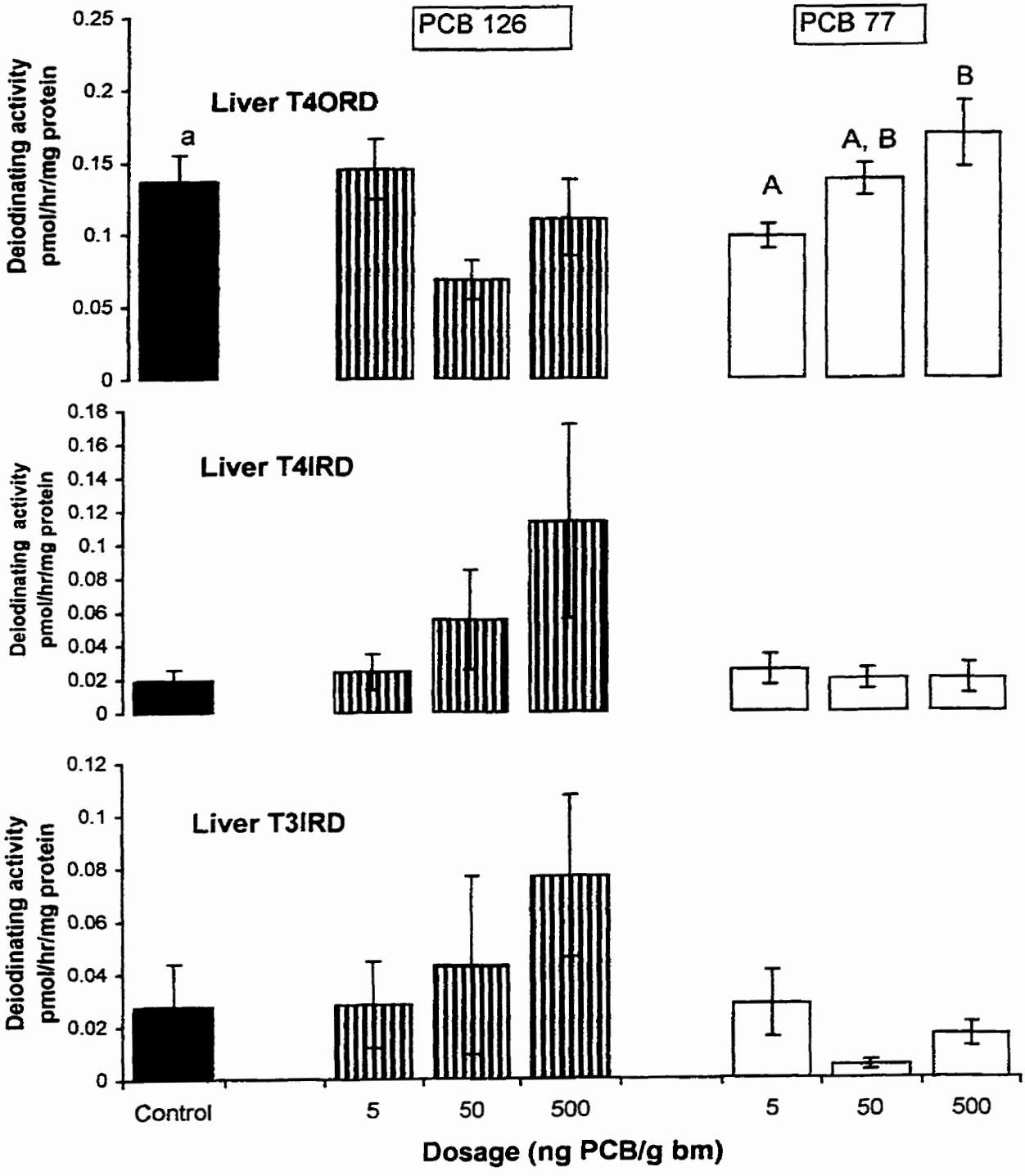
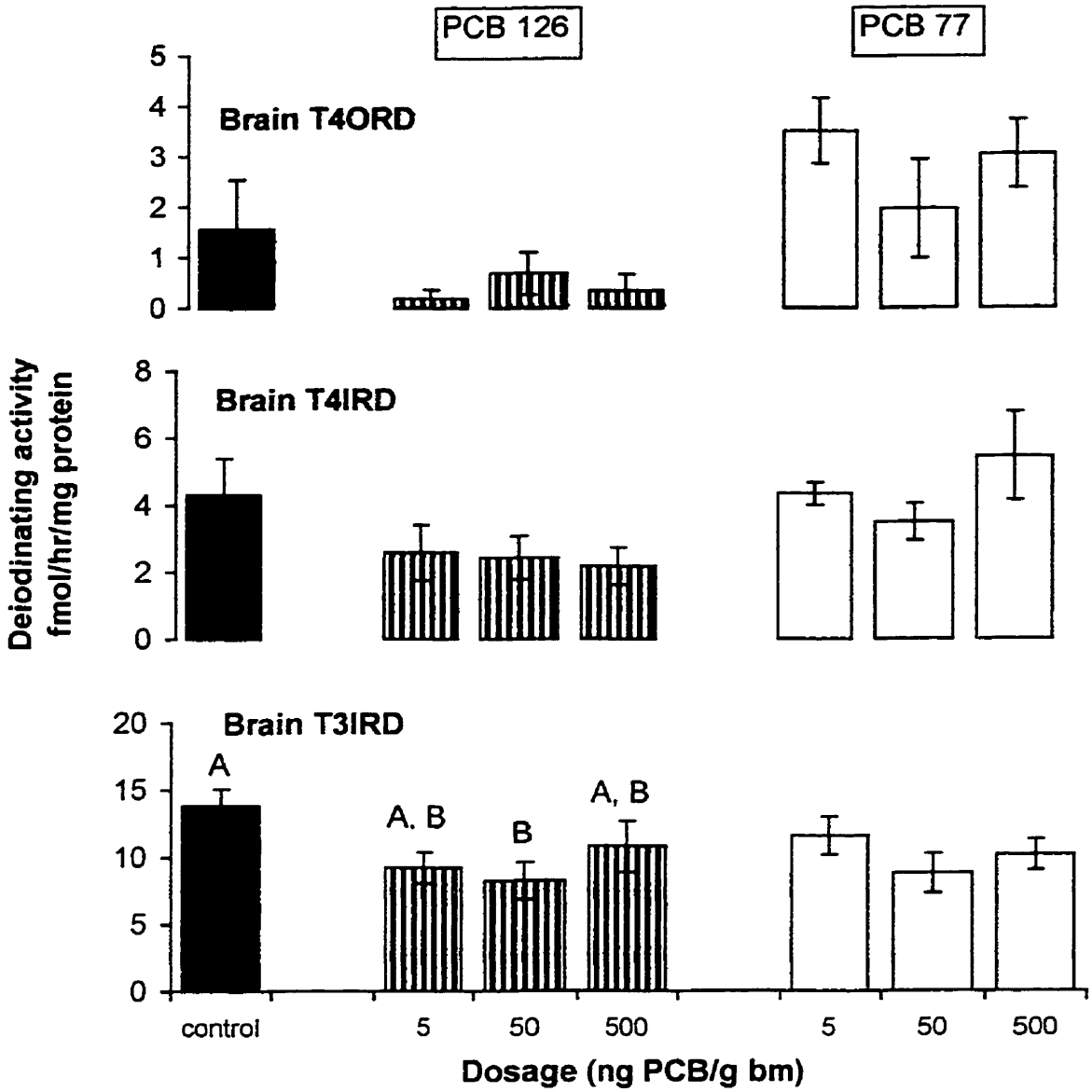

PCB 126
PCB 77
Liver T4ORD
Liver T4IRD
Liver T3IRD
Deiodinating activity pmol/hr/mg protein
a
A
A, B
B
Control
5
50
500
5
50
500
Dosage (ng PCB/g bm)

Figure 4-6: Brain T4ORD, T4IRD or T3IRD activities in plaice injected with 0 (n=6), 5 (n=5), 50 (n=5) or 500 (n=6) ng of PCB 126 or 5 (n=7), 50 (n=7) or 500 (n=7) ng of PCB 77 per g bm and sampled after one week. Differences ($p \leq 0.10$) among means are indicated with different uppercase superscripts.


PCB 126
PCB 77
Brain T4ORD
Brain T4IRD
Brain T3IRD
Deiodinating activity
fmol/hr/mg protein
A
A. B
B
A, B
control
5
50
500
5
50
500
Dosage (ng PCB/g bm)

that in Experiment 2, suggesting the fish may be of a different year class. The HSI of fish in Experiment 1 was larger than that of fish in Experiment 2 indicating a different physiological condition. These differences in physiological condition could lead to differences in thyroidal status in the fish used in the two experiments and hence in differences in response to PCB treatments. Finally, while both groups of fish were from the St. Lawrence Estuary, the fish from Experiment 1 were captured in the Fall, while those of Experiment 2 were captured in the Spring.

PCB residues exist in biota all over the world, including the St. Lawrence Estuary (Gagnon et al, 1990; Martineau et al, 1987) and the fish used in these experiments may have had previous exposure to PCBs. However, fish from the St. Lawrence Estuary have low or zero levels of PCBs 77 or 126 (Gagnon et al, 1990). Furthermore, the American plaice used in these experiments came from areas known to be "clean", that is, that are not heavily contaminated by PCBs or other contaminants. Also, the fish were young and therefore had less time to accumulate tissue burdens of contaminants.

The average level of plasma T4 in control plaice used in Experiment 1, 3 ng/ml, is about one third of that reported in winter flounder (Eales and Fletcher, 1982) and about half that of European plaice (Osborn and Simpson, 1978) sampled in the wild at a similar time of year. American plaice plasma T3 was about one fifth of that found in the above species. Lower levels of THs in American plaice may be explained by a species difference or perhaps by the transition to the laboratory. However, winter flounder maintained in the laboratory were found to have higher T4 and lower T3 values than flounder in the field (Eales and Fletcher, 1982).

Effects of PCBs on plasma TH levels.

Plasma T4 represents the systemic availability of prohormone and has feedback effects on the brain and pituitary. Plasma T3 represents the systemic availability of active hormone, the level of which is regulated systemically by liver TH enzymes and locally by peripheral tissues such as the brain.

The effects of PCBs on plasma TH levels of American plaice were evident only for PCB 77 and not PCB 126 at one and four weeks after injection. In both experiments treatment with PCB 77 increased plasma T4 levels. Plasma T3 levels were reduced at one week in the first experiment. However, this was not the case at four weeks or in Experiment 2 after only one week. In Experiment 2, plasma T3 levels for controls were already so low that there may be little scope for further reduction.

Plasma T4 levels have been shown to increase in most other PCB-exposed fish species. Flounder exposed to a PCB mixture (Besselink et al, 1996), coho salmon exposed to PCBs and fuel oil (Folmar, 1982), and white suckers exposed to mill effluent (Munkittrick et al, 1991) all showed increases in plasma T4. In contrast, trout species exposed to PCBs showed decreases in plasma T4 (Leatherland and Sonstegard, 1980b) or no change in plasma T4 (Leatherland and Sonstegard, 1979; Brown et al, 1997).

Mammals and birds often show a reduction in levels of plasma T4 and T3 when exposed to 2, 3, 7, 8- tetrachlorodibenzo-p-dioxin (TCDD) or a TCDD-like compound such as PCB 77. Mice exposed to PCB 77 experience decreases in total plasma T4 (Darnerud et al, 1996), while ring doves experience reduction in both total plasma T4 and T3 levels (Spear and Moon, 1985).

Besides deiodination, changes in circulating levels of THs may be related to changes in the storage or release of TH including production and release of T4 from thyroid follicles or TH release from muscle stores (Eales, 1997). Whole body levels of T4 have been shown to increase in turbot exposed to oil fractions (Stephens et al, 1997a, b), while tissue T3 levels have been reduced in rainbow trout stressed by low pH and aluminum exposure (Fok et al, 1990). Changes in plasma TH levels may also be related to increased or decreased excretion of THs, or an increased uptake of T3 for hormone action.

Effects of PCBs on Deiodination

Liver and brain tissues were selected for deiodination analysis for a number of reasons. Liver deiodinase activity is responsible for the systemic regulation of T3, while brain deiodinase activity represents local regulation of T3. Also, plaice liver had the highest ORD activity while brain had the highest IRD activity (Chapter 3). Furthermore, contaminants tend to accumulate in the liver of many vertebrates including flatfish species (Goksoyr et al, 1991), likely due to the fact the blood is routed to the liver from the gut and liver has a high lipid content and can accumulate PCBs. Finally, the brain is key to the brain-pituitary-thyroid axis for coordinating both feedback and new information as it relates to the thyroid system, and is critical for normal central nervous system development and function.

In Experiment 1, PCB 77 caused an increase in liver ORD activity at one week and a possible increase from the low to high dose treatments at four weeks. This may relate to a change in local TH status in the liver or a systemic change in TH levels. Liver T4ORD increased with increasing concentration of contaminant in fish injected with PCB

77 in Experiment 2. PCB 126 induced exceptional hepatic T4IRD and T3IRD activity in a few plaice. This was a dose dependent increase in IRD pathways.

Brain T4ORD activity was decreased by four weeks in Experiment 1. This may be an adjustment by the brain to accommodate for changes in the changing circulating levels of THs (Morin et al, 1995; Fines et al, 1999). ORD activity is responsible for the conversion of T4 to T3, while IRD converts T4 to rT3 or T3 to T2. This would suggest there is less emphasis on production of T3. Since there is more T4 in the plasma, the brain ORD activity may be reduced to maintain a standard supply to the local brain tissue. In Experiment 2, a 50 ng PCB 126/g bm injection caused a reduction in brain T3IRD activity.

There are four reports of ORD measurements in fish exposed to contaminants. Catfish exposed to lead showed a decrease in ORD (Chaurasia et al, 1996), rainbow trout exposed to PCB 126 showed no change in ORD (Brown et al, 1997), while aluminum exposure decreased ORD in rainbow trout (Brown et al, 1990) and increased ORD in brown trout (Waring and Brown, 1997).

Rats exposed to metals have experienced decreases in deiodinating activities (Paier et al, 1993; Gonzalez-Pondal et al, 1995), while TCDD and PCBs have caused an increase in type II deiodinase in rats (Morse et al, 1996; Raasmaja et al, 1996). While few studies have considered the impact of xenobiotics on brain IRD activity, one report found IRD activity altered by methylmercury in fetal mice (Watanabe et al, 1999).

Direct and indirect effects of PCB effects on the thyroid cascade

PCBs have the potential to influence the plaice thyroid system through a variety of mechanisms. There are direct mechanisms that depend on the similarity in structure of

THs to PCBs, which could theoretically affect the binding of THs to their normal protein binding sites in the thyroid cascade. There are also several indirect mechanisms by which PCBs might influence the thyroid system. These include a mechanism that depends on the stimulation or inhibition by PCBs of other body processes that secondarily influence the thyroid cascade

Due to the similarity in structure of halogenated biphenyl compounds to THs, there is the possibility these compounds can compete directly for protein binding sites for THs. PCBs theoretically can mimic THs at any point in the thyroid cascade. There may be more free hormone in the plasma if PCBs compete for plasma binding protein sites. This may result in a shift in the normal free hormone homeostasis, making more TH available for uptake into peripheral tissues or altering feedback processes. PCBs could interact directly with the deiodinase enzyme(s), resulting in inhibition of normal TH homeostasis or increasing the amount of enzyme metabolizing iodothyronine and iodothyronine-like compounds. Also, PCBs could exert both nuclear or genomic and non-genomic effects by binding to receptors that induce specific nuclear or organelle events.

Indirect influences by PCBs include the activation of CYP450 (Phase I) and Phase II biotransformation systems. CYP450 results in the hydroxylation of compounds, to make them more soluble and available for Phase II biotransformation processes. Phase II actions include conjugation events and amidation.

One explanation for a reduction in plasma levels of T4 in mammals is that TCDD and TCDD-like compounds bind to the Ah receptor, a component of the CYP450 system, which induces hepatic UDP-glucuronosyltransferase (UGT), a Phase II pathway. This increases the glucuronidation and subsequent excretion of T4 (Curran and DeGroot,

1991; Kohn et al, 1996; Hotz et al, 1997). A similar mechanism may occur in other species (Brouwer, 1991), and has been shown to occur in lake trout exposed to PCB 126 (Brown et al, 1997). However, the activity of UGT and related enzymes varies considerably in fish species (Pathiratne and George, 1996). If UGT is considered the primary reason for altered TH levels in organisms, then variability in UGT enzyme induction and activity could result in different TH profiles for different fish species exposed to a given xenobiotic.

Compounds such as PCB 77 that bind to the Ah receptor often induce ethoxyresorufin-*O*-deethylase (EROD). In the plaice used in this study, there was no induction of EROD activity at either one or four weeks due to the injection of PCB 77 (P. Raymond, unpublished data). Since no induction of EROD occurred, there was perhaps no induction of UGT. Without an induction in UGT, it is less likely plasma T4 or T3 levels would decrease, and indeed plasma T4 levels tended to increase in PCB 77 treated plaice.

Finally, PCBs may act indirectly on the thyroid cascade by having a general debilitating effect on fish. The associated stress can result in a change in health and susceptibility to parasites. Rice and Schlenk (1995) found PCB 126 to decrease the immune function of channel catfish, and American plaice exposed to contaminated sediments have an altered parasite profile (Marcogliese et al, 1998). Stressful changes as these can cause decreased state of health and an altered physiology, including changes in thyroid function.

In this regard the trend for PCB 126 to increase the hepatic IRD activity may be relevant. Hepatic IRD pathways are increased in rainbow trout moved from a hatchery to

the laboratory setting, perhaps as part of a stress response of the fish (Johnston et al, 1996). It is unlikely the increase seen here in plaice liver IRD was due to stress, as the stress response in trout occurred sooner than a week. There are few other reports of IRD pathway disruption by contaminants. IRD was decreased in the brain of fetal mice exposed to methylmercury, while ORD increased (Watanabe et al, 1999). These results warrant further study of IRD pathways in organisms, including fish, exposed to contaminants.

Conclusions

It may not be possible to directly compare the two experiments, even for results of PCB 77 treatment only, as the experiments were done at different times of the year on plaice of different ages, sizes and physiological state. Furthermore, TH levels and deiodinase activities of North Atlantic marine teleosts cycle during the year (Osborn and Simpson, 1978, Eales and Fletcher, 1982; Cyr et al, 1998).

The effects of PCBs on plaice thyroid function were found in all parameters measured. After one week there were significant effects of PCB 77 to increase the plasma T4 level in both experiments, decrease plasma T3 and increase hepatic while decreasing brain T4ORD activity. There is an up-regulation or increase in production and release of T4 by the thyroid system in response to PCB 77 at one week. This is indicated by effects of PCB 77 at both central (plasma T4 and T3) and peripheral (liver and brain deiodination) levels of the thyroid cascade and points to the liver as a key peripheral site. The brain responds by altering activity likely to maintain tight local regulation over T3. It remains to be demonstrated whether these effects occur by direct or indirect actions on the thyroid system.

Chapter 5: *In vitro* experiments.

Introduction

The *in vivo* experiments showed that PCB 77 at one week after injection could depress plasma T3 and augment hepatic T4ORD activity (Chapter 4). These effects may be the result of direct interaction of the PCB compound with the TH-binding plasma protein or with the deiodinase enzyme. However, the PCBs could also act through indirect routes. *In vitro* assays in which plasma or tissue microsomes are incubated with PCB compounds can be used to determine whether direct action is likely.

Metabolites of PCBs may also interfere with hormone systems. Methylsulfonyl PCBs interact with the glucocorticoid receptor (Johansson et al, 1998) and hydroxylated PCBs (OH-PCBs) have both estrogenic and antiestrogenic activity (Connor et al, 1997; Kramer et al, 1997) and also affect the binding of THs to transport proteins (Brouwer et al, 1990; Klasson-Wehler et al, 1998).

OH-PCBs found in blood have two common structural elements; either a 4-hydroxy-3,5-dichlorophenyl ring (or more chlorine atoms in the ring) or a 3-hydroxy-2,4-dichlorophenyl ring (or more chlorine atoms in the ring) and chlorine atoms in at least 3- and 4-positions in the other phenyl ring (Klasson-Wehler et al, 1998). OH-PCBs are structurally similar to T4, and more similar to T4 than PCBs that are not hydroxylated. Several OH-PCBs can compete with T4 for a binding site on the mammalian transport protein, transthyretin (TTR) (Klasson-Wehler et al, 1998). Competition by chlorinated compounds with T4 for TTR binding is based on the presence of a hydroxy-group, preferably in a meta or para position, as well as the substitution of chlorine ortho to this hydroxy-group (Brouwer, 1991) (Fig. 2-5).

The present goal was to determine using *in vitro* exposure whether PCBs 77 and 126 or three OH-PCBs have direct effects on liver deiodination and on plasma TH-binding proteins in plaice or rainbow trout.

Materials and Methods.

Chemicals. The non-ortho substituted tetrachlorobiphenyl PCB 77 and pentachlorobiphenyl PCB 126 were used in this set of assays (Fig. 2-5). The OH-PCBs utilized in this study contained a single hydroxy and variable chlorine substitution (Fig. 2-6). OH-PCBs were selected based on the position of the hydroxy group and chlorine group substitutions (Klasson-Wehler et al, 1998) and what was available from manufacturers.

PCB 77 (3,3',4,4'-tetrachlorobiphenyl), PCB 126 (3,3',4,4',5-pentachlorobiphenyl) (Fig. 2-5) and OH-PCBs (3,3',5,5'-tetrachloro-4,4'-biphenylol (OH-A); 2',3',4',5'-tetrachloro-4-biphenylol (OH-B); and 3,4',5-trichloro-4-biphenylol (OH-C)) (Fig. 2-6) were obtained from UltraScientific (Kingston, RI). These PCBs and OH-PCBs were tested by the manufacturers and stated as >99% pure. The PCBs and OH-PCBs were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical, St. Louis, MO). Supplies of *T4 (specific activity (SA) = 1250 uCi/ug) was obtained from New England Nuclear (NEN) Life Science Products. Unlabelled T4 and were obtained from Sigma (St. Louis, MO, USA).

Preparation of microsomes. Microsomes were prepared as describe previously (Chapter 3) using non-experimental plaice that had been caught and kept under similar conditions. In order to more effectively determine the PCB effects on IRD activities, trout liver microsomes were prepared from trout fed 12 ppm T3 for two weeks. This treatment

induces IRD pathways responsible for degrading T4 to rT3 and T3 to T2 and reduces T4ORD (Fines et al, 1999). Microsomes from at least three different fish were pooled for any assay, although not every assay used microsomes from the same three fish.

Deiodinase assays. The deiodinase assays were performed as in Chapter 3, with some modifications. The effect of each of five compounds on liver deiodinase function was determined by individually incubating the PCB or OH-PCB with T4 substrate and calculating the deiodination activity based on the amount of I-125-derived compounds and comparing with control activities. A 0.5-ml liver microsomal preparation was used. After a 30-minute pre-incubation period, PCBs or OH-PCBs suspended in DMSO (final volume 10 ul) were added for a desired final concentration (0.1 to 10 000 nM) of PCB or OH-PCB, followed immediately by the 10-ul addition of the T4 substrate (0.3 nM). The incubation temperature was 12°C. Three different controls were used: a 10-ul addition of buffer with no PCBs or OH-PCBs (control 1); a 10-ul addition of DMSO with no PCBs or OH-PCBs (control 2); and ATG (1000 nM) which served as a negative control. All incubations were prepared in triplicate from the pool of microsomes prepared for the assay.

T4 binding to plasma proteins: Dialysis was performed according to Eales and Shostak (1985, 1986) with some modifications. Plaice or trout plasma was diluted 1:9 in buffer (100 mM HEPES). PCB 77 or an OH-PCB dissolved in DMSO was added in a 10-ul volume to a desired final concentration to 1 ml diluted plasma and gently mixed. *T4 (~100 000 cpm) in a 10-ul volume were then added to the diluted plasma-PCB (or OH-PCB) mixture and gently mixed. These mixtures were immediately added to Spectraphor 2 membrane (MW exclusion = 14 000) dialysis sacs for dialysis against 20 ml of buffer.

Dialysis was performed over night (at least 16 hours) in a 12°C water bath. Following incubation, two samples were drawn from both inside the dialysis bag and outside the dialysis bag and each sample collected was loaded onto separate LH-20 columns. Free radio-iodide was removed with a 3-ml elution of ddH_2O and collected. Columns were transferred to new tubes and the *T4 component was eluted from the column with ethanolic-ammonia. The ratio of free *T4 (outside the dialysis bag) to total *T4 (free and bound inside the dialysis bag) was calculated. This dialysis ratio represents the proportion of *T4 in the system that is free. Any increase in this ratio would indicate displacement of TH by added PCB or OH-PCBs. Means of each treatment were compared with the control.

Statistics: Differences among groups were determined using one-way ANOVA. A significance level of $p = 0.05$ was used. All analyses were done using SigmaStat or SPSS 7.5 for Windows.

Results:

Deiodinase activity: Incubation with PCB 77 or 126 had no significant effect on plaice liver T4ORD or T4IRD activities (Fig. 5-1), or on trout liver T4ORD or T4IRD activities (Fig. 5-2). Furthermore, there was no significant effect of two of the OH-PCBs on plaice liver T4ORD activity (Fig. 5-3) or the three OH-PCBs on trout liver T4IRD activity (Fig. 5-4). ATG (1000 nM) served as an effective negative control, reducing deiodinase activity to near zero.

T4 binding to plasma proteins: Neither PCB 77 nor OH-PCB-A competed for T4 binding sites on plaice plasma proteins based on the dialysis method (Fig. 5-5). PCB 77, OH-A,

Figure 5-1: Deiodinating activity (T4ORD and T4IRD) in plaice liver microsomes incubated with 100, 1000 or 10000 nM PCB 126 or PCB 77.

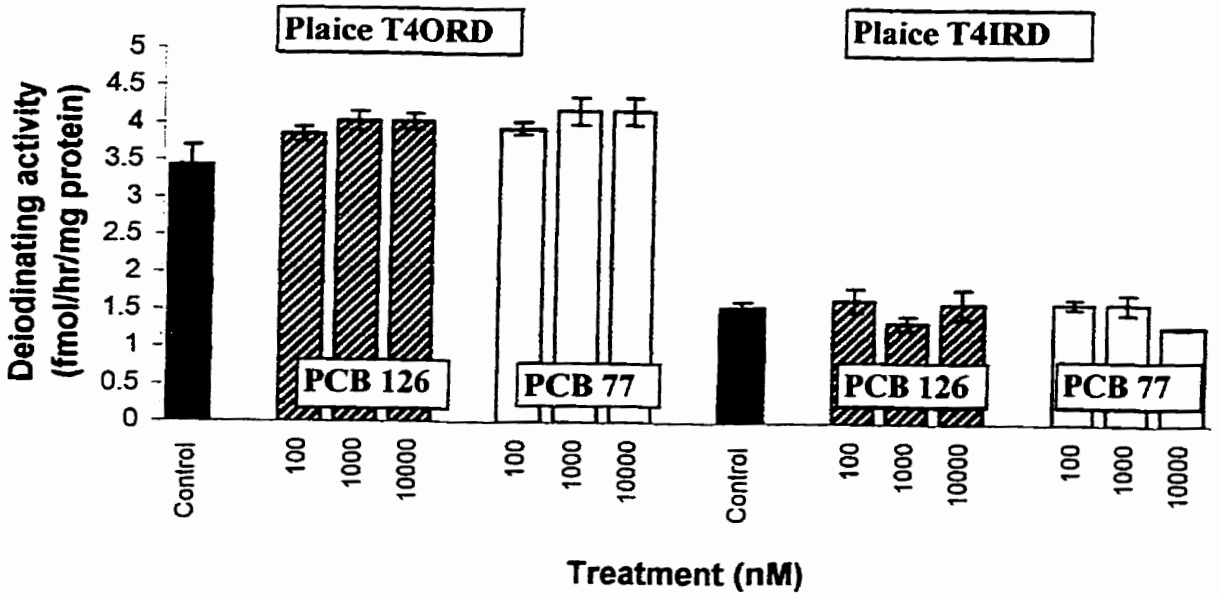

Plaice T4ORD
Plaice T4IRD
Deiodinating activity (fmol/hr/mg protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
PCB 126
PCB 77
PCB 126
PCB 77
Control
100
1000
10000
100
1000
10000
Control
100
1000
10000
100
1000
10000
Treatment (nM)

Figure 5-2: Deiodinating activity (T4ORD and T4IRD) in rainbow trout liver microsomes incubated with 100, 1000 or 10000 nM PCB 126 or PCB 77.

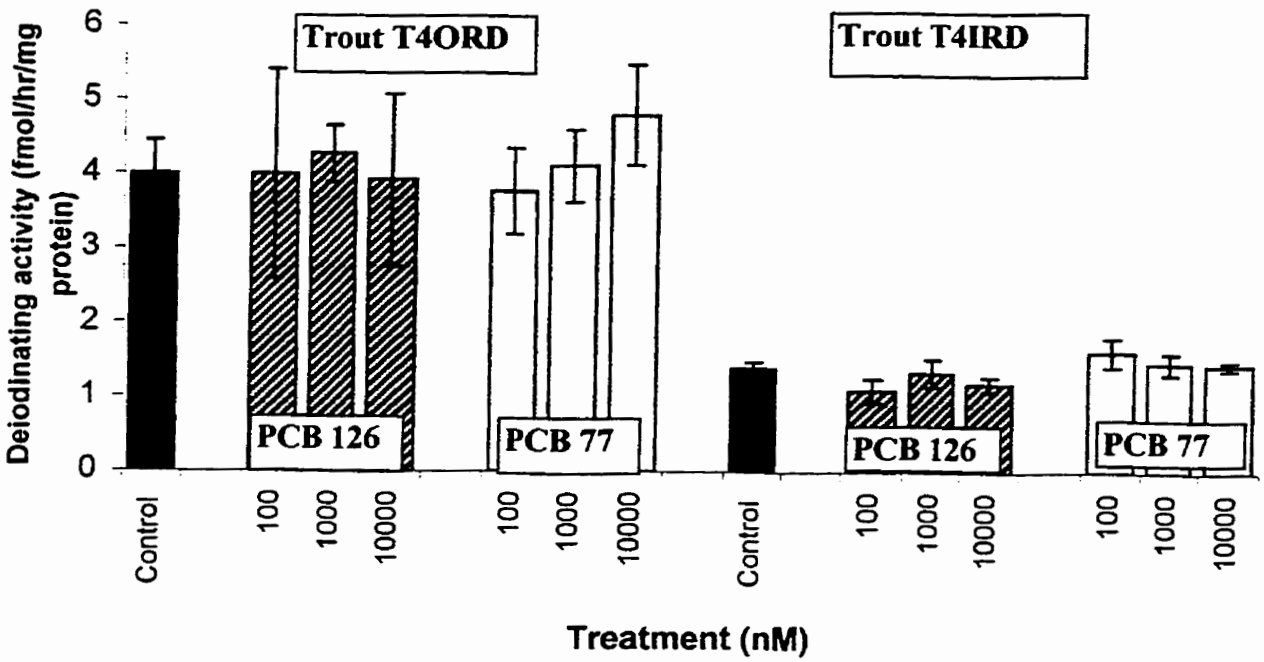

Trout T4ORD
Trout T4IRD
Deiodinating activity (fmol/hr/mg protein)
0
1
2
3
4
5
6
PCB 126
PCB 77
PCB 126
PCB 77
Control
100
1000
10000
100
1000
10000
Control
100
1000
10000
100
1000
10000
Treatment (nM)

Figure 5-3: Deiodinating activity (T4ORD) in plaice liver microsomes incubated with OH-A, OH-B or OH-C (0.1 – 10000 nM).

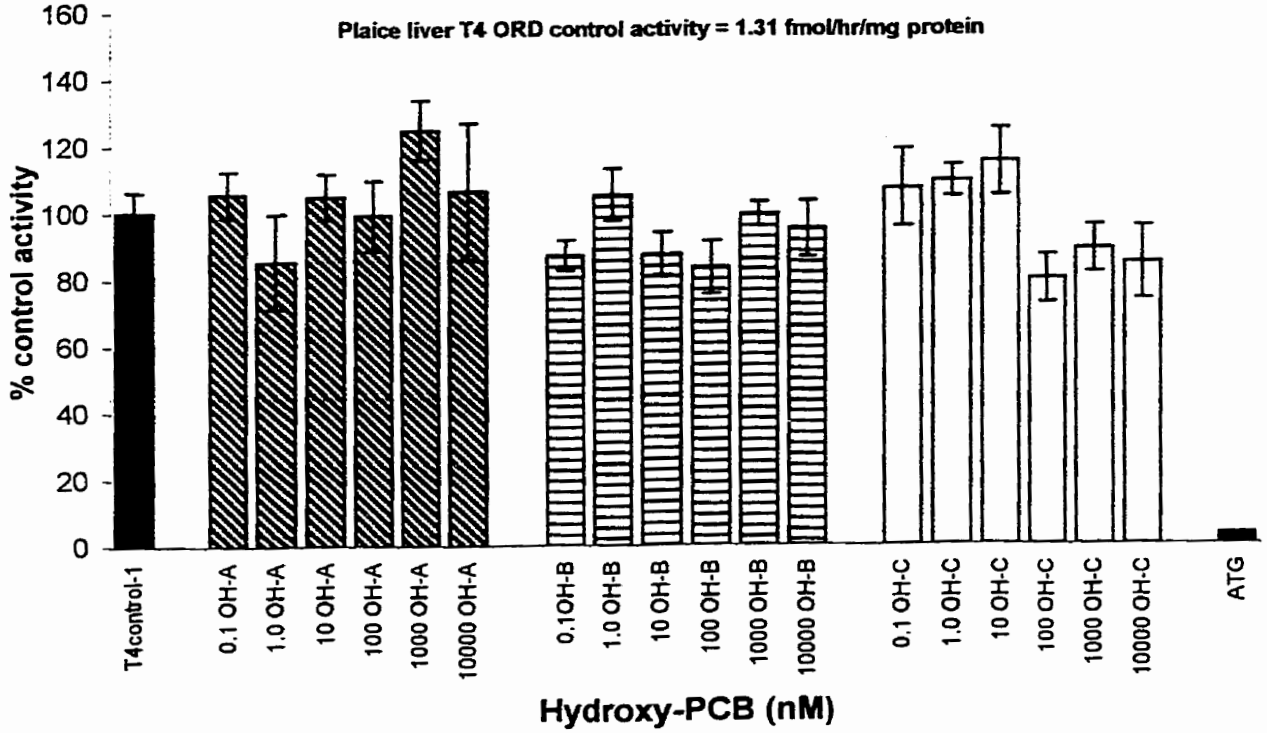

Plaice liver T4 ORD control activity = 1.31 fmol/hr/mg protein
% control activity
160
140
120
100
80
60
40
20
0
T4control-1
0.1 OH-A
1.0 OH-A
10 OH-A
100 OH-A
1000 OH-A
10000 OH-A
0.1OH-B
1.0 OH-B
10 OH-B
100 OH-B
1000 OH-B
10000 OH-B
0.1 OH-C
1.0 OH-C
10 OH-C
100 OH-C
1000 OH-C
10000 OH-C
ATG
Hydroxy-PCB (nM)

Figure 5-4: Deiodinating activity (T4IRD) in rainbow trout liver microsomes incubated with OH-A, OH-B or OH-C (0.1 – 10000 nM).

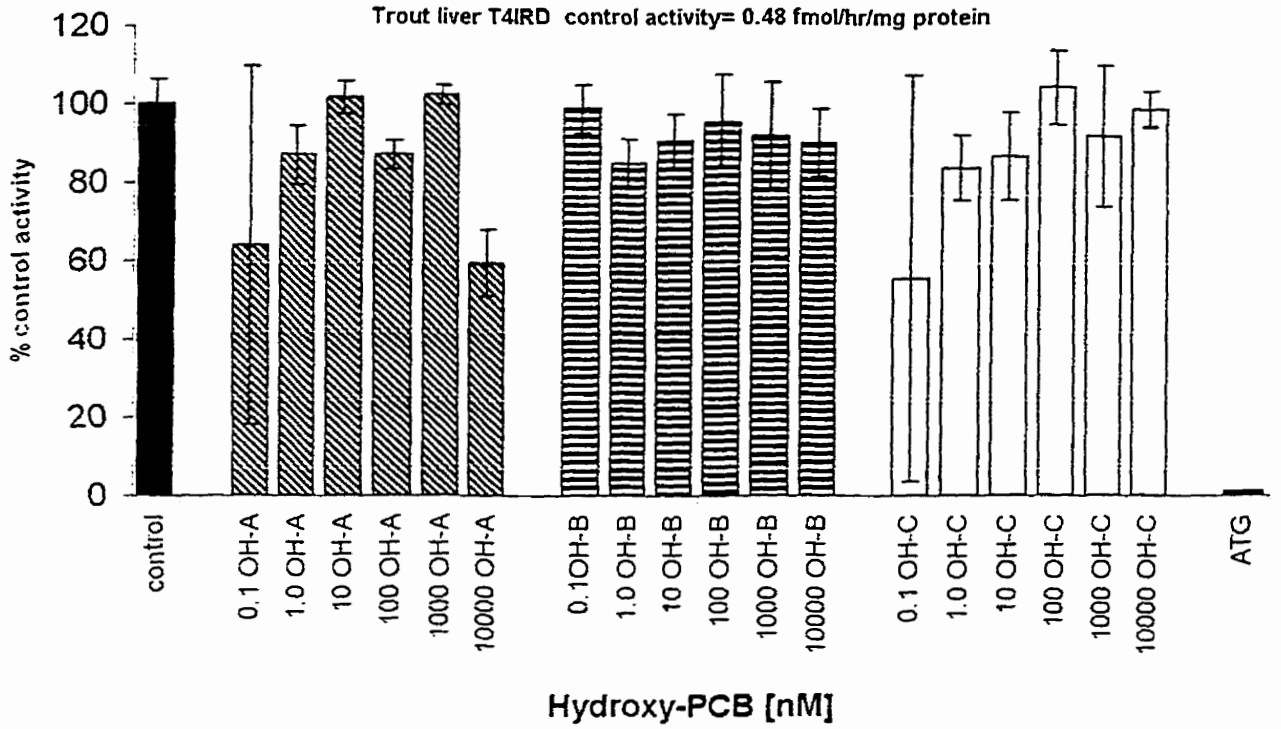
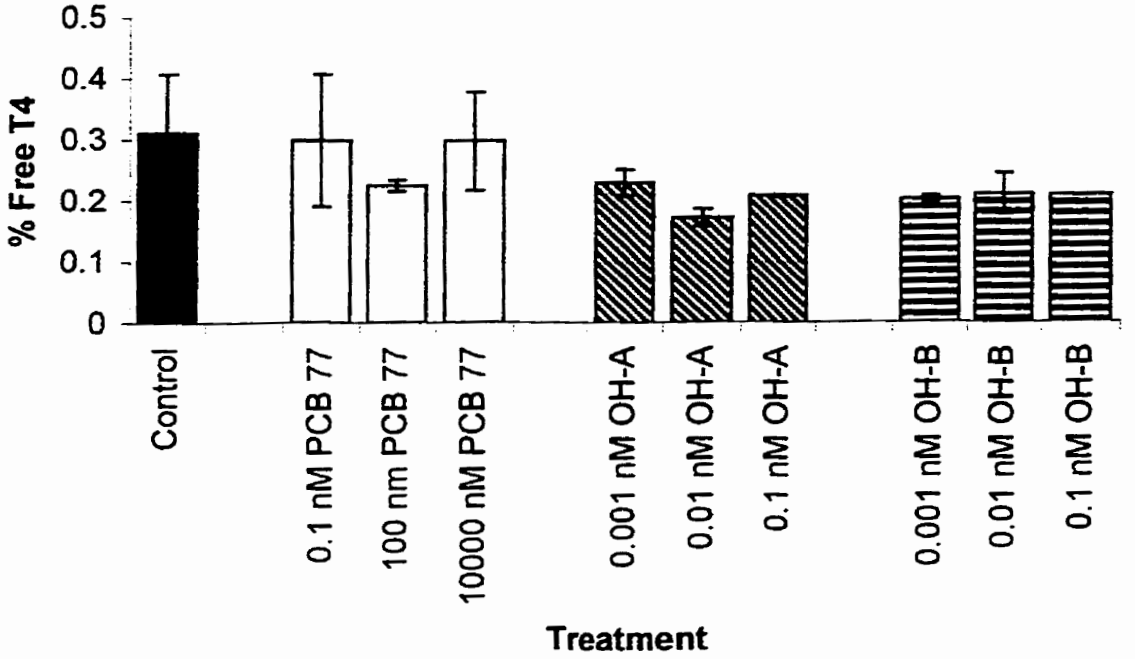

Trout liver T4IRD control activity= 0.48 fmol/hr/mg protein
% control activity
120
100
80
60
40
20
0
control
0.1 OH-A
1.0 OH-A
10 OH-A
100 OH-A
1000 OH-A
10000 OH-A
0.1OH-B
1.0 OH-B
10 OH-B
100 OH-B
1000 OH-B
10000 OH-B
0.1 OH-C
1.0 OH-C
10 OH-C
100 OH-C
1000 OH-C
10000 OH-C
ATG
Hydroxy-PCB [nM]

Figure 5-5: % FT4 in plasma incubated with 0.1, 100 or 10000 nM PCB 77 or 0.001, 0.01 or 0.1 nM OH- A or OH- B per ml plaice plasma.


0.5
0.4
0.3
0.2
0.1
0
% Free T4
Control
0.1 nM PCB 77
100 nm PCB 77
10000 nM PCB 77
0.001 nM OH-A
0.01 nM OH-A
0.1 nM OH-A
0.001 nM OH-B
0.01 nM OH-B
0.1 nM OH-B
Treatment

OH-B and OH-C were also ineffective in competing for T4-binding sites in trout plasma (Figs. 5-6 and 5-7).

Discussion

There were two main objectives for this set of experiments. The first was determine if PCB 77, PCB 126 or three OH-PCBs could affect *in vitro* American plaice or rainbow trout liver microsomal deiodination. This was assessed by incubating liver microsomes from either plaice or trout with a PCB or an OH-PCB over a range of concentrations.

The second objective was to determine if the PCBs or OH-PCBs can compete for T4 binding sites in plaice or trout plasma. This was assessed by adding *T4 and PCB or OH-PCB to plasma in a dialysis sac and allowing the system to incubate overnight, and then measuring relative amounts of *T4 inside and outside the dialysis sac.

There is little information on the specific pathways of metabolism of parent PCB isomers in fish. What data are available suggest there are differences in metabolic routes, particularly hydroxylation, in fish when compared to other vertebrates (Huntzinger et al, 1972). However, the CYP1A1 component of the CYP450 enzymes responsible for hydroxylation, is highly conserved in vertebrates (Goksoyr and Forlin, 1992). CYP1A enzymes are induced by PCBs similarly in all vertebrates (Goksoyr and Forlin, 1992) and PCBs in fish may get hydroxylated. It has been suggested the hydroxylation of PCBs in vertebrates depends on the specific congener rather than a general and similar response for hydroxylation for all PCB congeners (Matsusue et al, 1996; Elskus et al, 1994). It is uncertain how much of each parent PCB compound gets hydroxylated, and, what the metabolic products of PCB 77 or 126 would be in American plaice. However, it is

Figure 5-6: % FT4 in plasma incubated with 0.1, 100 or 10000 nM PCB 77 or 0.001, 0.01, 0.1, 100, or 10000 nM OH- A per ml trout plasma.

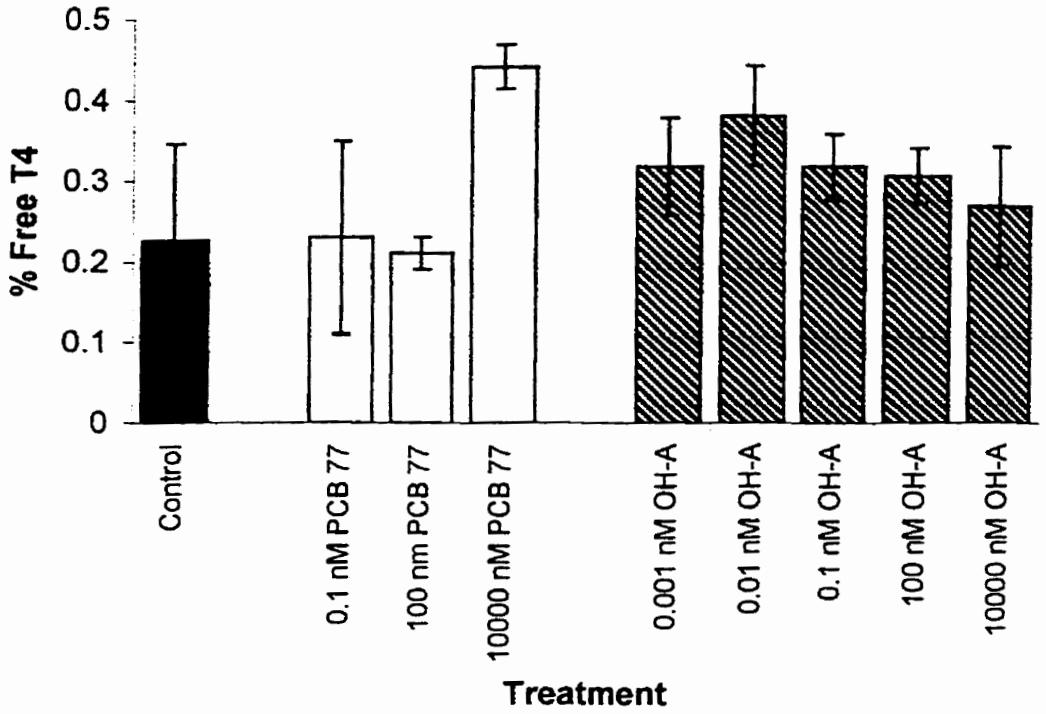
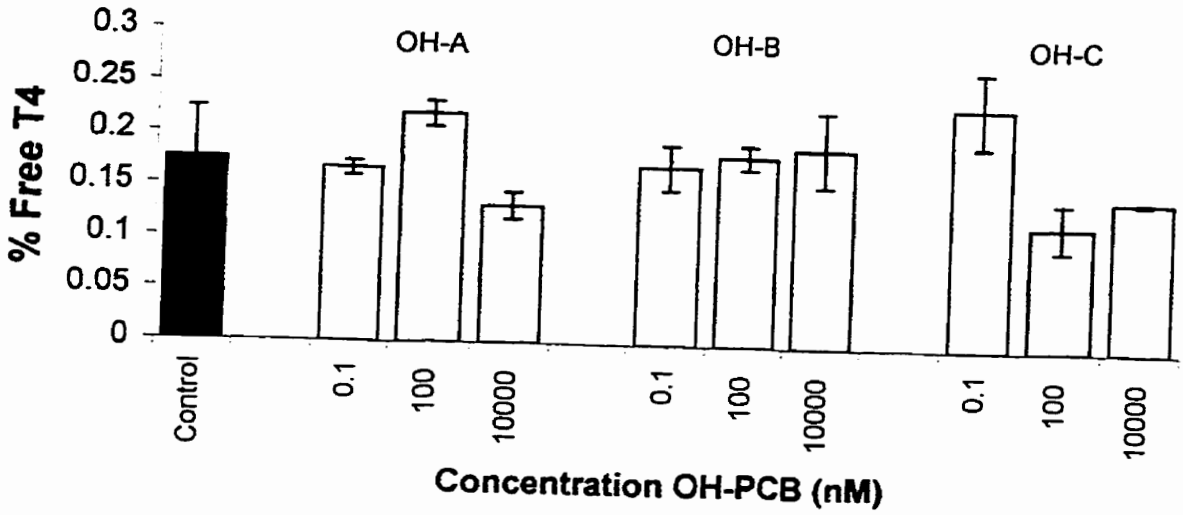

0.5
0.4
0.3
0.2
0.1
0
% Free T4
Control
0.1 nM PCB 77
100 nm PCB 77
10000 nM PCB 77
0.001 nM OH-A
0.01 nM OH-A
0.1 nM OH-A
100 nM OH-A
10000 nM OH-A
Treatment

Figure 5-7: % FT4 in plasma incubated with 0.1, 100 or 10000 nM OH-A, OH-B or OH-C per ml trout plasma.


% Free T4
0.3
0.25
0.2
0.15
0.1
0.05
0
OH-A
OH-B
OH-C
Control
0.1
100
10000
0.1
100
10000
0.1
100
10000
Concentration OH-PCB (nM)

common to find OH-PCBs in vertebrates with a similar chemical structure to those used in this study.

The lack of statistically significant difference from controls in some of the deiodination assays may be due to the large variation due to low enzyme activity. There was low deiodinase activity in plaice and trout microsomes used in this set of assays. Low enzyme activity makes it more difficult to determine significant trends or changes in an experiment. A larger sample size should be used in the future to reduce the variation in data and determine if there is indeed a positive effect of the OH-PCBs. In addition, microsomes from fish with higher deiodinase activity could be used to determine whether OH-PCBs may be having an effect. As well, assays could be performed at a temperature optimum (25°C for plaice), that significantly increases enzyme activity. This would increase the activity of the enzyme and perhaps allow for detection of changes in deiodinase activity in the presence of a contaminant. However, it would not necessarily reflect the *in vivo* response of the fish at natural water temperatures which are much lower. The activity level of the deiodinase being used is important when assessing the potential of a contaminant to interfere with deiodination.

Hydroxylated metabolites of PCB 77 have been found to have an affinity that is the same or higher than that for T4 for rat transthyretin (TTR) (Brouwer et al, 1991). This study showed that neither PCB 77 nor OH-PCBs altered T4-binding by plaice or trout plasma. In fish, a prealbumin is the predominant TH plasma transport protein, although TTR has recently been reported in juvenile sea bream (*Sparus aurata*) (Santos and Power, 1999) and many T4 plasma binding sites exist in salmonids (Shostak and Eales, 1985; McLeese et al, 1998). The binding sites on plaice prealbumin may not be

conducive to binding by PCB 77 or OH-PCBs. This would suggest there is a rather large difference in the mechanism of action for the toxicity of PCBs and related compounds to the thyroid system of fish compared to that of mammals.

My conclusions from this chapter are that there are no significant effects *in vitro* of PCB 77, PCB 126 or three OH-PCBs on the liver deiodinase function or on the binding of T4 to plasma proteins in either American plaice or rainbow trout. Therefore the *in vivo* effects of PCB 77 and PCB 126 likely reflect indirect effects of these substances on the plaice thyroidal system.

CHAPTER 6: Partial cloning of a fish deiodinase

Introduction

In fish, the primary role of the brain-pituitary-thyroid axis is to regulate the thyroidal secretion of T4 prohormone. The production and regulation of homeostasis of the more active T3 hormone is mainly controlled in peripheral tissues by adjusting the action of deiodinase enzyme pathways.

In mammals, cDNAs for the type I enzyme have been cloned in rat (Berry et al, 1991), dog (Mandel et al, 1992) and human (Toyoda et al, 1994). Cloning of type I deiodinase has also been achieved in the chick (Van Der Geyton et al, 1997). A deiodinase with some type I characteristics but differing in its insensitivity to the normally inhibiting propylthiouracil (PTU) has recently been cloned from the kidney of tilapia (*Oreochromis niloticus*). Cloning of a type II deiodinase was first achieved with the frog, *Rana catesbeiana* (Davey et al, 1995). It has since been cloned in mammals including the rat (Croteau et al, 1996), human (Salvatore et al, 1996) and in one fish species, *Fundulus heteroclitus* (Valverde-R et al, 1997). However, the mammalian type II deiodinases are quite different structurally from the frog type II (Leonard et al, 1999) and maybe type II deiodinases. The type III deiodinase enzyme has also been cloned in *Xenopus laevis* (St. Germain et al, 1994), *Rana catesbeiana* (Becker et al, 1995), the rat (Croteau et al, 1995), the chick (Van Der Geyton et al, 1997), and humans (Salvatore et al, 1995). Cloning of a type III deiodinase has also been reported in tilapia (Van Der Geyton et al, 1998), although the sequence has not been made available either through publication or submission to Genbank, and is therefore unavailable for comparison to other vertebrate deiodinase enzymes. Common to all these cDNAs for deiodinases is an

in-frame TGA coding for selenocysteine, which is necessary for maximal enzyme activity (Berry et al, 1992).

The objective here was to clone plaice and cod deiodinases based on conserved amino acid sequences to use as a tool to measure pretranscriptional affects of PCBs on thyroid hormone action in American plaice. In order to optimize the chances of amplifying a transcript, I also used Atlantic cod (*Gadus morhua*) tissues which have high deiodinase activity compared to American plaice and other fish species (Cyr et al, 1998).

Materials and Methods

Animals: American plaice and Atlantic cod (*Gadus morhua*) brain and liver tissue were used. Both plaice and cod had been caught by trawl in the St. Lawrence estuary off the coast of Matane, QC, and transported in aerated seawater to the Maurice Lamontagne Institute (Mont-Joli, QC). American plaice were maintained in 3.6 X 1.2 X 0.45 – m (L X W X D) fiberglass tanks. Atlantic cod were held in 9- or 13-kl tanks cylindrical tanks. Both tanks contained recirculated natural seawater (28 ppt) under artificial lighting (intensity 8 lx), adjusted to provide a photoperiod of natural duration. Fish were fed to satiation with capelin twice weekly, with vitamin-enriched food pellet supplements supplied every sixth feeding for plaice and every four weeks for cod.

RNA preparation: Total RNA was isolated from plaice and cod brain and liver tissues using the cesium chloride gradient ultracentrifugation method (Sambrook et al, 1989) as modified by Nagler and Cyr (1997). Frozen samples were first homogenized in a guanidium isothiocyanate buffer solution (4.0 M guanidium isothiocyanate, 0.1 M Tris, pH 7.5, and 1% betamercaptoethanol). These homogenates were centrifuged through a cesium chloride gradient (5.7 M CsCl, 0.01 M EDTA, pH 7.5) in 4 ml Konical TM

polyallomer tubes (Beckman, Mississauga, ON, Canada) at 274 000 g in a Beckman SW41 rotor for 4 h. RNA was diluted in RNase, DNase-free water (Sigma, St. Louis, MO), verified for purity by spectrophotometry (A280/260) and stored at –20°C.

RT-PCR: Based on conserved amino acid sequences in the then available frog, rat, and human Type III deiodinase sequences, primers were designed for RT-PCR of cod and plaice liver and brain tissue total RNA (Table 6-1). One to five ug of total RNA was reverse transcribed with either primer 4 or the oligo dT primer provided. The following cycling conditions for PCR were used: 94°C for 45 s; 52°C for 45 s; 72°C for 60 s, with a 2-s extension at 72°C for each of 30 cycles, and all of this was followed by a final 10-min extension at 72°C.

RT-PCR products were run on 1% agarose gels. Distinct bands of expected size were extracted from the agarose. The DNA was then ligated into a pCR 2.1© vector which was subsquently inserted by electroporation and cloned using a TA Cloning Kit (Invitrogen, San Diego, California). An attempt was also made to obtain the 3' and 5' ends of RT-PCR products using the rapid amplification of cDNA ends (RACE) procedure as recommended by the manufacturer (Life Technologies, Inc.), except that a modified universal amplification primer was used (Davey et al, 1995). Cycling conditions for RACE were those of the manufacturer.

Sequencing results (Sheldon Laboratory, McGill University) were compared with other sequences by using the BLAST sequence similarity searching program (Altschul et al, 1997) of the National Centre for Biotechnology Information of the National Institute of Health, USA.

Table 6-1: Primers used in a RT-PCR strategy to obtain deiodinase partial cDNAs from cod and plaice brain and liver total RNA.

Primer 1: 5'-ACCATTTCAAAATCGGTCGTCA-3'
Primer 2: 5'-ATTTCGGCAGCTGCACCTGACC-3'
Primer 3: 5'-GGTCAGGTGCAGCTGCCGAAAT-3'
Primer 4: 5'-CCAGGACGATGTAAAGTCTCTC-3'
(primer reference: XL5DIII sequence – St. Germain et al, 1994)

Primer 5: 5'-CTCAGCGTGGACCTGTTGATC-3'
Primer 6: 5'-AGCCAGTTGCCAGCCTTCA-3'
Primer 7: 5'-ATGAGCCTCATCGATGTAGAC-3'
Primer 8: 5'-CCTTACTTCTTGGAGGTTGTA-3'
(primer reference: RC5DII sequence – Davey et al, 1995)

Primer 9: 5'-AAAACGGTCCCTTTCCTCCCAGGTA-3'
Primer 10: 5'-ATCCTGCCCGGCTTCTTCTCCAACT-3'
(primer reference: FhDII sequence – Valverde-R et al, 1997)

Results and Discussion

A 306-bp product (Figure 6-1) was obtained using primers 2 and 4 (Table 6-1) that has high homology with vertebrate type III deiodinase sequences, both on the nucleotide (Table 6-2) and deduced amino acid sequences (Figure 6-2, Table 6-3). The highest homology is with *Xenopus laevis* type III deiodinase, 79% over 306 bp, which is likely due to the closer phylogenetic grouping of amphibians and fish. The cDNA contains a TGA codon at position X that codes for a selenocysteine, required for maximal activity of the deiodinases in other species (Berry et al, 1992). The conclusion that this 306 bp product is a partial cDNA coding for an Atlantic cod type III deiodinase is based on indirect evidence, as a complete sequence was not obtained and expression studies were not possible.

This cDNA may used to obtain a full length cDNA for a deiodinase in cod, or perhaps closely related fish species, by using it to screen a cDNA library prepared from liver or brain. The complete cDNA can then be characterized and used to contribute to a better understanding of the action and regulation of deiodination in fish.

Figure 6-1: Nucleotide sequence of 306 bp cDNA obtained from RT-PCR of cod liver tRNA using primers 2 and 4 (Table 6-1).

1 ATTTCGGCAG CTGCACCTGA CCGCCGTTCA TGACGCGCCT GGCCGCGTTC
51 CGGCGCGTGG CGAGCCAGTA CGCGGACATC GCGGACTCG CTGCTCGTGTA
101 CATCGAGGAG GCGCATCCGT CCGACGGCTG GGTGAGCTCC GACGCGCCGT
151 ACCAGATCCC CAAGCACCGC TGCATCGAGG ACCGGCTCCG CGCGGCGCAG
201 CTGATGCTGT CCGAGGTGCC GGGCAGCCAG GTGGTGATCG ATACCATGGA
251 CTACTCGTCC AACGCGGCGT ACGGCGCCTA CTTTGAGAGA CTTTACATCG
301 TCCTGG

Table 6-2: Percent nucleotide identities of cod 306-bp cDNA with deiodinases.

Species	Deiodinase Type (I, II or III)	% Identity	Reference
Xenopus laevis	type III	79 (243/306)	(St. Germain et al, 1994)
Gallus gallus	type III	80 (164/205)	(Van Der Geyton et al, 1997)
Rana catesbeiana	type III	73 (224/306)	(Becker et al, 1995)
Homo sapiens	type III	71 (217/305)	(Salvortore et al, 1995)
Rattus norvegicus	type III	66 (204/305)	(Croteau et al, 1995)
Fundulus heteroclitus	type II	68 (91/134)	(Valverde-R et al, 1997)
Rattus norvegicus	type I	58 (77/132)	(Berry et al, 1991)

Figure 6-2: Deduced amino acid sequence of the 306-bp cod liver cDNA.

FGSCT*PPFMTRLAAFRRVASQYADIADSLLVYIEEAHPSDGWVSSDAPYQIPKH
RCIED

Table 6-3: Percent amino acid identities and similarities of 306 bp cod cDNA with deiodinases.

Species	Deiodinase (I, II or III)	% Identity	% Similarity	Reference
Xenopus laevis	type III	75	86	(St. Germain et al, 1994)
Gallus gallus	type III	70	83	(Van Der Geyton et al, 1997)
Rana catesbeiana	type III	70	82	(Becker et al, 1995)
Rattus norvegicus	type III	58	81	(Croteau et al, 1995)
Homo sapiens	type III	58	81	(Salvortore et al, 1995)
Rana catesbeiana	type II	52	71	(Becker et al, 1997)
Rattus rattus	type II	53	68	(Croteau et al, 1996)
Fundulus heteroclitus	type II	49	70	(Valverde-R et al, 1997)
Rattus norvegicus	type I	46	64	(Berry et al, 1991)
Homo sapiens	type I	47	64	(Toyoda et al, 1994)
Tilapia nilotica	type I	45	66	(Sanders et al, 1997)

CHAPTER 7: General Discussion and Conclusions

There is considerable evidence that the thyroid system is a target of disruption by contaminants, but there has been little concentrated effort on elucidating the mechanisms of this disruption. Studies generally only measure one aspect of the thyroid system of an organism exposed to a xenobiotic. Earlier studies considered the changes in histology of the thyroid follicles and/or plasma TH levels. More recent studies report plasma TH levels and a few measure the changes in deiodinase enzyme activity as an indicator of altered thyroidal status. The objective here was to determine the effect of PCB 77 and 126 on the thyroidal status and TH action in American plaice, using measures of plasma TH as well as deiodinase function and deiodinase transcript levels.

Deiodination pathways in plaice overall are similar to those that have been described in other fish species, with respect to Km, substrate affinity, effects of inhibitors, as well as pH, temperature and thiol cofactor optima. There were, however, some differences in temperature optima, substrate affinities and pH optima compared with other teleosts. Of the plaice tissues surveyed, ORD activity was highest in liver and IRD activity was highest in brain. These tissues were subsequently the focus of deiodinase activity for the duration of the study. Liver tissue serves as a choice tissue for study of deiodinase alteration by xenobiotics, since contaminants such as PCBs tend to accumulate in the liver and it is has the highest potential for T4 conversion.

Injection of plaice with PCB 77 increased liver T4ORD activity, decreased brain T4ORD activity, increased plasma T4 levels and decreased plasma T3 after one week. A second experiment with PCB 77 or PCB126 was performed. After one week, PCB 77 increased plasma T4 and liver T4ORD activity and PCB 126 increased liver IRD

activities in some fish, while PCB 126 caused a decrease in brain T3IRD activity. *In vitro* studies involving incubation of plaice or rainbow trout liver microsomes with PCB 77, PCB 126 or three hydroxylated PCBs (OH-PCBs) showed no significant effect by these compounds on deiodination or on plasma T4 binding.

An increase in liver ORD may be a response specifically to the presence of the PCB or to an increase T4 metabolism. The brain decreases T4ORD activity so as not to upset the local supply of T3. If the brain did not decrease T4ORD, it is possible too much T3 would be produced locally. Also, since the brain is involved in a negative feedback system with the thyroid, a high level of T4 may be used by the brain to signal a down-regulation at the pituitary for reducing T4 production or release by the thyroid follicles. PCBs are fat-soluble and tend to accumulate in tissues of animals high in fat such as the liver. While nervous tissue is also high in fat, there may be greater barriers in the brain for protection from foreign compounds. Since there is no increase in plasma levels of T3, however, it is possible that T3 is being produced and stored in or cleared from the plaice.

Secondly, the liver is a key organ for biotransformation pathways including those important for TH metabolism in vertebrates including glucuronidation and sulfation. There is limited information on these non-deiodinating pathways in rainbow trout (Finnson and Eales, 1997; 1998; Finnson et al, 1999) and no information on these pathways in American plaice. There are some differences in mammals and fish in the functions of these pathways. In rainbow trout, glucuronidation is important in excretion of TH and sulfation is believed to be responsible for both excretion and cellular storage of TH (Finnson and Eales, 1998). In rainbow trout these pathways are subject to dietary influences and, therefore, have the potential to alter the total pool of TH for the fish, thus

affecting the overall thyroidal status of the fish. It is likely that similar pathways of TH metabolism exist in American plaice. There is only a small set of evidence which suggests that glucuronidation pathway may be influenced by the presence of a contaminant in fish species, while deiodination pathways are not altered (Brown et al, 1997). However, all the major enzymatic pathways, including deiodination, have been induced in rats treated with PCBs (Boll et al, 1998).

The *in vivo* experiments indicate an up-regulation of the thyroid system in response to PCB 77 at one week. This is indicated by effects of PCB 77 at both central (plasma T4 and T3) and peripheral levels (liver and brain deiodination) of the thyroid cascade and points to the liver as a key peripheral site.

It was not clear from the *in vivo* experiment if the actions of PCBs were the result of direct effects of the PCB or indirect effects of a PCB metabolite on the deiodinase enzymes. To help determine this, plaice liver microsomes were exposed to the PCB 77, PCB 126 and hydroxylated forms of these PCB compounds *in vitro*. These experiments suggest there is likely no direct effect by the parent compounds or OH-PCBs that I tested on deiodinase function. Based on these experiments, the *in vivo* effects on thyroidal status of plaice is likely the result of indirect actions of PCBs and PCB-metabolites on other enzymes system components that may influence thyroidal status, rather than direct interaction by the PCBs and related compounds directly on thyroid system components. Also, no direct PCB or OH-PCB action in the plasma TH-binding proteins, and therefore no direct effects on the distribution of TH between free and bound plasma fractions.

The development of cDNAs for fish deiodinases will be useful for determining whether contaminants can induce deiodinase mRNA, which would indicate a direct effect

on TH action. Attempts to clone full length deiodinase enzymes for plaice were not achieved. However, this is the first report of a cloning of a partial cDNA in Atlantic cod, which is likely a type III deiodinase, based on sequence homology. This is only the second report of a cDNA for fish type III deiodinase.

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