Oxidative Stress in Lake Trout (*Salvelinus namaycush*) Exposed to Organochlorine Contaminants that Induce the Phase I Biotransformation Enzyme System

by

VINCENT P. PALACE

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

Department of Zoology
Winnipeg, Manitoba
April 1996

ISBN 0-612-13429-6

Canada

Name ___

Dissertation Abstracts International and *Masters Abstracts International* are arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation or thesis. Enter the corresponding four-digit code in the spaces provided.

ENVIRONMENTAL SCIENCES.

SUBJECT TERM

0768

SUBJECT CODE

UMI

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology ... 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology .. 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics ... 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

THE UNIVERSITY OF MANITOBA

FACUTY OF GRADUATE STUDIES

COPYRIGHT PERMISSION

OXIDATIVE STRESS IN LAKE TROUT (*Salvelinus namaycush*) EXPOSED TO ORGANOCHLORINE CONTAMINANTS THAT INDUCE THE PHASE I BIOTRANSFORMATION ENZYME SYSTEM

BY

VINCENT P. PALACE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Oxidative Stress in Lake Trout (*Salvelinus namaycush*) Exposed to Organochlorine Contaminants that Induce the Phase I Biotransformation Enzyme System

by

Vince P. Palace

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

Department of Zoology
Winnipeg, Manitoba
April 1996

Table of Contents

Abstract

Lake trout from Lake Ontario are among the most heavily contaminated freshwater fish in the world. The most toxic contaminants that they contain are planar, lipophilic organochlorine molecules that interact with the Ah cellular receptor. Interaction with the Ah-receptor begins a series of cellular events culminating in the induction of Phase I or mixed-function oxygenase (MFO) enzymes. Although these enzymes are sensitive indicators of exposure to planar organochlorines, relationships between their induction and mechanisms of cellular toxicity are unknown. Recent evidence suggests that elevated Phase I activity could increase the proliferation of oxyradicals, deplete cellular antioxidants and increase oxidative membrane breakdown. To examine this relationship, lake trout and lake sturgeon were exposed to organochlorines in the laboratory, and lake trout were collected from organochlorine contaminated sites in the Great Lakes. Lake trout and lake sturgeon exposed to organochlorines in laboratory experiments and lake trout collected from contamianted sites in the Great Lakes had elevated Phase I activity that was correlated with biochemical measures of oxidative membrane breakdown. While activities of the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) or glutathione peroxidase (GPx) do not appear to be sensitive indicators of oxidative stress, tocopherol (vitamin E) and retinoids (vitamin A) were depleted in fish that had

induced Phase I activity and elevated indices of oxidative stress. Mechanisms of retinoid depletion were examined in the laboratory by injecting ^{3}H-retinol into the circulation of PCB dosed lake trout with induced Phase I enzyme activity. Retinol declined due to direct metabolism by Phase I and Phase II (conjugation) enzymes and not due to increased utilization of the vitamin as an antioxidant. In addition to high contaminant burdens, offspring from a high percentage of Lake Ontario lake trout exhibit an early mortality syndrome (EMS) that results in almost complete mortality of fry just before the swimup stage of development. Studies of lake trout from Lake Ontario indicate that although oxidative stress in adult females may predispose her offspring to exhibit EMS, mortality is not related to depleted concentrations of vitamins A,E or C in the embryos.

Acknowledgements

This thesis could not have been completed without the patient assistance of many people at the University of Manitoba's Department of Zoology and at the Department of Fisheries and Oceans' Freshwater Institute and Canadian Center for Inland Waters. C. Baron, Dr. S. Brown, Dr. T. Dick, B. Evans, Dr. J. Fitzsimmons, S. Harrison, M. Keir, R. Kiriluk, Dr. L. Lockhart, R. McNichol, D. Metner, Dr. D. Muir, L. Vandenbyllardt and K. Wautier all made vital contributions to various experiments described in this thesis. I would especially like to thank the members of my advisory and examining committees: Dr. D. Burton, Dr. E. Huebner, Dr. T. Wiens and Dr. S. Swanson for their thorough evaluations and constructive criticisms.

It has been my privilege to work under the supervision of Dr. J.F. Klaverkamp who has truly been a mentor to me. Words of thanks seem inadequate to express my gratitude, and I can only hope that in the future I have the opportunity to make as large a difference in someone's life as Jack has made in mine.

I would also like to acknowledge the unwavering moral support of my wife Barbara who never doubted that her husband would someday not be a student. Your encouragement and the promise of a better life for both of us (now three) has been the momentum behind this project. Thank you also to my mother, Marie, who taught me about perseverance and hard work and who

instilled in me the confidence to pursue this goal. Finally, this project is for Daniel, who at the time of this writing is a sausage of only three and a half months. I hope that you too will discover the satisfaction of pursuing and achieving your goals.

Abbreviations

Ah	aryl-hydrocarbon receptor
AHH	aryl-hydrocarbon hydroxylase enzyme
Bq	Becquerel
CAT	catalase enzyme
Ci	Curie
cyp1A	cytochrome P4501A protein
DDW	double-distilled water
DNA	deoxyribonucleic acid
ECOD	ethoxycoumarin-o-deethylase enzyme
EMS	early mortality syndrome
EROD	ethoxyresorufin-O-deethylase enzyme
g	gravitational force
gm	gram
GPx	glutathione peroxidase enzyme
IJC	International Joint Commission
kg	kilogram
L	litre
LSC	liquid scintillation counting
M	molarity
MDA	malonaldehyde
MFO	mixed-function oxygenase enzymes
ml	millilitre
mmol	millimole
MS222	tricaine methanesulfonate anesthetic
mV	millivolts
nm	nanometres
nmol	nanomoles
NOAEL	no adverse effect level
PAH	polyaromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCDD	polychlorinated dibenzo-dioxins
PCDF	polychlorinated dibenzo-furans
PHAH	polyhalogenated aromatic hydrocarbons
PUFA	polyunsaturated fatty acid lipids
SOD	superoxide dismutase enzyme
TCDD	tetrachlorodibenzodioxin
TCDF	tetrachlorodibenzofuran
UGT	uridine glucuronyltransferase enzyme
µl	microlitre

List of Figures and Tables

Chapter 5 cont'd

Chapter 6

Appendix A

Chapter 1

General Introduction

Lake trout populations of the Great Lakes drastically declined beginning in the 1930's so that most of the natural stocks were considered extinct by the 1960's. Lake Superior is the exception, maintaining several native, self-sustaining stocks. In an attempt to re-establish whole-lake populations, hatchery stocking of lake trout began in the Great Lakes about 1958 and continues today with over 150 million lake trout fry having been introduced into the Great Lakes (Eschenroder 1990). Except for Lake Superior, where some stocks have become self-sustaining, and isolated Lake Huron and Lake Ontario reports of fry capture (Nester and Poe 1984, Marsden et al. 1988), there has been little success in reestablishing lake trout in the Great Lakes.

The initial stages of the decline of lake trout populations are generally attributed to the invasion of the sea lamprey through the Erie Barge Canal (Curtis 1990). However, the continued failure of recruitment may involve overfishing and spawning habitat destruction (Leatherland 1993), increased predation (Fitzsimmons 1990), effects of newly introduced exotic species (Mills et al. 1993), failure of introduced strains to utilize man-made spawning shoals (Eschenroder 1990), toxic contaminants (Walker and Peterson 1990) or a combination of these factors (Hartman 1988).

Lake trout are high trophic level predators with high lipid content and a relatively long life span (Scott and Crossman 1973). They therefore have great potential to

accumulate lipophilic organic contaminants such as polychlorinated biphenyls (PCBs), dioxins and furans, which are present in the Great Lakes (Morrison et al. 1985, De Vault et al. 1989) and are not readily metabolized once assimilated (Walker et al. 1991, Smith et al. 1990). Larger fish captured in the Great Lakes, in fact, do have higher concentrations of these compounds than smaller fish of the same species (Miller et al. 1992, Borgmann and Whittle 1991).

Because of their persistence and ubiquitous presence in lake trout tissues, organic contaminants such as PCBs, dioxins and furans have received increasing consideration as a primary concern related to reproductive failure of lake trout in the Great Lakes. Inputs of these compounds were greatest during the 1960s and 1970s and have since steadily declined (Janz et al. 1992). Lake trout contaminant concentrations showed a similar initial decline in the 1970s and early 1980s. However, in recent years, PCB concentrations in whole lake trout have stabilized near 5 µg/gm (Borgmann and Whittle 1991), while total dioxins and furans are currently near 290 ng/gm and 67 ng/gm, respectively (Niimi and Oliver 1989).

Toxic effects have been shown in fish exposed to concentrations of organochlorine contaminants similar to those noted above. These include reproductive impairment as well as wasting syndrome and immune suppression (Smith et al. 1990), altered liver function and lipid metabolism (Leatherland and Sonstegard 1982), aberrant swimming (Mac et al. 1985),

necrotic epithelia (Frimith 1990), increased prevalence of tumours (Metcalfe et al. 1988), altered steroid profiles (Van der Kraak et al. 1992, McMaster et al. 1991), smaller gonads (Thornburn 1994), edema, vacuolization of the liver and other teratogenic effects (Helder 1981).

Female lake trout produce relatively large eggs. A large dose of contaminants is often delivered by contaminated females to the fry from the high-lipid yolk, which is used as an energy source before feeding begins (Niimi 1983). Organochlorine contaminants present in fry are derived from their egg yolk material and not from incubation water (Walker et al. 1991). High mortality rates have in fact been associated with the swimup stage (Skea et al. 1985) when yolk resorption has been completed and the contaminants have been fully assimilated (Giesy et al. 1986). Mortality at this stage, referred to as swimup syndrome, is often preceded by distinct behavioural and physical symptoms including loss of equilibrium, corkscrew swimming or lack of swimming activity, unresponsiveness to touch stimuli, empty stomachs and swim bladders and emaciation at death (Mac et al. 1985).

Organochlorines found in the Great Lakes, including PCBs, dioxins and furans, have all been shown to inhibit lake trout fry development in laboratory experiments (Walker et al. 1991). A characteristic syndrome resulting in near 100% fry mortality, and known as early mortality syndrome (EMS), is also more prevalent in fry from the more heavily contaminated

Lake Ontario than from the less contaminated Lake Superior (Mac et al. 1985, Marquenski 1990). Investigations to elucidate factors involved in organochlorine contaminant mediated recruitment failure in fish have focused on a number of molecular mechanisms of toxicity in mammals. These include the two groups of metabolic enzymes, Phase I or mixed-function oxygenase enzymes (MFO), which are found in the endoplasmic reticulum (Boon et al. 1992), and Phase II or conjugation enzymes that are associated with the cell membrane (Antoine et al. 1988).

There are 10 families of MFO enzymes, also known as cytochrome P-450 monooxygenases, in mammals but only the P450IA subfamily is prominent in fish (Goksoyr et al. 1991, Elskus and Stegeman 1989). Activity of this group is induced by exposure to chlorinated hydrocarbons that interact with the cytosolic aryl-hydrocarbon (Ah) receptor (Goksoyr and Forlin 1992). Activity is generally measured, in fish, using assays for 7-ethoxyresorufin-O-deethylase (EROD), ethoxycoumarin-O-deethylase (ECOD) or aryl hydrocarbon hydroxylase (AHH) (Goksoyr et al. 1991). Whether the MFO activity is induced by exposure to contaminants or represents constitutive activity that is responsible for metabolizing endogenous steroids, prostaglandins or fatty acids, the reaction mechanisms are the same. One atom of oxygen is inserted into the substrate in the first step (Phase I), rendering the substrate more polar and, therefore, more readily excreted (Goksoyr and Forlin 1992).

Because they are easily and sensitively measured, MFO enzymes have become an important tool for assessing environmental exposure to chlorinated hydrocarbons. Variations of MFO enzyme activity with season (Luxon et al. 1987, Edwards et al. 1988), water temperature (Stegeman 1979), pH (Willis et al. 1991), fish age (Addison and Willis 1982, Goksoyr and Larsen 1991), species (Goksoyr et al. 1991), fish sex (Forlin and Hansson 1982), feeding status (Jimenez and Burtis 1989), storage conditions of tissues before analysis (Forlin and Andersson 1985) and even interlaboratory comparisons (Munkittrick et al. 1993) have all been quantified, making comparisons of data sets possible.

After the initial addition of oxygen by MFO enzymes, conjugation of the lipophilic substrate (Phase II) with a number of polar endogenous molecules further increases aqueous solubility and excretion (Antoine et al. 1988). In fish, these endogenous molecules usually include glutathione, glucuronic acid and sulfate groups. The presence of each conjugate has been confirmed in tissues and bile of fish exposed to chlorinated hydrocarbons (Malins and Ostrander 1991). Like MFOs, Phase II enzymes are also induced by exposure to contaminants. However, their activity in fish is less responsive to induction and long contaminant exposures are often required to yield significant differences in activity from control groups (Jimenez and Stegeman 1990).

Despite considerable research on these metabolic enzyme

activities in fish, mechanistic relationships between enzyme induction, recruitment failure and fry mortality in the Great Lakes have not been clearly established (Payne et al. 1987). Nevertheless, a relevant link between reproduction and activity of these enzymes exists in that reproductive hormones are metabolized by the same enzymes that respond to, and metabolize, environmental contaminants (Haux and Forlin 1988). Several studies have identified lower circulating concentrations of steroid hormones in fish exposed to contaminants (Larsson et al. 1988, Munkittrick et al. 1991, Forlin and Haux 1985, Van der Kraak et al. 1992). Concurrently with lower steroid concentrations, induced fish also show evidence of impaired gonad development and lower fertilization rates (Andersson et al. 1988, Spies et al. 1985). The link is further strengthened by observations that these same metabolic enzymes naturally show lower activity corresponding to seasonal spawning times when steroid hormone levels are at their highest (Andersson and Forlin 1992). Again, however, relationships among contaminant exposure, enzyme induction and population level effects have not been demonstrated. Reasons for this deficiency are likely associated with our poor understanding of all the toxic mechanisms initiated as part of, or concurrently with, induction of metabolic enzymes.

Recently, studies examining a process known as oxidative stress, have been gaining increased attention (Winston and Di Giulio 1991). Oxidative stress is a condition which can result

in damage to cellular components, including lipid membranes and DNA (Stohs et al. 1990). When cellular and subcellular membranes are oxidatively damaged the process is known as lipid peroxidation (Singh et al. 1992). Lipid peroxidation can be initiated by any reactive species capable of removing a hydrogen atom from a carbon atom in polyunsaturated fatty acid (PUFA) membrane lipids (Fig. 1.1). Carbon atoms in methylene interrupted polyenoic acids are particularly susceptible to having their hydrogen atoms removed and since PUFA contain a large number of carbon atoms in this configuration, they are especially vulnerable to lipid peroxidation (Suguhara et al. 1994). PUFA lipids become relatively unstable lipid radicals once a hydrogen atom has been removed. Lipid radicals can cross-link with another lipid radical; however, in aerobic organisms they most often combine with molecular oxygen to produce a lipid peroxyl radical (Kappus 1986). Although lipid peroxyl radicals are more stable than lipid radicals, they are sufficiently reactive to abstract a second hydrogen from an adjacent PUFA lipid, forming a lipid hydroperoxide from the initial lipid and beginning the process anew in the second lipid. Therefore, the initial removal of a single hydrogen atom can propagate the reaction in hundreds of other lipids (Gutteridge and Halliwell 1990).

Examples of the reactive species capable of initiating lipid peroxidation include transition metals which undergo redox reactions (ie. Cu and Fe), the superoxide radical (O_2^-)

produced by mitochondria as a direct reduction of molecular oxygen, and the hydroxyl radical ($OH^{\cdot}$) evolved from metal or enzyme reduction of hydrogen peroxide or by irradiation of aqueous solutions (Gutteridge and Halliwell 1990). Aerobic respiration routinely produces trace amounts of hydrogen peroxide, the hydroxyl radical and the superoxide anion, making removal of a hydrogen atom from PUFA membrane lipids a perpetual danger (Babaich et al. 1993).

Unobstructed lipid peroxidation results in loss of membrane fluidity and function, including inactivation of membrane-bound receptors and enzyme proteins, and an increase of overall membrane permeability (Horton and Fairhurst 1987). Lipid hydroperoxides can further decompose to hydrocarbon gases such as ethane and pentane (Kohlmüller and Kochen 1993) or to cytotoxic carbon fragments including malonaldehyde (Esterbauer et al. 1991). The lipid peroxidation process in oxidatively stressed organisms can be measured by the amount of ethane and pentane gases exhaled in the breath (Kohlmuller and Kochen 1993) or by determining the concentration of malonaldehyde (Ichinose et al. 1994) or lipid hydroperoxides (Williams et al. 1992) in liver tissue.

Lipid peroxidation is not normally allowed to proceed unobstructed within the cell. Instead, cellular factors referred to as antioxidants intercede, by re-reducing and stabilizing damaging free radicals before they can initiate or propagate lipid peroxidation (Winston 1991). Two groups of

antioxidants are present in cells. The first group of antioxidants are enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Mather-Mihaich and Di Giulio 1986). All three of these enzymes metabolize oxygen derivatives that are capable of increasing oxidative stress (Winston and Di Giulio 1991). SOD is a membrane bound metalloenzyme consisting of two homodimers (total MW = 32,000) and incorporating either Cu, Zn, Fe or Mn in its structure, depending on its cellular location and the species in question (Canada and Calabrese 1989). SOD catalyzes reaction 1, reducing the highly reactive superoxide anion to hydrogen peroxide (McCord and Fridovich 1988):

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{Reaction 1}$$

Although SOD removes the superoxide radical, the resultant hydrogen peroxide is also capable of increasing oxidative stress by producing the reactive hydroxyl radical ($OH^{\cdot}$) in the Fenton reaction (reaction 2) (Babaich et al. 1993):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \qquad \text{Reaction 2}$$

However, CAT and GPx prevent formation of the hydroxyl radical by metabolizing hydrogen peroxide (Wendel 1988). It has long been known that CAT, consisting of 4 iron-containing subunits (total MW = 240,000) and localized in mitochondria and

peroxisomes, catalyzes reaction 3 (Aebi 1974):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{Reaction 3}$$

Meanwhile, GPx activity, localized in the soluble fraction of the cytosol and in the mitochondria of the cell (4 subunits, total MW = 88,000), also metabolizes hydrogen peroxide by reaction 4 (Wendel 1988):

$$2NADH + H_2O_2 \rightarrow H_2O + H_20 + 2NAD^+ \qquad \text{Reaction 4}$$

GPx also reduces lipid hydroperoxides, cytotoxic products of peroxidizing lipids (Esterbauer et al. 1991), to hydroxy fatty acids using the reducing power of glutathione (reaction 5) (Günzler and Flohé 1986):

$$LOOH + GSH_{(red)} \rightarrow H_2O + LOH + GSSG_{(ox)} \qquad \text{Reaction 5}$$

In addition to enzymatic antioxidants, cells also contain non-enzymatic antioxidants. This grouping includes α-tocopherol (vitamin E), ascorbic acid (vitamin C), β-carotene, astaxanthin, canthaxanthin and other retinoids (vitamin A), low molecular weight scavengers of oxyradicals like glutathione and metallothionein, and melatonin which is produced by the pineal gland (Pallozza and Krinsky 1991,

Thomas 1987, Rousseau et al. 1992, Sato and Bremner 1993, Melchiorri et al. 1994). Molecular structures for these lipid-soluble and water-soluble antioxidants are depicted in Figures 1.2 and 1.3 respectively. Antioxidant capacity is conferred upon each of these molecules by the structures encircled in shaded areas. In the case of lipid-soluble antioxidants, the shaded portions encircle only an approximation of the antioxidant active area. The reason for this is that reaction with peroxyl radicals or oxyradicals often involves quenching of the radical within the immediate area of the hydrophobic carbon chain of the antioxidant molecule followed by complex molecular rearrangements (Burton and Ingold 1984, Gonzalez Flecha et al. 1991).

Despite subtle differences in the mechanisms of reactions with radicals, all of these cytoprotective molecules exert their protective effect by the same general mechanism. Reducing power is sacrificed to potentially damaging oxidants before they can oxidize and damage essential cellular components (Winston and Di Giulio 1991). For example, α-tocopherol (α-TOH), which is embedded directly in the lipid membrane bilayer, quenches lipid hydroperoxide radicals (ROO·) in the manner of reaction 6 (Packer 1991):

$$\alpha\text{-TOH} + \text{ROO}\cdot \rightarrow \alpha\text{-TO}\cdot + \text{ROOH} \qquad \text{Reaction 6}$$

The reaction rate for lipid radicals with α-tocopherol is

several orders of magnitude faster than with other adjacent PUFA lipids (Liebler 1993). Additionally, the resultant α-tocopheroxy radical (α-TO·) is more stable than the lipid hydroperoxide, and is less likely to damage cellular components, including neighbouring lipids (Burton and Traber 1990). Liebler (1993) reviewed several studies which have shown that lipid peroxidation cannot occur when α-tocopherol is above a certain threshold concentration. This threshold concentration ranges from one α-tocopherol molecule per 500 to 1000 membrane lipid molecules, depending on the content and saturation character of the lipids (Kornbrust and Mavis 1980). Maximal antioxidant activity in the membrane is ensured because α-tocopherol molecules are distributed homogeneously rather than in clusters (Serbinova et al. 1991).

Although ascorbic acid is soluble in the aqueous portion of the cell, it may actually exert its antioxidant activity at the lipid bilayer margin by re-reducing the α-tocopheroxy radical to α-tocopherol (Liebler et al. 1986, Geetha et al. 1989). In fact, *in vitro* studies show that α-tocopheroxy radicals do not appear until much of the available ascorbic acid is oxidized (Kagan et al. 1990). Glutathione may re-reduce oxidized ascorbic acid (Frei et al. 1989), or vice versa (Koul et al. 1989), or glutathione may interact directly with α-tocopherol to re-reduce that molecule (Leedle and Aust 1990). Conclusions from these studies are based largely on sparing of one molecule in the presence of the other

antioxidant molecule. Therefore, it is just as likely that sparing of α-tocopherol by glutathione, for example, occurs because they both consume similar substrates (i.e., lipid radicals by α-tocopherol and lipid hydroperoxides by glutathione in the GPx enzyme system) rather than because one molecule specifically re-reduces the other (Liebler 1993). While much work remains to be done regarding the interactive effects of glutathione, ascorbic acid and α-tocopherol, it is clear that all are important factors conferring cellular protection against oxidant damage.

Metallothionein is a heat-stable, low molecular weight protein which is induced in fish exposed to the heavy metals Cd, Zn, Cu, Co, Ni, Mn, Cr and Fe (Roesijadi 1992). The protein contains 61 amino acid residues, of which approximately 20 are cysteine (Kägi and Schäffer 1988). It has been well established that metallothionein is important for sequestering selected heavy metals away from potential sites of cellular toxic action and for absorption, storage and regulation of essential trace metals (Bremner and Beattie 1990). Recently, metallothionein has also been identified as a potentially active cellular antioxidant (Bremner 1987). Metallothionein appears to reduce oxidative stress by quenching hydroxyl and superoxide radicals and possibly by binding to organic radicals; however, the specific reactions have yet to be clarified (Sato and Bremner 1993). In a review of the experimental evidence supporting metallothionein as a

radical scavenger, Sato and Bremner (1993) note several suggestions for the protective antioxidant reactions of metallothionein. These include reduction of oxyradicals by metallothionein's cysteine residues, enhanced delivery of Zn by metallothionein to prevent lipid peroxidation at affected membrane sites, and chelation of Fe by metallothionein to reduce the formation of oxyradicals in Fenton type reactions.

The pineal and retinal hormone melatonin has recently been shown to possess antioxidant activity (Halliwell et al. 1988), in addition to its function as a messenger for conveying photoperiodic information (Vivien-Roels and Pévet 1993). Protection against oxidative damage is generally attributed to melatonin's ability to scavenge the hydroxyl radical, although other reports indicate that peroxyl radicals may also be stabilized by melatonin (Reiter et al. 1994). As a lipophilic molecule, melatonin can augment vitamin E protection of lipids in most cellular compartments (Melchiorri et al. 1995). Melatonin may actually be twice as potent as vitamin E for scavenging peroxyl radicals, and more effective than glutathione for quenching hydroxyl radicals (Pieri et al. 1994).

While the antioxidants discussed to this point are among the most active and important for protecting cells from oxidative damage, there is a growing list of additional molecules identified as antioxidants. The cumulative activity of all antioxidant molecules is normally sufficient to protect

cellular components from oxidative damage arising during aerobic metabolism. Oxidative stress arises only when prooxidant concentrations overcome antioxidant capacity (Babich et al. 1993). The ratio of pro-oxidants to antioxidants increases as an organism ages and after exposure to radiation, ischemic reperfusion, alcohol ingestion or exposure to certain xenobiotics (Winston and Di Giulio 1991). In particular, organochlorine xenobiotics that induce Phase I and Phase II enzymes have received increasing attention for their oxidative stress generation in mammals. Recently, this process has been investigated in fish (Lemaire et al. 1994, Peters et al. 1994, Otto et al. 1994, Livingstone et al. 1993, Rodriguez-Ariza et al. 1993, Thomas and Wofford 1993, Mather-Mihaich and Di Giulio 1991).

Phase I enzyme activity relies on the transfer of electrons in membrane bound transport chains to convert substrates (Guengerich 1990). Because of this mechanism, Phase I enzyme activity can promote oxidative stress by increasing the production of free-radical species that are derived from molecular oxygen (Lehtinen 1990). Phase I biotransformation of some substrates, including the reproductive hormone estrogen, can create metabolites that cyclically remove hydrogens atoms from cellular molecules and surrender them to other weak oxidants present in the cell (i.e., redox cycling) (Liehr and Roy 1990). Redox cycling by transformed metabolites further increases oxyradical production and oxidative stress. Other

metabolites of exogenous contaminant molecules, including PCBs, can mobilize iron that in turn increases oxyradical proliferation in Fenton type reactions (Smith and De Matteis 1990). Thus, organisms exposed to compounds that induce Phase I biotransformation enzymes have greater transformation of xenobiotics and endogenous molecules, and greater oxidative stress (Lehtinen 1990). This process was confirmed in mammalian systems (Al-Bayati et al. 1987, Shara and Stohs 1987). In fact oxidative stress was, in one study of rats, shown to be dependent on activation of the Ah receptor complex (Mohammadpour et al. 1988). This toxic mechanism has not been evaluated in fish or, specifically, in lake trout exposed to contaminants present in the Great Lakes.

Examination of the role of oxidative stress in adult freshwater fish, including lake trout, exposed to organic contaminants that induce Phase I and Phase II enzyme activity is the central focus of this thesis. Additionally, since biotransformation enzyme activity and lesions indicative of oxidative stress have been simultaneously identified in lake trout fry, and since fry represent the most sensitive cohort to contaminants (Metcalfe et al. 1988, Walker et al. 1991), the role of oxidative stress in fry mortality and recruitment failure of lake trout from the Great Lakes is also examined. In addition to the general introduction (Chapter 1) and general conclusions (Chapter 7) chapters, the thesis consists of five chapters describing experimental studies.

Additionally, there is an appended methodological section, describing a sensitive HPLC method which was developed to facilitate quantification of retinoids and tocopherol in fish tissues. Each of the five experimental chapters are written in manuscript style for submission to primary scientific journals.

In the second chapter, a laboratory experiment designed to test the hypothesis that "lake trout exposed to an organochlorine contaminant that induces Phase I enzyme activity will have increased hepatic oxidative stress marked by altered antioxidant concentrations and elevated concentrations of membrane breakdown by-products" is described. To test this hypothesis, juvenile lake trout were intraperitoneally injected with a single environmentally relevant dose of coplanar 3,3,4,4,5-pentachlorobiphenyl (PCB congener 126). Oxidative stress, quantified by changes to antioxidant concentrations and increases in membrane breakdown, was monitored in 3 dose groups and a control group for up to 30 weeks following exposure. Results from this experiment support the hypothesis by showing a strong correlation between hepatic MFO activity and oxidative stress. Depletions of tococopherol and retinol antioxidant concentrations in livers of the PCB exposed and oxidatively stressed lake trout suggest that these vitamins are important molecules for protecting against oxidative degradation of membranes.

After a link between MFO enzyme activity and oxidative stress was established in the second chapter, the objective of the third chapter was to examine this relationship in a field setting. The existing gradient of lowest organic contamination in Lake Superior to highest in the lower Great Lakes, with a corresponding gradient of MFO activity in lake trout (Luxon et al. 1987), offered an excellent opportunity to study this relationship. Phase I and Phase II enzyme activities and indicators of oxidative stress were, therefore, assessed in lake trout populations from Lake Ontario and Lake Superior over three years. The third chapter addressed the following hypothesis: "Lake trout from Lake Ontario will have higher Phase I and Phase II enzyme activities and greater indices of oxidative stress than lake trout from Lake Superior, because they are exposed to higher concentrations of organochlorine contaminants."

Earlier studies showed dramatic differences in Phase I enzyme activity between lake trout from Lake Ontario and Lake Superior (Luxon et al. 1987). However, declining concentrations of organochlorine contaminants since that study (Whittle et al. 1992) may explain why only marginally higher Phase I enzyme activity in lake trout from Lake Ontario and similar Phase II activities between the two populations were found in the studies of Chapter 3. Although Phase I and Phase II activities were comparable to lake trout from Lake Superior, lake trout from Lake Ontario had significantly

higher oxidative membrane breakdown. Oxidation resulting from exposure to inorganic contaminants, or lower concentrations of antioxidants in lake trout from Lake Ontario that were not considered in this study, could all be involved in producing the higher membrane breakdown in lake trout from Lake Ontario (Winston and Di Giulio 1991). Regardless of the source of increased oxidative stress, a consideration of the antioxidants in lake trout from the Great Lakes revealed that tocopherol concentrations and the ratio of didehydroretinol to retinol were both promising factors for determining the amount of oxidative membrane breakdown. This was similar to the laboratory results from Chapter 2.

Continued recruitment failure of a number of salmonid species in the most contaminated Great Lakes has been attributed, at least in part, to early mortality syndrome (EMS) (Mac and Gilbertson 1990). Loss of equilibrium, a corkscrew swimming pattern and death of the affected fry at the swimup stage are the most readily identified characteristics of EMS. However, edema resulting from membrane damage and increased permeability (Walker and Peterson 1990) also suggest that oxidative stress may be related to EMS.

All of the EMS features noted above have been reproduced in the laboratory by injecting lake trout eggs with organochlorine contaminants representative of those found in the Great Lakes (Walker and Peterson 1991, Walker and Peterson 1990). Additional evidence for organochlorine involvement in

EMS includes the death of affected fry immediately after the yolk sac has been resorbed, when the body burden of contaminants is highest, and more prevalent EMS in the fry of female lake trout from Lake Ontario than in the upper Great Lakes (Mac 1990). The associations noted among organochlorine contaminant exposure, conditions resembling oxidative stress in the fry from Lake Ontario and greater EMS in offspring of lake trout from Lake Ontario, gave rise to the working hypothesis of chapter 4: "EMS is related to increased oxidative stress in adult female lake trout from Lake Ontario and their offspring." To test the hypothesis, female lake trout captured from Lake Ontario, and from a less contaminated reference site in Lake Manitou, were assessed for Phase I and Phase II enzyme activities and indices of oxidative stress. Liver concentrations of enzymatic and non-enzymatic antioxidants as well as lipid hydroperoxides, an indicator of oxidation in cellular and subcellular membranes (Ohishi et al. 1985), were included in this evaluation of the adult fish. In addition, eggs stripped from each corresponding female were fertilized, reared separately in the laboratory, and monitored throughout their development to the swimup stage for survival, concentrations of non-enzymatic antioxidant vitamins, cyp1A protein content (a measure related to Phase I enzymatic activity) and the appearance of EMS. Correlations between the hepatic concentrations of lipid hydroperoxides and EMS were evident in the lake trout from Lake Ontario whose offspring

exhibit >50% EMS. While there were other alterations in antioxidant defenses in adults which suggested that oxidative stress may be related to EMS, Phase I activity and antioxidant vitamin concentrations in the embryos were similar between EMS, non-EMS and reference populations of lake trout. However, since only the survivors within the EMS population could be analyzed, additional study is required to evaluate links between Phase I activity, oxidative stress and EMS. In particular, stages of embryonic development closer to the appearance of mortality should be considered.

Vitamin A compounds (retinoids) proved to be among the most sensitive biochemical parameters in organochlorine-exposed fish and may be important indicators of oxidative stress in fish from both the laboratory and field experiments. While retinol may be depleted because of increased utilization as an antioxidant following organochlorine exposure (Ribera et al. 1991), other mechanisms have also been proposed to explain the depletion of vitamin A. For example, there is evidence to suggest that vitamin A may be directly metabolized by Phase I and Phase II enzymes (Gilbert et al. 1995). Organochlorine metabolites produced by Phase I metabolism of these molecules may also disturb the binding of retinol to its carrier protein in plasma resulting in loss of the relatively small retinol molecule through the glomerular membrane of the kidney (Brouwer and van den Berg 1986). Exposure to compounds that induce Phase I and Phase II enzymes has also been correlated

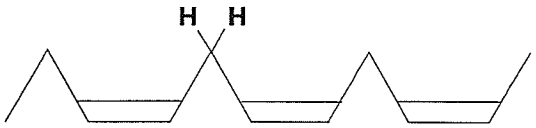
with declining vitamin A intake due to loss of appetite (Spear et al. 1994).

To investigate the mechanism of retinoid depletion and the significance of differing forms of retinoids in determining oxidative stress, radiolabelled ^{3}H-retinol was injected directly into the circulation of lake trout that had been pre-exposed to PCB 126. Phase I and Phase II enzyme activity, plasma and bile radioactivity recovery profiles as well as tissue distributions of ^{3}H-retinol and its metabolites were examined for each PCB dose group in order to test the following working hypothesis of Chapter 5: "retinol is depleted in fish exposed to organochlorines due to its increased utilization as an antioxidant." However, the hypothesis was not supported by results from this experiment, which indicated that direct metabolism of retinol by Phase I and Phase II enzymes is likely responsible for retinol depletion in fish exposed to PCBs.

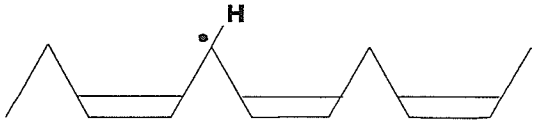
After oxidative stress, reproductive failure, antioxidant dynamics and the metabolism of retinol as an antioxidant in lake trout exposed to organochlorines were examined, confirming the applicability of these results to other species of fish was of interest. In particular, benthic species of the Great Lakes would be expected to be most heavily impacted because of their closer association with organochlorine contaminated sediments. Lake sturgeon (*Acipenser fulvescens*) are benthic fish whose populations have declined steadily in

the Great Lakes and European freshwaters for at least 100 years (Birstein 1993). Sturgeon have high lipid content and a long age to sexual maturity contributing to their great potential for accumulating lipophilic contaminants (Rousseaux et al. 1995). Interestingly, previous studies have also shown that sturgeon captured from sites contaminated with organochlorines have lower hepatic retinoid concentrations than sturgeon from less contaminated sites (Ndayibagira et al. 1995). In Chapter 6 the hypothesis that "sturgeon exposed to an organochlorine will have induced Phase I and Phase II enzymes and greater indices of oxidative stress, similar to the results described from earlier experiments with lake trout" was tested. Juvenile sturgeon were exposed in the laboratory to low concentrations of 2,3,7,8-tetrachlorodibenzofuran (TCDF), relative to those found in tissue of fish from the Great Lakes (Safe 1990). Relationships between organochlorine exposure, Phase I and Phase II enzyme activity and oxidative stress were examined in the sturgeon tissues 10 and 27 days after exposure. Results showed that the breakdown of lipid membranes in liver tissue increased while hepatic tocopherol concentrations declined, indicating that TCDF treated sturgeon were subject to greater oxidative stress than sturgeon from the control group. The hypothesis was, therefore, supported, demonstrating that results obtained for lake trout in Chapters 2 and 3 may be generally applicable to other species of freshwater fish.

Figure 1.1: Lipid peroxidation (after Gutteridge and Halliwell 1990).

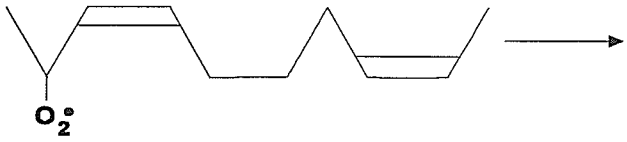


Removal of a hydrogen atom initiates lipid peroxidation



Creates a lipid radical.
- oxidize proteins
- combine with molecular oxygen

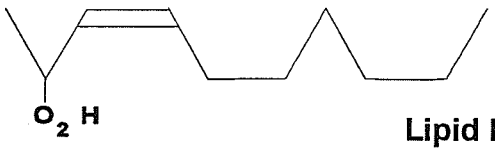
O_2



Reaction of two lipid peroxyl radicals
- singlet oxygen

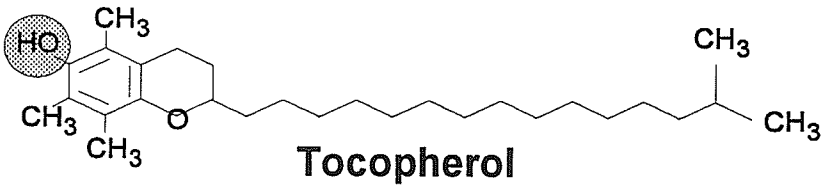
O_2

Lipid peroxyl radicals are unstable.
- oxidize adjacent PUFA.
  1) reinitiates oxidation
  2) produces lipid hydroperoxide

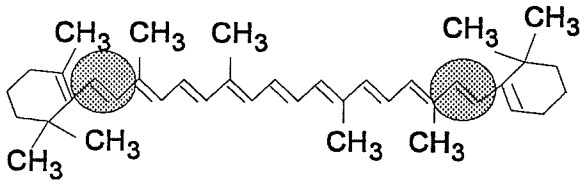


Lipid Hydroperoxide

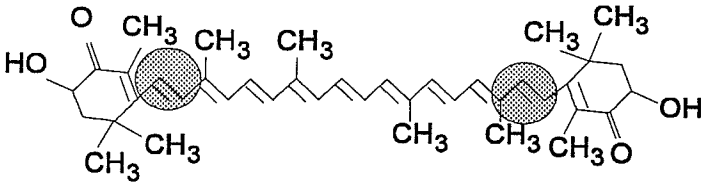
Figure 1.2: Structures of lipid soluble antioxidants.



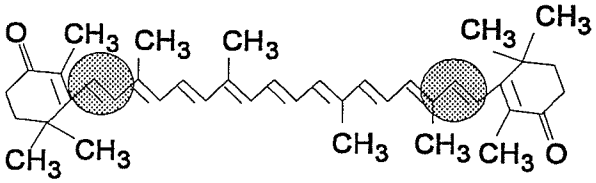
Tocopherol



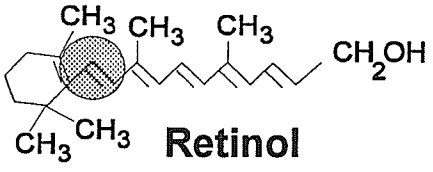
β-Carotene



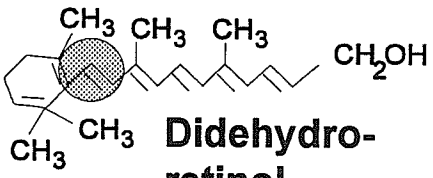
Asta-xanthin



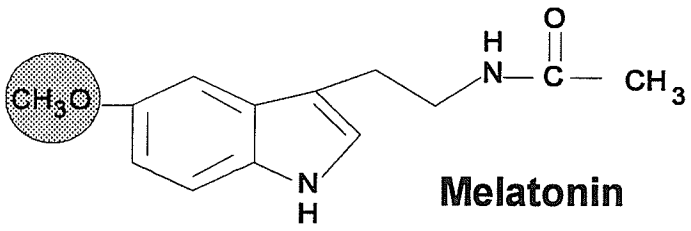
Cantha-xanthin



Retinol



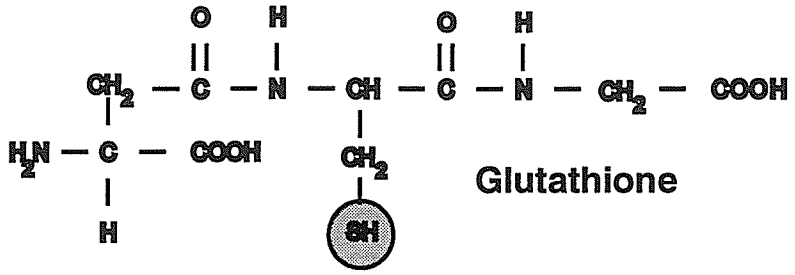
Didehydro-retinol



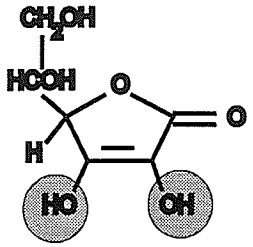
Melatonin

Vitamin A Compounds

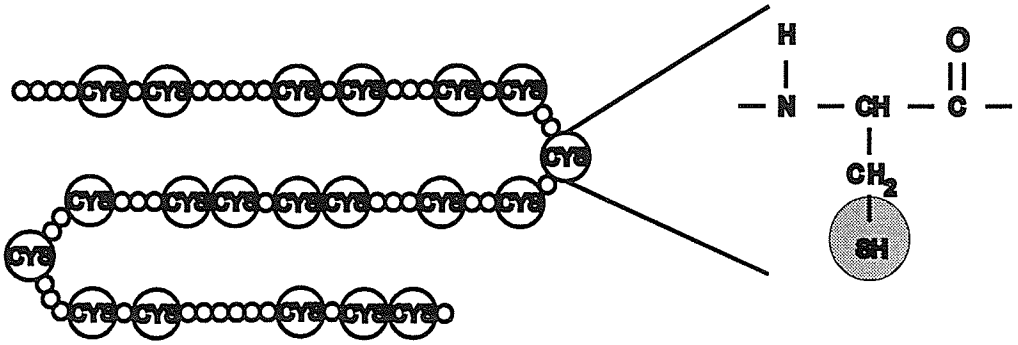
Figure 1.3: Structures of water soluble antioxidants.



Glutathione



Ascorbic Acid



Metallothionein

Chapter 2

Oxidative Stress in Lake Trout (*Salvelinus namaycush*) Exposed to 3,3',4,4',5-Pentachlorobiphenyl (PCB 126) Doses that Induce Mixed Function Oxidase Enzyme Activity.

Abstract

Juvenile lake trout were intraperitoneally injected with corn oil containing nominal concentrations of 0, 0.6, 6.3 or 25 µg [^{14}C]-3,3',4,4',5-pentachlorobiphenyl (PCB 126) per gm of body weight. PCB accumulated in the liver in a dose-dependent manner reaching a maximum concentration by 6 weeks and remained elevated for the 30 week experimental period. Mixed function oxidase enzyme activity, measured as ethoxyresorufin-O-deethylase (EROD), was elevated in the two highest dose groups over that of the control group but not in the low dose group throughout the 30 weeks. Oxidative stress, measured by the thiobarbituric acid-reactive substances test (TBARS), was also elevated in liver of the two highest PCB dose groups but not the low dose group. The activities of the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were not affected by PCB 126 exposure. The nonenzymatic antioxidant tocopherol was depleted to approximately 75% of the control concentration in liver of all three PCB-dosed groups. Ascorbic acid concentration in liver was not different in any of the treatment groups. Retinol was depleted by an order of magnitude in liver of the two highest dose groups but not in the low dose group. Further study is required to confirm the antioxidant activity of retinol.

Introduction

Non-ortho substituted polychlorinated biphenyls (nPCBs) are of ecotoxicological interest because of their persistence and involvement in reproductive effects, weight loss and immune system disturbances (Safe 1990). Previous work has established that fish from the Great Lakes are contaminated with PCBs in concentrations as high as 20 ppm (Mac et al. 1993, Miller et al. 1993). A group of enzymes embedded in the smooth endoplasmic reticulum known as mixed function oxidase (MFO) enzymes have shown utility as indicators of exposure to PCBs (Dillon et al. 1990). PCB 126 (3,3',4,4',5-pentachlorobiphenyl) is one of the most toxic PCB congeners in the Great Lakes (Hong et al. 1992), and has been identified as an environmental concern based on its toxicity, frequency of occurrence and abundance (McFarland and Clarke 1989). PCB 126 is also a potent inducer of MFO enzymes (Safe et al. 1987).

Although MFO enzymes are sensitive indicators of exposure to a variety of organic contaminants, there is little information directly linking their induction to specific toxic mechanisms within the physiology of cells. Recent evidence has correlated the induction of MFO enzymes with proliferation of oxygen radicals and a rise in the membrane degradative process known as lipid peroxidation (Lehtinen 1990). Lipid peroxidation is a free-radical generated process by which polyunsaturated fatty acid lipids (PUFA), essential for

maintaining membrane fluidity, can be degraded in both cellular and sub-cellular membranes. The cellular Ah receptor can bind organic contaminants that are approximate stereoisomers of 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD), including nPCBs (Mantyla and Ahotupa 1993). Binding of the organic contaminant begins a series of cellular events resulting in induction of MFO activity that can then result in the proliferation of radicals that initiate lipid peroxidation (Mohammadpour et al. 1988). If unchecked, lipid peroxidation can result in erythrocyte lysis, membrane-bound enzyme inhibition, altered ionic balance and can create potentially mutagenic and carcinogenic by-products (Horton and Fairhurst 1987).

Cellular defense mechanisms against damaging free radical generation and lipid peroxidation include both enzymatic and non-enzymatic antioxidants. Enzymes important in the detoxification of oxygen radicals involved in lipid peroxidation include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Winston and DiGiulio 1991). Previous studies have shown that antioxidant enzyme activity can be altered with exposure to organic pollutants that induce MFO enzymes (Pohjanvirta et al. 1990). Antioxidant vitamins, including tocopherol (Vitamin E), ascorbic acid (Vitamin C) and retinol (Vitamin A), can also be consumed while detoxifying oxyradicals that propagate lipid peroxidation (Ribera et al. 1991).

To gain an understanding of the linkages among nPCB exposure, MFO induction and oxidative stress, lake trout were exposed to environmentally relevant doses of the nPCB congener 126. MFO activity, oxidative membrane breakdown, and enzymatic and vitamin antioxidant tissue stores were monitored for 30 weeks after exposure.

Materials and Methods

Fish Holding

Juvenile lake trout (*Salvelinus namaycush*) (391g ± 7) were acclimated in 130 L fibreglass tanks for 4 weeks while being fed Martin Feeds trout food at a ration of 1% of bodyweight every second day. Each tank received at least 2 L of aerated water (11.5 to 13.5°C) per gram of fish per day with a photoperiod of 12 h light and 12 h darkness.

Chemicals

Uniformly ring-labelled [^{14}C]-3,3',4,4',5-pentachlorobiphenyl (IUPAC PCB congener 126, specific activity 8.7 X 10^{11} Bq mol^{-1}) was obtained from Sigma Chem. Co (St. Louis, MO.). Unlabelled PCB-126 was purchased from Ultra Scientific. Reverse-phase HPLC with radiochemical detection and capillary GC analysis with electron-capture detection showed a purity in terms of ^{14}C and for the product as a whole of >99%. Injection solutions were prepared by evaporating a hexane solution of PCB-126 to near dryness under N_2 and then

suspending it in corn oil.

Dosing of Fish

After acclimation, fish were lightly anesthetized in water containing tricaine methanesulfonate (MS222) (0.38 mmol L^{-1}) neutralized to tank pH with ammonium hydroxide and NaCl (150 mmol L^{-1}) approximately isosmotic with fish plasma. Length and weight were recorded for each fish and visual implant tags (Northwest Marine Technology) were inserted. Fish (n=30) were then randomly dosed with corn oil injected intraperitoneally at a dose of 0.6, 6.3 or 25 ng gm^{-1} body weight. One group of fish, serving as controls, was injected with corn oil containing no PCB. Fish recovered from the procedure in anesthetic-free water within 3 minutes. Both control and treated fish were housed together in each tank. Secondary uptake of excreted PCB was limited by removing feces daily and by continuous filtration of the holding water with activated charcoal.

Sampling

After 1,3,6,13,20 and 30 weeks post-injection, 5 fish from each dose group were anesthetized with pH-neutralized MS222 as described earlier. After equilibrium was lost, fish were removed form the anesthetic solution and their tissues were dissected. Liver tissue to be analyzed for MFO enzyme activity was cooled on ice before immediate preparation of

microsomes. Other tissues were placed in sterile plastic bags and frozen between slabs of dry ice before being transferred to a -120 ± 10°C freezer for storage before analysis (Palace et al. 1990).

Analyses

MFO, measured as ethoxyresorufin-O-deethylase (EROD) activity, and nPCB 126 concentrations were determined as previously described (Muir et al. 1990). Briefly, liver (0.5 to 1 gm) was homogenized in 4 volumes of cold HEPES-KCl buffer pH=7.8 (0.02M HEPES, 0.15M KCl) using 5 to 7 passes of a motor-driven Teflon pestle. Centrifugation of the homogenate at 2°C for 20 minutes at 12,000 X g yielded a supernatant which was recovered and recentrifuged at 2°C for 75 minutes at 105,000 X g. The microsomal pellet from the last centrifugation was recovered and resuspended in 2 ml of 0.05 M Tris-HCl resuspension buffer with 1mM dithiothreitol, 1mM ethylenediamine tetracetic acid (EDTA) and 20% glycerol (v/v). Resuspended microsomes were frozen and stored in liquid nitrogen until analysis. Aliquots of these microsomes were slow-thawed on ice and used in the EROD assay which fluorometrically follows the disappearance of ethoxyresorufin as it is converted to resorufin. Protein content of the resuspended microsomes was determined by the method of Peterson (1977) using bovine serum albumin as a standard. nPCB 126 concentrations were quantified based on extractable

radioactivity which was determined for each sample by homogenizing freeze-dried liver in toluene and assaying portions of this extract by liquid scintillation counting.

SOD, CAT and GPx were assayed by the methods of Sun and Zigman (1978), Rabie et al. (1972) and Tappel (1978) respectively. Ascorbic acid was determined by the colorimetric method of Jagota and Dani (1982). Tocopherol and retinol were quantified by the HPLC method of Palace and Brown (1994). Because tocopherol may vary in its spatial distribution in tissues (Staats and Colby 1989), liver tissue from the same vicinity was analyzed in each fish and values are expressed relative to the control group, weighted for lipid content. Thiobarbituric reactive substances (TBARS) was measured by Uchiyama and Mihara's (1978) method using malonaldehyde as a standard.

All data were analyzed using ANOVA with statistical significance set at $P<0.05$. Statistical differences were verified using Duncan's multiple-range and Tukey's studentized range tests.

Results and Discussion

Doses of PCB 126 used in this experiment are environmentally relevant. Fish from sites in the Great Lakes and Baltic regions are known to contain concentrations of PCB 126 in the ng gm^{-1} range (Smith et al. 1990, Falandysz et al. 1994). Niimi and Oliver (1989) reported PCB 126 concentrations

of 7 to 19 ng gm^{-1} in muscle and whole lake trout from Lake Ontario. Janz et al. (1992) correlated muscle and liver PCB 126 concentrations in an approximately 1 to 1 ratio in lake trout from Lake Ontario. They found lake trout liver to contain below 1 ng gm^{-1} of PCB 126 but noted that PCB 126 content can vary widely between sample sites in Lake Ontario as has been noted for PCB content in other Great Lakes salmonids (Madenjian et al. 1994).

PCB 126 accumulated in liver in a dose dependent manner (Fig. 2.1). Maximum concentrations were attained in the liver after 1 week in the two lowest dose groups and after 6 weeks in the highest dose group. The liver concentration of PCB 126 did not change significantly between 6 and 30 weeks in any of the treatments, confirming previous reports of slow elimination of pentachlorobiphenyls in salmonids (Niimi and Oliver 1989). Additionally, radioactive counts in control fish held in the same tanks as dosed fish indicated no secondary uptake of PCB 126 excreted by dosed fish (data not shown), further supporting the absence of significant metabolism and excretion in dosed fish during the period of this exposure.

The hepatic cytochrome P4501A enzyme system catalyzes the introduction of an oxygen atom into lipophilic substrates during the first phase of xenobiotic metabolism (Andersson and Forlin 1992). This system is induced by exposure to certain contaminants, including PCB 126, that interact with the cellular Ah receptor (Boon et al. 1992). Induction of P4501A

proteins is conventionally measured in liver and other tissues by mixed function oxidase (MFO) enzyme assays. In fish, the most common of these measures is the de-ethylation of the artificial substrate 7-ethoxyresorufin by the enzyme known as ethoxyresorufin-o-deethylase (EROD) (Munkittrick et al. 1993).

Both the 6.3 and 25 ng gm^{-1} dose groups had significantly higher hepatic EROD enzyme activity than the control group throughout the experiment (Fig. 2.2). Maximal induction in the two highest PCB dose groups was attained by 6 weeks and maintained to 30 weeks and, except for the 30 week sample period, activity was not significantly different between these dose groups. Janz and Metcalfe (1991) estimated the 50% effective dose (ED50) for PCB 126 to be 329 ng gm^{-1} in rainbow trout (*Oncorhynchus mykiss*) and a "no adverse effects level" (NOAEL) of 0.47 ng gm^{-1} has been established for P4501A induction in rats exposed to PCB 126 (Van Birgelen et al. 1994). Not surprisingly then, hepatic EROD activity was not consistently induced in the 0.6 ng gm^{-1} dose group compared to the controls during our experiment.

Although EROD has become a popular biomonitoring tool for evaluating exposure to organic contaminants in fish, its relevance as an actual toxicological mechanism is not clear. Evidence linking induced biotransformation of Ah-active substrates and oxidative stress, however, has accumulated in the last several years (Thomas and Wofford 1993, Mantyla and Ahotupa 1993). Oxidative stress can be quantified by measuring

membrane breakdown products in the thiobarbituric reactive substances (TBARS) assay, also known as the malonaldehyde (MDA) test (Kappus 1986). The *in vivo* relevance of this test has been questioned because of its non-specificity and amplification of membrane peroxidation, but it remains a popular method. Liver tissue is heated in an acidic incubation that allows peroxidation in the lipid fraction to be amplified so that oxidative stress potential in membranes can be examined (Gutteridge and Halliwell 1990).

Similar to the EROD activity data, the two highest dose groups in this experiment had significantly elevated liver TBARS measures that reached maximum concentrations after 6 weeks and remained elevated to 30 weeks (Fig. 2.3). Again, the 0.6 ng gm^{-1} group did not differ from the control group in its TBARS content throughout the experiment. Concentrations of TBARS in liver from dosed fish in this experiment are similar to those earlier reported from liver of carp exposed to the herbicide paraquat, which also induces oxidative stress (Matkovics et al. 1984). Our results indicate greater oxidative stress in EROD-induced lake trout, supporting similar previous work in fish and mammals exposed to contaminants that interact with the Ah receptor (Thomas and Wofford 1993, Stohs et al. 1990).

Although oxidative stress can be initiated by MFO biotransformation of PCBs (Winston and Di Giulio 1991), results presented in Figure 1 indicate that PCB 126 tissue

concentrations did not significantly decline during the study period. Elevated MFO activity may increasingly metabolize other endogenous substrates, however, such as steroid hormones, forming reactive intermediates and resulting in greater oxidative stress and membrane breakdown measured by TBARS (Liehr and Roy 1990).

In addition to increased TBARS concentrations, oxidative stress can affect the enzymatic activities or concentrations of protective antioxidants in organisms exposed to PCBs. For example, the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Rodriguez-Ariza et al. 1993) as well as the non-enzymatic antioxidant vitamins C (ascorbic acid), E (tocopherol) and A (retinol) (Mantyla and Ahotupa 1993, Ribera et al. 1991) have been shown to be sensitive indicators of increased oxidative stress. In this experiment, however, there were no significant differences in SOD, CAT or GPx activities in the liver of lake trout for any of the treatments (Fig. 2.4). In other studies, Roberts et al. (1987) found greater SOD activity in liver of spot (*Leiostomus xanthurus*) from a PAH-contaminated site compared to a less contaminated site. Similarly, CAT activity increased in channel catfish (*Ictalurus punctatus*) exposed to bleached kraft mill effluent (Mather-Mihaich and Di Giulio 1991) and SOD, GPx and CAT were all elevated in liver of grey mullet (*Mugil sp.*) from contaminated sites (Rodriguez-Ariza et al. 1993). Variable responses in the activity of all three

enzymes have been shown in carp (*Hypothalmichthys molitrix*) depending on the level of exposure to the herbicide paraquat (Matkovics et al. 1984). Additional results regarding the activities of these three enzymes in fish exposed to contaminants have been characterized as inconclusive (Winston and Di Giulio 1991). Data from this experiment indicate that these enzymes may not represent sensitive indicators of oxidative stress resulting from PCB 126 exposure in lake trout.

Ascorbic acid and tocopherol act as antioxidants to quench oxidative radicals produced in the aqueous and lipid compartments of the cell respectively (Koul et al. 1989, Packer 1991). It is known that pollutant stress may result in depletion of one or both of these nonenzymatic defenses (Neff 1985). Ascorbic acid may transfer aqueous soluble reducing power from substrates such as glutathione to re-reduce tocopherol from the tocopheroxy radical produced during the quenching action of tocopherol in the lipid fraction of the cell (Geetha et al. 1989, Stocker and Peterhans 1989). Thomas (1987) showed declines of liver concentrations of ascorbic acid in striped mullet (*Mugil cephalus*) exposed to fuel oil. Depletion of ascorbic acid has been attributed to increased use of ascorbic acid as an antioxidant (Ribera et al. 1991). Because of the importance of ascorbic acid in collagen synthesis and wound healing, increased use of the vitamin for detoxification could lead to deficiency for other metabolic

processes and ultimately result in decreased disease resistance or skeletal deformations (Lehtinen 1990). On the other hand, ascorbic acid concentrations in liver of fish have been reported to increase in response to bleached kraft mill effluent exposure (Andersson et al. 1988). These authors postulated that increasing ascorbic acid concentrations in liver of perch (*Perca fluviatilis*) may be indicative of increased ascorbic acid synthesis and general metabolic dysfunction. However, subsequent work has shown that some teleost fish, including lake trout, lack gulonolactone-oxidase, the terminal enzyme in the ascorbic acid synthetic pathway (Dabrowski 1990). Our results indicate that no consistent significant differences in ascorbic acid in liver were seen for any of the treatments (Fig. 2.5). Ascorbic acid content in liver of Atlantic croaker exposed to a mixture containing PCB 126 (Thomas and Wofford 1993) and English sole (*Parophrys vetulus*) exposed to CCl_4, which induces oxidative stress (Casillas and Ames 1986), also exhibited no reduction of hepatic ascorbic acid.

Tocopherol is recognized as the most important cellular compound preventing oxidative damage (Serbinova et al. 1991). Whereas the cellular role of ascorbic acid is multidimensional, the only function currently attributed to tocopherol is that of an antioxidant (Packer 1991). Tocopherol is especially important in fish because they contain high concentrations of polyunsaturated fatty acid (PUFA) lipids.

PUFA are susceptible to the oxidative radical process known as lipid peroxidation in which PUFA lipids are degraded. Unchecked by the antioxidant action of tocopherol this process could compromise membrane integrity and cellular function (Singh et al. 1992). Tocopherol supplementation reduced the mutagenicity resulting from exposure to the classic P4501A inducer benzo(a)pyrene (BaP) in rats, mussels and fish (Michel et al. 1993) and depletion of tocopherol in liver of trout was correlated with greater susceptibility to BaP toxicity (Williams et al. 1992).

Tocopherol concentrations in liver of all three PCB-exposed groups were significantly lower than in the control group beginning at the 13 week sample period and extending to 30 weeks (Fig. 2.6). Maximal depletion was achieved by 20 weeks after PCB 126 injection in all groups but depletion did not differ between dose groups at any sample time. Tocopherol probably declined due to its increased utilization as an antioxidant to quench oxidative radicals generated in PCB-exposed fish (Palozza and Krinsky 1991). Similar tocopherol reductions have been documented in mussels exposed to oxidative stress (Ribera et al. 1991) and in mammals exposed to MFO-inducing organics (Mantyla and Ahotupa 1993).

Although induced P4501A enzymes can deplete tocopherol by increasing the production of oxidative radicals, this depletion can then negatively feed back resulting in lower P4501A activity. This effect is produced because of radical

scavenging by tocopherol in the microsomal membranes where the P4501A system is embedded. Any depletion of tocopherol in these membranes results in proliferation of oxyradicals and greater oxidative conversion of cytochrome P450s to their inactive P420 forms (Serbinova et al. 1991). Therefore, while the current experiment suggests a link between oxidative stress and MFO induction, there may actually be a balance between induced MFO activity and radical production and a decrease in MFO activity due to depletion of antioxidants.

Retinol and its pro-vitamin form β-carotene have a number of important functions and have long been recognized as having antioxidant activity (Wolf 1984). Retinol concentrations in liver of the two highest dose groups were significantly lower than both the control and low dose groups beginning at 13 weeks post-exposure and extending to the 30 week sample period (Fig. 7). The high dose group showed the greatest depletion of liver retinol, being more than an order of magnitude lower than the control group at the 30 week sample period. Depletion of retinol in liver of lake trout exposed to PCB 126 has previously been reported (Palace and Brown 1994). Similar results have been shown for mink (Hakansson et al. 1992), rats (Mantyla and Ahotupa 1993) and birds (Spear et al. 1989) exposed to PCBs.

While retinol may be depleted due to increased usage as an antioxidant during MFO induction (Ribera et al. 1991) there remain other possible mechanisms to explain retinol depletion

following PCB exposure. For example, retinol binding to the retinol-binding-protein, transthyretin complex, may be disturbed by PCB metabolites so that retinol is increasingly filtered through glomerular membranes (Brouwer and Van den Berg 1986). It has also been suggested that retinol may be directly catabolized by a specific P450 and conjugation enzyme system that is induced by exposure to PCBs (Vanden Bossche and Willemsens 1991, Zile 1992). This mechanism seems unlikely, however, given that Brouwer and van den Berg (1986) reported retinol depletion in mice exposed to PCB concentrations that did not induce MFO enzymes. Finally, liver depletion of retinol may reflect net movement of retinol from the liver to other compartments, such as the kidney, and may result from elevated esterifying enzyme activity in the kidney in an attempt to limit retinol loss (Jurek et al. 1990). Evidence for this mechanism is also lacking as Mercier et al. (1990) have shown lower rather than enhanced esterifying enzyme activity in rats treated with a PCB congener. Clearly, additional research is required to confirm the antioxidant activity of retinol and elucidate the mechanism of depletion in PCB-exposed fish.

Summary and Conclusions

Lake trout exposed to PCB 126 exhibit induced MFO activity in liver that is reflective of the uptake of the

contaminant into liver. Fish with induced MFO enzymes also exhibited greater indices of oxidative stress. These included elevated concentrations of membrane breakdown products measured as TBARS, and depleted tocopherol and retinol in liver. Activities of the antioxidant enzymes SOD, CAT and GPx and concentrations of the non-enzymatic antioxidant ascorbic acid do not appear to be sensitive indicators of exposure to PCB 126 in lake trout. Confirmation of retinol as an important antioxidant in fish is required. Research on PCB congeners that are more readily metabolized to free-radical intermediates will also produce additional insights on the role of oxidative stress in the toxicology of MFO-inducing contaminants.

Figure 2.1: PCB 126 in liver of lake trout dosed with 0.6 µg PCB gm^{-1}, 6.3 µg PCB gm^{-1} or 25 µg PCB gm^{-1} based on recovery of ^{14}C-radioactivity by scintillation counting. Means that are statistically different from the control group (not shown) are denoted with letters. Statistical differences between means of dose groups are denoted with different letters. Data are expressed (mean with standard error) as µg PCB 126 per gm of wet liver weight (n=5, α=0.05).

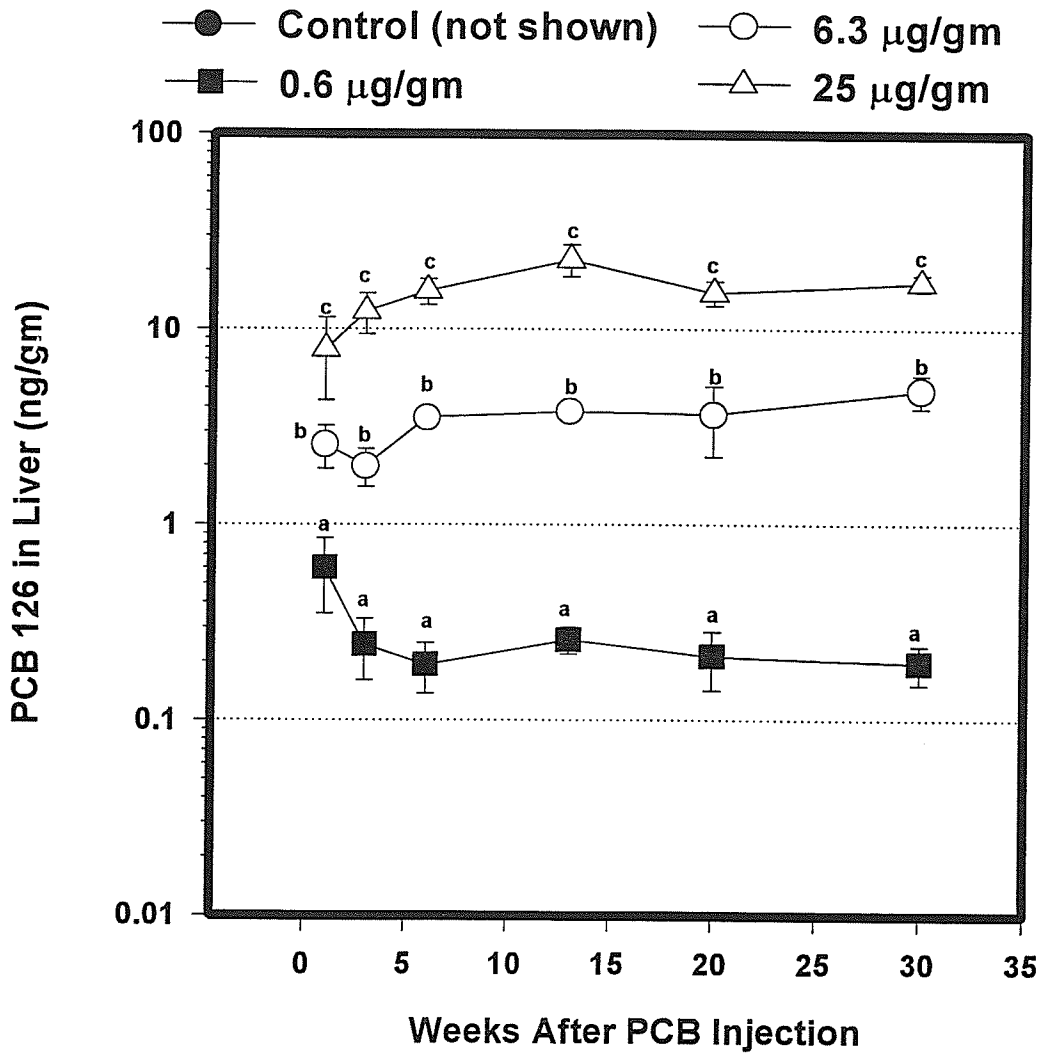

Control (not shown)
6.3 µg/gm
0.6 µg/gm
25 µg/gm
PCB 126 in Liver (ng/gm)
100
10
1
0.1
0.01
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.2: Ethoxyresorufin-O-deethylase (EROD) activity in liver of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as nmol of resorufin produced per mg of microsomal protein. Graphic and statistical notation are as in Fig. 2.1 (n=5, α=0.05).

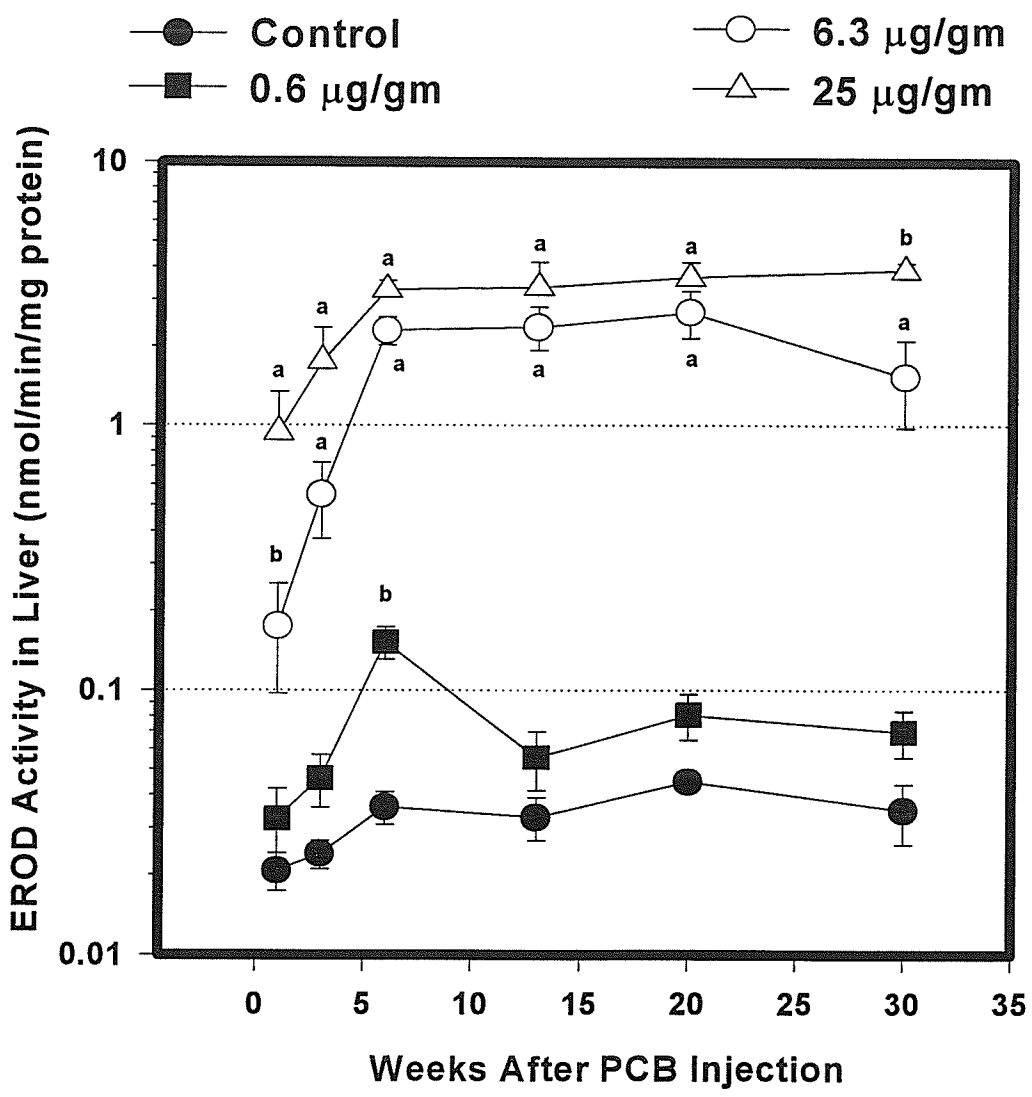

Control
6.3 µg/gm
0.6 µg/gm
25 µg/gm
EROD Activity in Liver (nmol/min/mg protein)
10
1
0.1
0.01
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.3: Thiobarbituric reactive substances (TBARS) in liver of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as nmol of TBARS produced per gm of wet liver weight. Graphic and statistical notation are as in Fig. 2.1 (n=5, α=0.05).

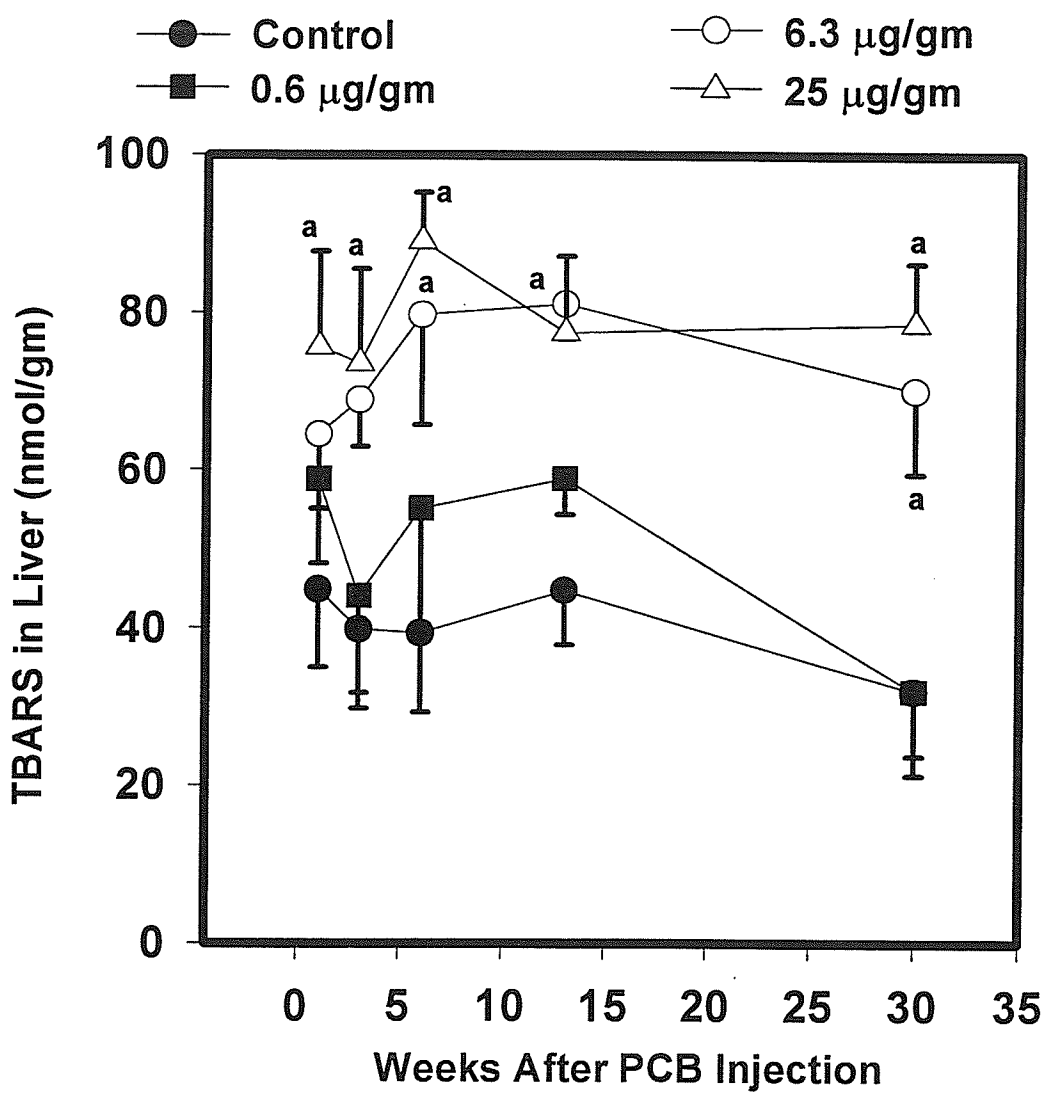

Control
0.6 µg/gm
6.3 µg/gm
25 µg/gm
TBARS in Liver (nmol/gm)
100
80
60
40
20
0
a
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.4: Superoxide dismutase (panel A), catalase (panel B) and glutathione peroxidase (panel C) enzymatic activity in liver of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as units of activity per gm of wet liver weight. One unit of SOD inhibits autoxidation of epinephrine by 50% at pH=7.0, 25°C. One unit of catalase decomposes 1.0 µmol of H_2O_2 per minute at pH=7.0, 25°C. One unit of GPx catalyzes the oxidation of 1.0 µmol of reduced glutathione by H_2O_2 to oxidized glutathione per minute at pH=7.0, 25°C. Graphic and statistical notation are as in Fig. 2.1 (n=5, α0.05).

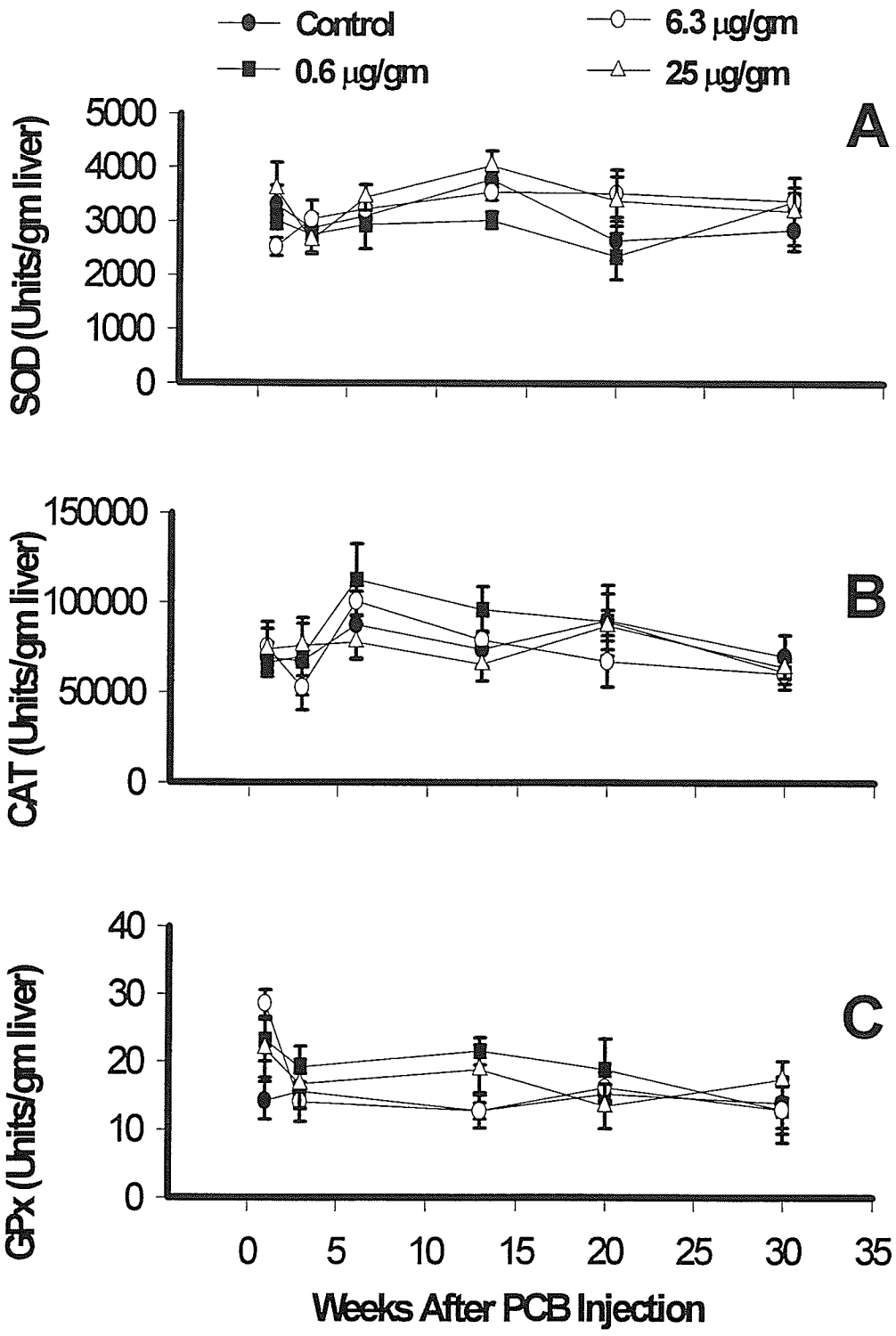

Control
6.3 µg/gm
0.6 µg/gm
25 µg/gm
A
SOD (Units/gm liver)
5000
4000
3000
2000
1000
0
B
CAT (Units/gm liver)
150000
100000
50000
0
C
GPx (Units/gm liver)
40
30
20
10
0
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.5: Ascorbic acid concentrations in livers of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as µg ascorbic acid per gm of wet liver weight. Graphic and statistical notation are as in Fig. 2.1 ($n=5$, $\alpha=0.05$).

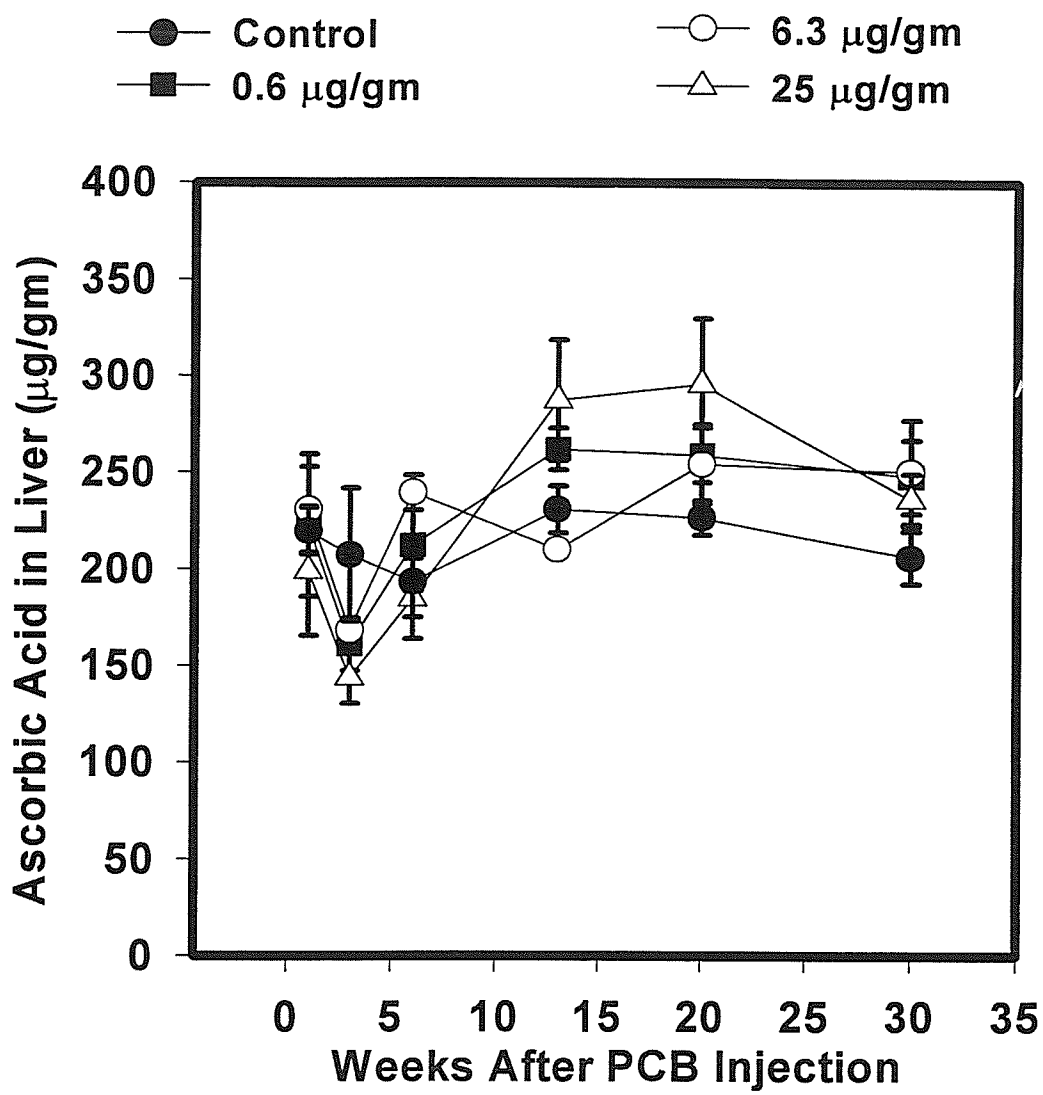

Control
0.6 µg/gm
6.3 µg/gm
25 µg/gm
Ascorbic Acid in Liver (µg/gm)
400
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.6: Tocopherol in liver of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as the percentage of tocopherol present in the control group at the same sample time. Tocopherol from all groups was quantified as µg of tocopherol per gm of liver lipid weight. Graphic and statistical notation are as in Fig. 2.1 (n=5, α=0.05).

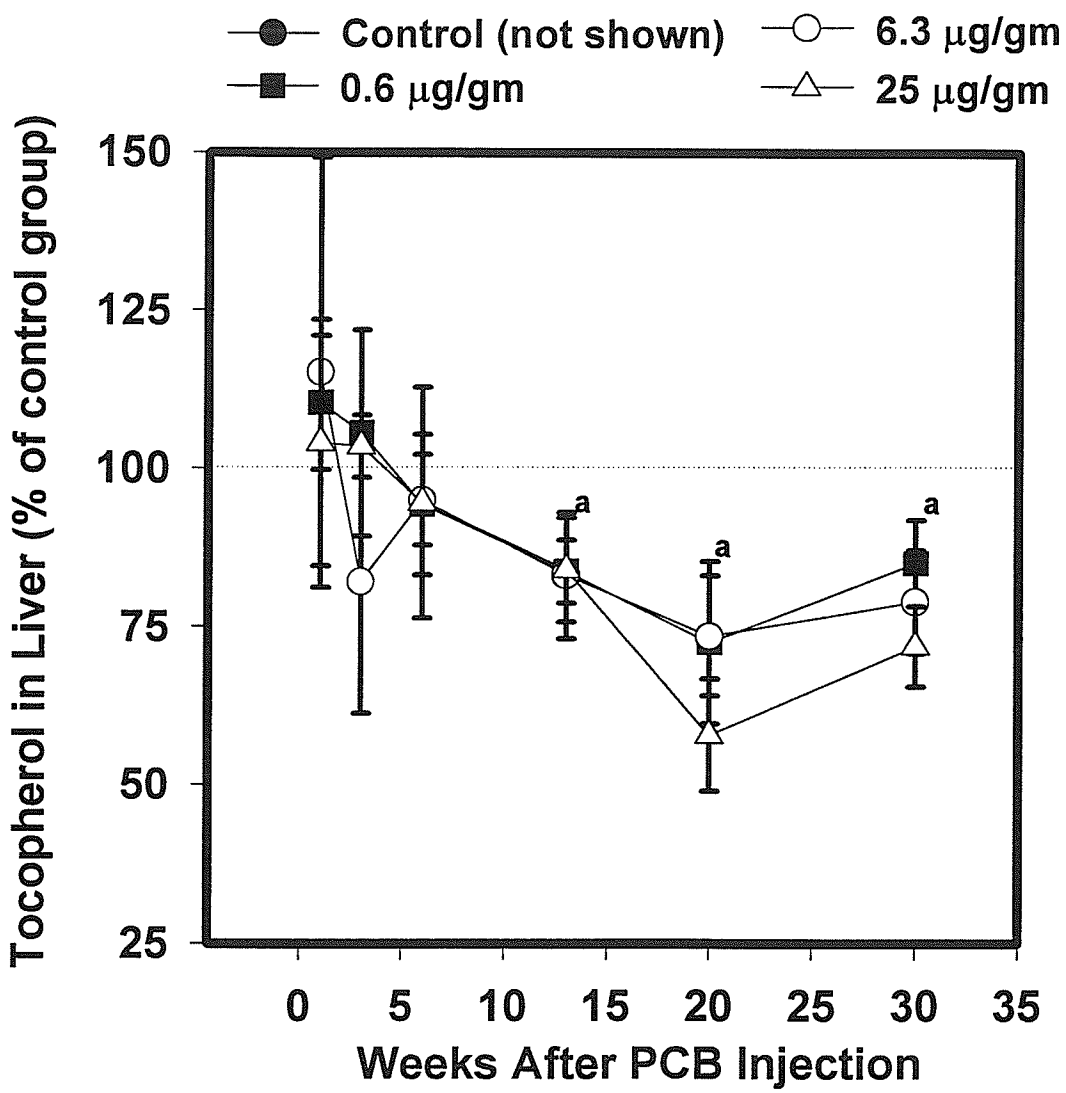
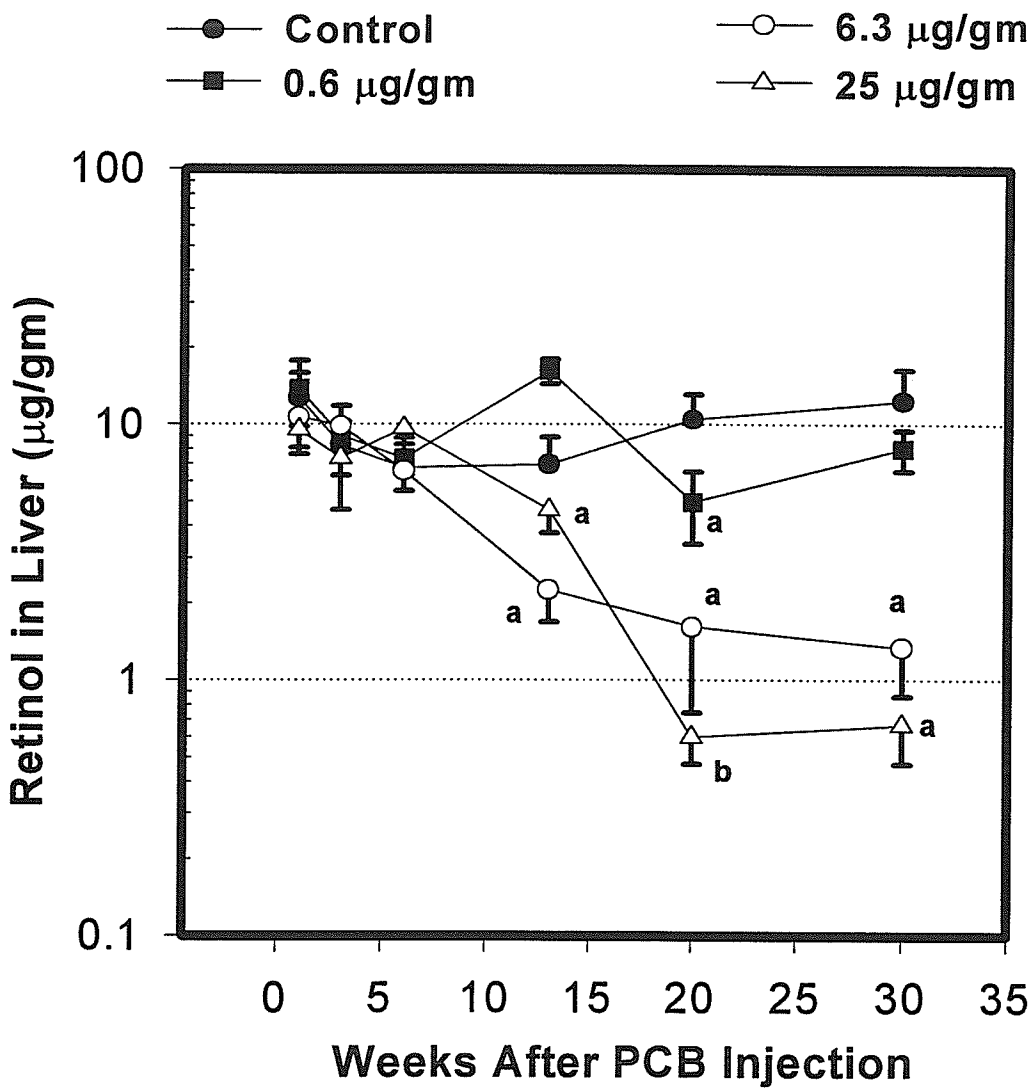

Control (not shown)
6.3 µg/gm
0.6 µg/gm
25 µg/gm
Tocopherol in Liver (% of control group)
150
125
100
75
50
25
a
a
a
0
5
10
15
20
25
30
35
Weeks After PCB Injection

Figure 2.7: Retinol in liver of control and PCB-dosed lake trout. Data are expressed (mean with standard error) as µg of retinol per gm of wet liver weight. Graphic and statistical notation are as in Fig. 2.1 (n=5, α=0.05).


Control
6.3 µg/gm
0.6 µg/gm
25 µg/gm
100
10
1
0.1
Retinol in Liver (µg/gm)
0
5
10
15
20
25
30
35
Weeks After PCB Injection
a
a
a
a
a
b
a

Chapter 3

Relationships between Phase I and Phase II Enzymatic Activities and Oxidative Stress in Adult Lake Trout (*Salvelinus namaycush*) from Lake Ontario and Lake Superior

Abstract

Relationships between MFO and Phase II enzymatic activities and the appearance of oxidative stress were investigated in the livers of lake trout collected during 1992-94 from Lake Ontario and Lake Superior, respectively the most and least contaminated of the Great Lakes. Lake trout from Lake Ontario had marginally greater hepatic EROD activity and equivalent UGT activity when compared to lake trout from Lake Superior. Renal EROD activity was significantly greater in lake trout from Lake Ontario. UGT activity in the kidney was similar in lake trout from both lakes except for those from Black Bay of Lake Superior which had significantly lower activity. Lake Superior populations had greater hepatic activites of the enzymatic antioxidants catalase and glutathione peroxidase and consistently greater concentrations of the non-enzymatic antioxidant tocopherol. Tocopherol concentrations and glutathione peroxidase activity were also greater in the kidney of lake trout from Lake Superior. Hepatic and renal ascorbic acid were not different between the two populations. The predominant vitamin A compounds in lake trout from Lake Superior were retinoids, whereas didehydroretinoids were more predominant in lake trout from Lake Ontario. Total alcohol and esterified concentrations of retinoids were significantly higher in the kidneys of lake trout from Lake Ontario. This suggests a compensatory mechanism, similar to that described for organochlorine-

exposed rats, whereby retinol which is released from transthyretin by organochlorine metabolites in the general circulation is resorbed by cells of the kidney. Lake Ontario lake trout had significantly greater hepatic concentrations of lipid hydroperoxides, a measure of oxidative stress. Studies of additional antioxidant and pro-oxidant factors, possibly contributing to the recruitment failure of lake trout from Lake Ontario, require further consideration.

Introduction

Historic declines of lake trout (*Salvelinus namaycush*) populations within the Great Lakes have been attributed to increased predation on lake trout by lamprey, introduced in the late 1940s or early 1950s, and to overfishing of lake trout by commercial fishermen (Hartman 1988). Application of lampricides over the last 30 years has been effective in reducing the lamprey population and associated predation on lake trout (Curtis 1990). Additionally, massive stocking efforts have restored lake trout population numbers substantially (International Joint Commission (IJC) 1990).

Despite these efforts, lake trout in the lower Great Lakes continue to exhibit low reproductive recruitment, and progress towards re-establishing self-sustaining populations has been slow (Gilbertson 1992). Destruction of habitat, continued sport and commercial fishing and lamprey predation pressures, as well as increased competition by other introduced exotic species, have all been cited as factors contributing to the slow recovery of lake trout in the Great Lakes (Hartman 1988). Toxic substances, particularly organic contaminants, are also suspected of playing a role in reproductive failure of lake trout in the lower Great Lakes (Mac et al. 1993).

Lake trout are particularly susceptible to accumulation of organic contaminants because of their top predator status,

relatively long life span and high lipid content (IJC 1990). Although concentrations of organic contaminants in tissues of lake trout from the lower Great Lakes have declined over the last two decades, the rate of decline appears to have slowed recently so that tissue concentrations are remaining relatively stable (Miller et al. 1992). Tissue polychlorinated biphenyl (PCB), polychlorinated dibenzodioxin (PCDD) and polychlorinated dibenzofuran (PCDF) concentrations remain high in lake trout from Lake Ontario and Lake Michigan compared with those from Lake Superior (Rowan and Rasmussen 1992, Whittle et al. 1992).

The most toxic organochlorine compounds are those with a planar configuration that allows them to interact with the cellular Ah receptor. Interaction with the Ah receptor begins a series of cellular events culminating in the induction of phase I or mixed-function oxygenase (MFO) enzymes (Safe 1990). The MFO enzyme system, embedded in the endoplasmic reticulum, catalyzes the introduction of an oxygen atom into steroids, fatty acids or xenobiotics during the first phase of their metabolism (Andersson and Forlin 1992). Phase II enzyme activity, like MFO activity, can be induced by exposure to certain contaminants and is an essential second step in excretion of many lipophilic substrates (Clarke et al. 1991). Following insertion of an oxygen molecule into the substrate by MFO enzymes, phase II enzymes conjugate the altered substrate with endogenous polar molecules such as glucuronic

acid, sulfates or glutathione, so that the entire complex can be more readily excreted (Jimenez and Stegeman 1990).

MFO enzymes are constitutively expressed in liver, kidney and other tissues at low activites, but with exposure to PCBs, PCDDs or PCDFs of a planar configuration their activity can be induced by as much as 40- to 60-fold (Hodson et al. 1991, Luxon et al. 1987). While MFOs are thought to be involved in metabolism of fatty acids, steroids and bile acids, they may increasingly metabolize PCBs, PCDDs and PCDFs following exposure when their activity has been induced (Larsen et al. 1992). Lake trout from the lower Great Lakes have greater MFO activity than those from Lake Superior (Luxon et al. 1987, Hodson et al. 1990). However, MFO activity has yet to be linked with reproductive failure of lake trout in the lower Great Lakes or to a specific mechanism of toxicity.

There is increasing evidence to support a link between induction of MFO enzymes and the appearance of oxidative stress in fish (Lehtinen 1990, Chapter 2 Palace et al. 1996a). Oxidative stress is produced when free radicals, generated by the activity of MFO enzymes, proliferate and react with vital cell components including lipids and proteins, potentially damaging them and disrupting cell function (Winston and Di Giulio 1991). The existing gradient of lowest organic contamination in Lake Superior to highest in the lower Great Lakes, with a corresponding gradient of MFO activity in lake trout (Luxon et al. 1987), offers an excellent opportunity to

study this relationship. MFO activity and indicators of oxidative stress were, therefore, assessed in lake trout populations from Lake Ontario and Lake Superior for three successive years. Examination of oxidative stress in lake trout with induced MFO and Phase II enzymatic activities may provide a mechanistic link to help understand the continued recruitment failure of Lake Ontario lake trout.

Materials and Methods

Sample Locations and Collection Methods

Our initial assessment of relationships between MFO and Phase II activities and oxidative stress utilized lake trout collected from Lake Superior at the Welcome Islands site in June, and from Lake Ontario near Port Weller in May of 1992 (Figure 3.1). Subsequent collections of pre-spawned lake trout were obtained from Lake Superior at the Welcome Islands site in September 1993 and 1994 and from the Black Bay site in September 1994. Pre-spawned lake trout from Lake Ontario were collected near Port Weller in September 1993 and October and November of 1994. All lake trout were between 5 and 8 years of age, according to otolith annuli determinations, and weighed 3,664 ± 197 gm from Lake Ontario and 1,167 ± 79 gm from Lake Superior.

Live lake trout were collected using 104 mm gill nets set overnight in water of approximately 10°C (depth range of 30 to

50 metres). After removal from the nets, fish were maintained on the boat in tanks supplied with fresh circulating lake water. Immediately after fish were killed by a blow to the head, their length and weight measurements were obtained and the liver was removed, placed in a sterile plastic bag and frozen between slabs of dry ice. For the 1994 collections, kidney was also dissected and preserved in the same manner. After transport to the laboratory, tissues were stored at -100°C until analysis.

Biochemical Analyses

i) Phase I and Phase II Enzymes

Microsomes were prepared by homogenizing liver or kidney in ice cold buffer containing 0.15 M KCL HEPES 0.1mM EDTA buffer (pH=7.5) with several passes of a Teflon pestle. The homogenate was centrifuged at 9,000 X g for 20 minutes at 4°C, supernatant was collected, and re-centrifuged at 105,000 X g for 75 minutes at 4°C to obtain a microsomal pellet. The pellet was resuspended in 0.1M phosphate buffer (pH=7.4). Activity of the MFO enzyme ethoxyresorufin-O-deethylase (EROD) and microsomal protein concentrations were determined simultaneously using Kennedy and Trudeau's (1994) modification of the Kennedy et al. (1993) fluorometric microplate method. Increasing fluorescence arising from EROD-catalyzed de-ethylation of ethoxyresorufin to resorufin was linear for at least 8 minutes, indicating that enzyme activity was

maintained in the assay system during this period.

Microsomes for measuring the activity of the phase II enzyme glucuronyltransferase (UGT) were prepared as above except that 0.01M sodium phosphate 0.15M KCl homogenization buffer (pH=7.4) and a 0.1M sodium phosphate resuspension buffer (pH=7.4) were used. UGT activity was determined at room temperature using p-nitrophenol as a substrate in a spectrophotometric assay (Burchell and Whetherhill 1981).

ii) Antioxidants

Tocopherol and retinoids (retinol, didehydroretinol, didehydroretinyl palmitate and retinyl palmitate) were extracted and quantified by the reverse phase HPLC method of Palace and Brown (1994 and Appendix A). Detection in this method was further improved by including propionic acid (1%) in the mobile phase and by using a 5 μM bead size 4.6 mm i.d., 250 cm Adsorbosphere HS C_{18} column. Run times were extended to 25 minutes and four detection channels are now monitored: 325 nm for detection of didehydroretinol, didehydroretinyl palmitate and the internal standard retinyl acetate; 292 nm for tocopherol and the internal standard tocopherol acetate; fluorescence excitation at 330 nm and detection at 480 nm for retinol and retinyl palmitate; 450 nm for the pro-retinoid pigments astaxanthin, canthaxanthin and β-carotene.

Ascorbic acid was assayed by the colorimetric method of Jagota and Dani (1982). Activities of the enzymatic

antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were determined as previously described (Palace et al. 1993).

iii) Membrane Breakdown Products

Thiobarbituric reactive substances (TBARS), a measure of oxidative membrane breakdown, were measured in 1992 liver samples using Uchiyama and Mihara's (1978) method, with malonaldehyde (Sigma Chemical Co., St Louis, Mo.) as a standard. The TBARS method was replaced with a more specific test for lipid hydroperoxides in 1993 and 1994 samples. This spectrophotometric method is commercially available in a kit (Kamiya Biomedical Co., Thousand Oaks, CA) that utilizes a derivative of methylene blue to specifically react with lipid hydroperoxides, which are derived from oxidized membranes (Ohishi et al. 1985).

iv) Data Analysis

All data within the same year and for the same sex were analyzed using a completely randomized one way ANOVA. Means were compared, where appropriate and as denoted for each figure, using Dunnett's one- and two-tailed t-tests with a level of confidence set at α=0.05. Relationships between EROD and oxidative stress indices were assessed within each year and across all years for the same sex using stepwise regression analysis with significance also accepted at the

α=0.05 level. Note that all variables left in the model were significant at the α=0.15 level. All statistical analysis was performed using SAS v. 6.08 (SAS Institute Inc, Cary, NC. 1989).

Results and Discussion

i) Phase I and Phase II Enzymes

MFO activities, measured in this study as ethoxyresorufin-O-deethylase (EROD), in liver of lake trout from Lakes Ontario and Superior (panel A, Fig. 3.2) were similar to recent activities found in liver of lake trout from the same lakes (L.Lockhart and D.A. Metner, unpub. data, Dept. of Fisheries and Oceans, Winnipeg, MB). The EROD activities from this study are approximately 10 fold greater than those reported by Munkittrick et al. (1992) from lake trout captured at reference and contaminated sites in Lake Superior. However, lower activities are expected for hepatic post-microsomal supernatant compared with the microsomal activities reported in this study.

Although Luxon et al. (1987) reported that lake trout from Lake Ontario had MFO activities 15 times greater than lake trout from Lake Superior, results from our collections indicate less dramatic differences between lake trout from the two lakes. Male lake trout collected from Lake Ontario in 1993 had 5.6 times greater EROD activity than male lake trout taken

from Lake Superior in the same year. Similarly, in 1994, females from Lake Ontario had 4.4 times greater EROD activity than females from Black Bay in Lake Superior. Aside from these differences, hepatic lake trout EROD activities were remarkably similar between the two lakes during all three years. Declining concentrations of organic contaminants (Whittle et al. 1992) since the 1983 collections of Luxon et al. (1987) may be partly responsible for explaining the similar EROD activities in trout from Lake Ontario and in those from Lake Superior. Additionally, except for the samples from Black Bay in 1994, lake trout from Lake Superior for this study were obtained near the Welcome Islands, close to Thunder Bay, Ontario. This site receives significant contaminant input from a nearby pulp and paper mill, coal stores and a municipal sewage treatment plant on the Kaministiquia River (Smith et al. 1990). All of these sources are capable of discharging organic contaminants which could elevate EROD and further contribute to less dramatic differences in hepatic EROD of lake trout from Lake Ontario and Lake Superior. Hodson et al. (1989) also reported relatively high MFO activites in lake trout from this area of eastern Lake Superior.

Hepatic MFO activity often declines in female fish just prior to spawning, probably in response to increasing circulating levels of estradiol (Forlin and Haux 1990). However, except for the aforementioned exceptions, EROD activities from 1992 samples collected during May and June

from both lakes were not consistently different from the activities of 1993 and 1994 collections made in October and November just prior to spawning. Lake trout caught in 1993 and 1994 were, therefore, obtained a sufficient time before spawning so that MFO activity reduction had not yet occurred. Luxon et al. (1987) also reported elevated MFO activities in lake trout from Lake Ontario during October, prior to spawning.

While MFO enzyme activity is usually greatest in liver tissue, extrahepatic activity is also present and is usually highest in the kidney (Melancon et al. 1988). Greater biotransformation capability by extrahepatic tissues, including kidney, could contribute to the overall concentration of oxidants for each fish. Consideration of these sources is especially important because induction may occur in extrahepatic tissues even when elevated activity cannot be detected in the liver (Payne et al. 1984). MFO activities of kidney tissue from our 1994 Lake Superior collections (Table 3.1) were lower than activities in corresponding livers. Alternately, MFO activity was higher in kidney than in liver of lake trout captured from Lake Ontario in 1994. Similar to results from the liver, kidney MFO activity was greatest in lake trout from Lake Ontario followed by the Thunder Bay and Black Bay sites of Lake Superior, respectively. EROD activity was 15 times higher in kidney of lake trout from Lake Ontario relative to lake trout from the

Black Bay site of Lake Superior and 5.5 times greater than in lake trout from the Welcome Islands site.

Activity of the phase II enzyme, glucuronyltransferase (UGT), was not significantly elevated, relative to Lake Superior samples, in livers of lake trout collected from Lake Ontario (Fig. 3.2 panel B). The activities of UGT described in the current study are consistent with those previously reported for hepatic microsomes from trout using the same p-nitrophenol assay substrate (Iannelli et al. 1994, Gregus et al. 1983). Evidence that indicates hepatic UGT activity is not affected by spawning activity and gonadal development has recently been reviewed (Clarke et al. 1991). Not surprisingly, there were no consistent differences between the hepatic UGT activity of samples collected in spring 1992 and the prespawning collections of 1993 and 1994. In the kidney, UGT activity was 3 to 3.5 times higher in lake trout from Lake Ontario and the Welcome Islands site of Lake Superior relative to lake trout from Black Bay during 1994 (Table 1).

Phase II enzymes are often not as responsive to contaminant induction as MFO enzymes (Jimenez and Stegeman 1990). Because trout have a relatively low capacity for glucuronidation compared with other vertebrates (Gregus et al. 1983), increased oxidative activity and toxicity may arise when oxidized substrates accumulate due to an imbalance between MFO activity and phase II conjugation (Smith et al. 1990).

ii) Antioxidants

Enzymatic and non-enzymatic antioxidant molecules offer protection from oxidative damage by quenching free radicals before they can react with and damage cellular components (Winston and Di Giulio 1991). Antioxidant protection by the three enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) involves consumption of oxidative radicals by mechanisms previously discussed (Palace and Klaverkamp 1993). The sensitivity of these enzymes as indicators of oxidative stress induced by contaminants, however, remains unclear. Elevated SOD activity has been reported in fish from organochlorine-contaminated sites (Roberts et al. 1987) and in fish exposed to hydrocarbon-contaminated sediments (Livingstone et al. 1993). Catfish (*Ictalurus punctatus*) exposed to pulp mill effluent had elevated CAT but not SOD or GPx activity (Mather-Mihaich and Di Giulio 1991). Otto et al. (1994) concluded that SOD and CAT were relatively insensitive indicators of oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to sediments contaminated by pulp mill effluent. However, mullet (*Mugil sp.*) captured from polluted sites had elevated hepatic activities of SOD, CAT and GPx relative to fish collected from a reference site (Rodriguez-Ariza et al. 1993). Confusion regarding these enzymatic activity alterations increases when one considers that activity differences present after short

term exposure to contaminants may dissipate over longer periods of exposure (Livingstone et al. 1993).

Lake trout from Lake Ontario and Lake Superior have similar activities of hepatic SOD, CAT and GPx enzymes (Fig. 3.3, panels A,B,C respectively) to those reported earlier from the same species captured in small Precambrian Shield lakes of northwestern Ontario (Palace and Klaverkamp 1993). Whereas lake trout populations from these small lakes exhibited consistent peaks of SOD activity in September, hepatic SOD activity was greater for samples collected from both Great Lakes in May and June of 1992 than in October of 1993 and 1994. No consistent differences of hepatic SOD activity in lake trout liver were found between the two Great Lakes. CAT activity was, with the exception of those samples collected during spring 1992, significantly higher in liver of lake trout from Lake Superior than those from Lake Ontario. While both lake trout populations had significantly higher hepatic GPx activity in 1994 than in the two previous years, the greatest activity during 1994 was assayed in Lake Superior trout. Activities of CAT in kidney were not different between any of the populations during 1994. GPx activity was significantly greater in kidneys of lake trout from both sites of Lake Superior compared to those from Lake Ontario (Table 3.1). The elevated activities of CAT and GPx enzymes in liver and GPx in kidney may afford greater protection against oxidant damage to lake trout from Lake Superior (Rodriguez-

Ariza et al. 1993). However, different activities within fish populations between lakes or in the same population during successive years, may also be reflective of temperature, seasonal and/or dietary differences (Gabryelak et al. 1983, Radi et al. 1987).

Although significant varibility in liver tocopherol content was evident in both populations, lake trout from Lake Superior had consistently greater concentrations of hepatic tocopherol than lake trout from Lake Ontario (Fig. 3.4, panel A). This difference was even more dramatic in kidney tissue, where lake trout from Lake Superior had at least 35 to 50 times higher concentrations of tocopherol than lake trout from Lake Ontario (Table 3.1). Tocopherol, or vitamin E, is thought to represent the most important protection against oxidant damage to cellular and subcellular membranes (Burton and Traber 1990). Scavenging of reactive free-radical species by tocopherol occurs directly within membranes, sparing lipid molecules from oxyradical-mediated breakdown (Burton and Traber 1990). In Chapter 2 we showed that exposure of lake trout in the laboratory to contaminants that induce MFO enzymes and increase oxyradical production also results in depleted tissue stores of tocopherol. Whether this mechanism or dietary differences are responsible for the lower hepatic and renal tocopherol of trout from Lake Ontario requires additional study. Regardless of the factors involved, greater concentrations of tocopherol in lake trout from Lake Superior

would afford them greater protection against oxidant-mediated cell injury (Maellaro et al. 1990).

No consistent differences were evident in the liver or kidney content of vitamin C between lake trout from Lake Ontario and Lake Superior, although female lake trout from Lake Ontario in 1994 had significantly greater hepatic ascorbic acid content (Fig. 3.4, panel B, Table 3.1). Other vitamins including ascorbic acid (vitamin C), found in the aqueous fraction of the cell, and retinoids (vitamin A), localized in the lipid fraction of the cell, contribute to the total antioxidant capacity of cells (Ribera et al. 1991). These vitamins may also be depleted when aquatic organisms are subjected to contaminant mediated oxidative stress (Thomas 1987, Ribera et al. 1991). Elevations, rather than depletions, of ascorbic acid have previously been documented in fish exposed to contaminants that induce oxidative stress (Andersson et al. 1988). Whereas these increases can be attributed to enhanced synthesis of the antioxidant, lake trout lack an essential enzyme of the biosynthetic pathway and are unable to synthesize ascorbic acid (Dabrowski 1990). Therefore, the high ascorbic acid concentration in female lake trout from Lake Ontario in 1994 must be attributed to greater dietary intake.

Lake trout from Lake Superior had greater hepatic concentrations of retinol than lake trout from Lake Ontario (Fig. 3.5, panel A). A given fish species or population can

most often be characterized as having either predominantly retinol or didehydroretinol and their corresponding esterified forms (Ndayibagara et al. 1995, S. Brown, unpub. obs., Dept. of Fisheries and Oceans, Winnipeg, MB). Therefore, differences between the two populations should not be interpreted as a deficiency of vitamin A in lake trout from Lake Ontario, as they had greater hepatic concentrations of the alternate retinoid, didehydroretinol, than trout from Lake Superior (Fig. 3.5, panel B). Dietary intake of the pro-retinoid pigments astaxanthin and canthaxanthin may explain some of these differences. While canthaxanthin is converted to β-carotene and then to retinol and didehydroretinol, astaxanthin may be preferentially converted to didehydroretinol in trout (Guillou et al. 1989). Lake trout from Lake Ontario have consistently greater hepatic concentrations of astaxanthin than lake trout from Lake Superior (data not shown), suggesting that vitamin A metabolism is primarily didehydroretinoid in lake trout from Lake Ontario. However, didehydroretinol has only 40 to 51% of the biological activity of retinol (Spear et al. 1992). When we correct for the differences in activity between retinol and didehydroretinol, lake trout from Lake Ontario do not have significantly lower hepatic free vitamin A concentrations than lake trout from Lake Superior.

Vitamin A is stored primarily as esters in trout liver. For example, retinol is stored mostly as retinyl palmitate

(Futterman and Andrews 1964). Not surprisingly since lake trout from Lake Ontario had low hepatic retinol concentrations, they also had significantly lower hepatic concentrations of retinyl palmitate than lake trout from Lake Superior (Fig. 3.5, panel C). Given the predominance of didehydroretinol in lake trout from Lake Ontario, it is reasonable to assume that their major hepatic storage form would be a didehydroretinyl ester, such as didehydroretinyl palmitate. Without standard samples of this and other compounds we are unable to positively identify these esters on the HPLC chromatogram. However, different absorbance/fluorescence properties can be exploited to identify peaks that we believe correspond to didehydroretinyl esters (Ndayibagara et al. 1995). A major peak eluting at 20.7 min is probably the primary didehydroretinol ester found in lake trout from Lake Ontario. It elutes at the predicted time with reference to the separation time of retinol and didehydroretinol and the relative elution time of retinyl palmitate. It has the same absorbance ratio between detection channels as didehydroretinol, exhibits limited fluorescence and has a characteristic didehydroretinol absorbance spectrum. When the suspected didehydroretinyl ester peak is quantified against the retinyl palmitate standard and corrected for the different UV absorbances of retinol and didehydroretinol (Stancher and Zonata 1984), it indicates that lake trout from Lake Ontario have approximately equivalent esterified stores

of vitamin A to lake trout from Lake Superior (data not shown).

Didehydroretinoids also predominate over retinoids in the kidneys of lake trout from Lake Ontario (Table 3.1). While renal retinol concentrations were not consistently different between any of the lake trout populations, both didehydroretinol and its esterified form were 2.5- to 7-times and 25- to 65-times, respectively, more concentrated in the kidneys of lake trout from Lake Ontario. It is particularly interesting to note that the total free alcohol (retinol + didehydroretinol) and esterified vitamin A concentrations are significantly higher in kidneys of lake trout from Lake Ontario than from Lake Superior. Greater renal concentrations of retinoids have previously been reported for rats exposed to TCDD (Jurek et al. 1990). The authors postulated that biotransformed metabolites of TCDD may interfere with the binding of retinol/didehydroretinol to the transthyretin protein complex in plasma and that, as a compensatory mechanism, elevated esterifying enzyme activity in kidney may help to retain the free retinol. With historically higher exposure to organochlorine contaminants than lake trout from Lake Superior (Luxon et al. 1987), lake trout from Lake Ontario may contain greater concentrations of the appropriate metabolites to facilitate this mechanism. Further study is required to evaluate the biological significance of higher kidney retinoid concentrations to lake trout in the Great

Lakes.

Retinol has previously been identified as an antioxidant molecule that quenches singlet oxygen, a reactive oxyradical (Roberfroid 1995). Conversion of retinol to didehydroretinol via dehydrogenation (Schiedt et al. 1985) could, therefore, account for some of the retinol depletion previously observed in the oxidatively stressed fish of Chapter 2. Relationships between the predominant retinoid forms and oxidative stress indicated by membrane breakdown will be considered later, in the discussion of membrane breakdown.

β-carotene and related carotenoid pigments operate preferentially as antioxidants only at low partial pressures of oxygen (Terao et al. 1992). β-carotene is, however, not efficiently absorbed by trout and usually represents a small fraction of the pro-retinoid compounds in trout liver (Schiedt et al. 1986). Both populations of lake trout from the Great Lakes had hepatic and renal β-carotene concentrations near or below detection limits using the current method. At these low concentrations and with limited antioxidant activity relative to tocopherol (Terao et al. 1992), β-carotene probably does not play a major role as an antioxidant in lake trout.

iii) Membrane Breakdown

The TBARS or malonaldehyde (MDA) test has been used to measure oxidative breakdown in lipid membranes (Gutteridge and

Halliwell 1990), and has specifically been applied to assessing oxidative stress in fish resulting from contaminant exposure (Thomas and Wofford 1993). Membrane breakdown in livers of lake trout collected during 1992 was assessed using the TBARS method (Fig. 3.6). Lake trout from both lakes had TBARS concentrations similar to those we previously reported for the lake trout exposed to PCB 126 in Chapter 2. While the means for both sexes were higher, only male lake trout from Lake Ontario had significantly greater TBARS concentrations compared to the unexpectedly high TBARS concentrations of lake trout from Lake Superior. This suggests that fish from the Thunder Bay site of Lake Superior are subjected to significant contaminant-induced stress.

Because of the large variability and questionable *in vivo* relevance of the TBARS measure (Gutteridge and Halliwell 1990), a direct assay utilizing a methylene blue derivative to quantify lipid peroxides (Ohishi et al. 1985) was used to assess oxidative membrane breakdown in lake trout collected during 1993 and 1994. Since lipid hydroperoxide content increases during long term storage, samples from 1992 could not be reanalyzed using the new method. However, we have observed a linear relationship between TBARS and lipid peroxide content of liver from lake trout in the laboratory (R^2= 0.81) (unpub. obs.).

Lipid peroxide content of liver tissue was significantly greater in lake trout from Lake Ontario than lake trout from

Lake Superior during both 1993 and 1994, indicating that the Lake Ontario population experiences greater oxidative stress. Oxidative stress is a product of antioxidant capacity and pro-oxidant forces, including those arising from contaminant exposure (Winston and Di Guilio 1991). We have previously established a link between oxidative stress, measured as TBARS, and induced MFO enzyme activity in lake trout exposed to organic contaminants in the laboratory (Chapter 2). However, a wider range of enzyme activities was induced in these laboratory fish (150 to 200 times greater than control fish) than for the current field collections (maximum 8 times greater activity in lake trout from Lake Ontario compared with fish from Lake Superior), which may explain why there was no significant relationship between MFO activity and TBARS or lipid hydroperoxide concentrations in the lake trout populations from the Great Lakes. Stepwise regression analysis of dependent variables did show that lower concentrations of CAT and tocopherol along with higher content of ascorbic acid and didehydroretinol were correlated with greater oxidative stress, measured by lipid hydroperoxides (R^2=0.5162, $p<0.05$) and TBARS (R^2=0.3917, $p<0.05$) in the liver of lake trout from Lakes Ontario and Superior. Similarly in the kidney, stepwise regression analysis correlated greater MFO activity with depleted vitamin E and elevated concentrations of didehydroretinol (R^2=0.5917, $p<0.05$). Note that ascorbic acid may actually act as a pro-oxidant, even at physiological

concentrations, when tocopherol is depleted (Staats and Colby 1989) and that female lake trout from Lake Ontario had elevated concentrations of hepatic ascorbic acid concurrent with lower tocopherol. This relationship, as well as the predominance of hepatic didehydroretinol and oxidative stress, requires additional study. Other cellular antioxidant stores, for example metallothionein and glutathione (Sato and Bremner 1993), and pro-oxidant forces, including tissue concentrations of redox active metals, should also be considered as potential sources contributing to the greater oxidation of liver cells from lake trout in Lake Ontario (Winston and Di Giulio 1991). The relationship between oxidative stress and recruitment failure of lake trout from Lake Ontario is examined further in Chapter 4.

Table 3.1: Biotransformation enzyme activity and antioxidant concentrations in kidney of lake trout captured from the Port Weller area of Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior during 1994. Data for male lake trout are contained in shaded rows while the non-shaded rows contain data for female lake trout. Dunnett's one-tailed t-test was used to test whether EROD, UGT, didehydroetinol and didehydroretinyl palmitate were higher, or vitamin E was lower, in kidneys of lake trout from Lake Ontario than in kidneys of lake trout from Lake Superior. Dunnett's two-tailed t-test was used to test whether CAT, GPx, vitamin C, retinol or retinyl palmitate were different between kidneys of lake trout from Lake Ontario and Lake Superior. Means from Lake Ontario labelled with the same letter as means from Lake Superior are not significantly higher (EROD, UGT, didehydroretinol and retinyl palmitate), lower (vitamin E) or different (CAT, GPx, vitamin C, retinol and retinyl palmitate) for comparisons within the same year and for the same sex (α=0.05).

Sample Location	EROD nmol/min/ mg of protein	UGT nmol/min/ mg of protein	CAT units/mg of protein	GPx units/mg of protein	Vit E µg/gm of kidney	Vit C µg/gm of kidney	Retinol µg/gm of kidney	Dehydro-retinol µg/gm of kidney	Retinyl Palm. µg/gm of kidney	Dehydro Retinyl Palm. µg/gm of Kidney
Lake Ontario	1.21[a] ±0.15	0.27[a] ± 0.05	716[a] ± 40	0.05[a] ± 0.01	<5[a]	62.9[a] ± 4.0	0.78[a] ± 0.06	3.41[a] ± 0.38	15.96[a] ± 1.56	291.46[a] ± 43.01
Black Bay Lake Sup.	0.08 ± 0.01	0.08 ± 0.01	671 ± 87	0.08 ± 0.01	181.29 ± 10.48	79.1 ± 11.2	0.57 ± 0.25	1.35 ± 0.44	26.67 ± 9.13	8.60 ± 1.60
	0.08[b] ± 0.01	0.08[b] ± 0.01	716[a] ± 77	0.08[b] ± 0.01	177.30[b] ± 6.66	67.67[a] ± 8.54	0.33[b] ± 0.22	0.81[b] ± 0.47	20.15[a] ± 5.31	5.34[b] ± 0.75
Thdr. Bay Lake Sup.	0.13 ± 0.05	0.28 ± 0.07	662 ± 70	0.10 ± 0.01	242.34 ± 22.31	75.02 ± 6.06	0.28 ± 0.06	<0.5	113.59 ± 20.89	7.81 ± 0.56
	0.22[b] ± 0.09	0.26[a] ± 0.10	630[a] ± 44	0.10[b] ± 0.01	191.16[b] ± 10.24	71.27[a] ± 7.88	1.10[a] ± 0.31	<0.5[b]	170.61[b] ± 67.29	11.09[b] ± 2.30

Figure 3.1: Sampling locations on the Great Lakes for 1992-94 lake trout collections.

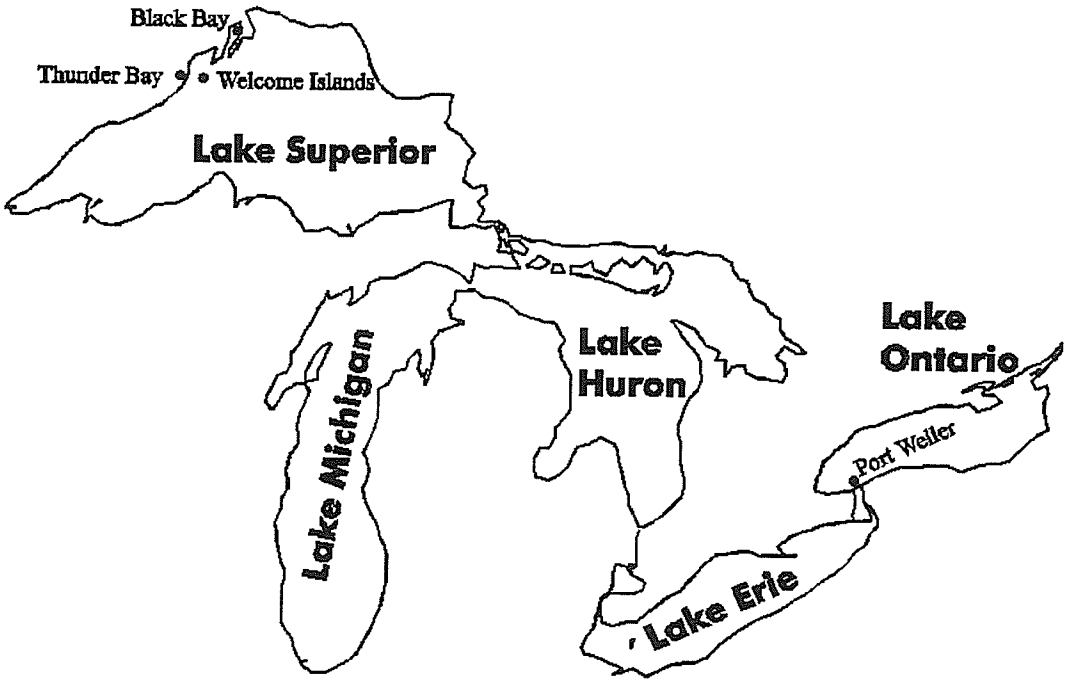

Black Bay
Thunder Bay
Welcome Islands
Lake Superior
Lake Michigan
Lake Huron
Lake Ontario
Port Weller
Lake Erie

Figure 3.2: Mixed function oxidase (MFO) activity, measured as EROD (panel A), and phase II enzyme activity, measured as UGT (panel B), in liver of lake trout collected from Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior during 1992-94. EROD activity appears as nmol of resorufin produced per min per mg of microsomal protein and UGT activity appears as nmol of p-nitrophenol conjugated per min per mg of microsomal protein. Data are presented as mean with standard error. Dunnett's one- tailed t-test was used to test whether the enzyme activity in livers of lake trout from Lake Ontario was greater than the enzyme activity in livers of lake trout from Lake Superior. Means from Lake Ontario labelled with the same letter as means from Lake Superior are not significantly higher for comparisons within the same year and for the same sex (α=0.05).

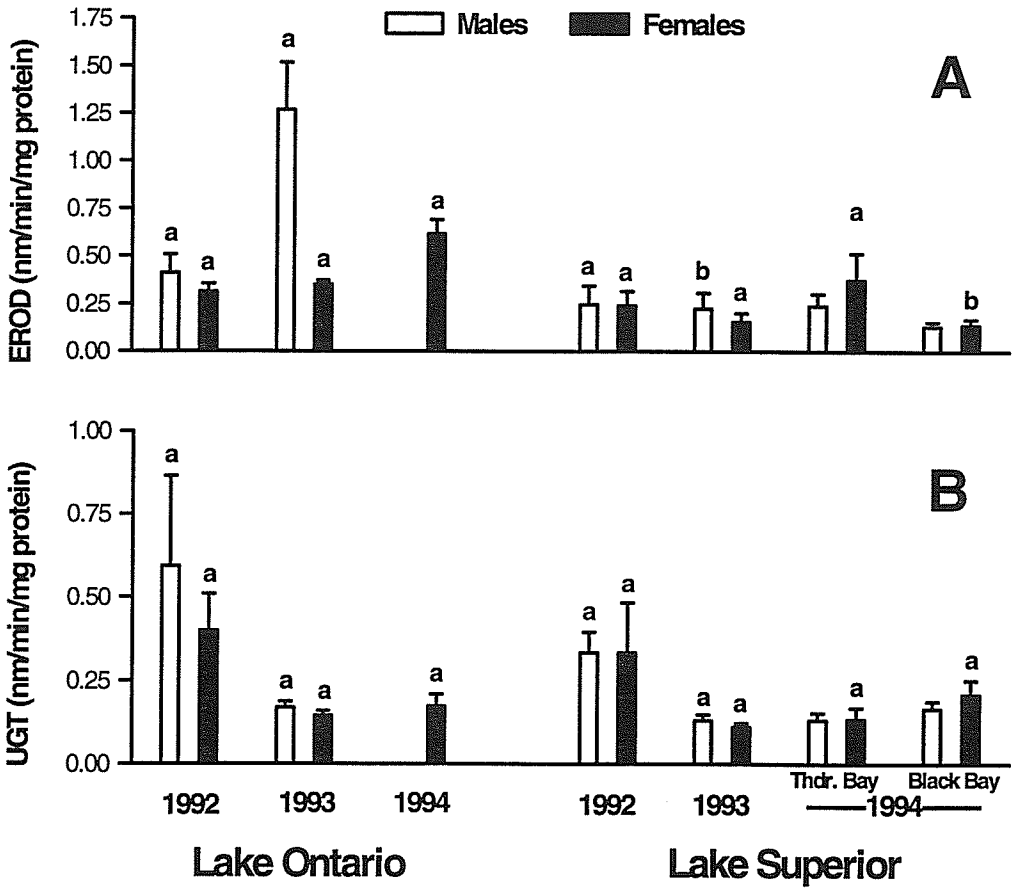

Males
Females
A
B
EROD (nm/min/mg protein)
UGT (nm/min/mg protein)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
b
1992
1993
1994
Thdr. Bay
Black Bay
Lake Ontario
Lake Superior

Figure 3.3: Activities of the enzymatic antioxidants SOD (panel A), CAT (panel B) and GPx (panel C) in liver of lake trout collected from Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior during 1992-94. Enzyme activites are presented as units per mg of protein. One unit of SOD inhibits autooxidation of epinephrine by 50% at pH=7.0, 25°C. One unit of CAT decomposes 1.0 µmol of hydrogen peroxide at pH=7.0, 25°C. One unit of GPx catalyzes the oxidation of 1.0 mmol of reduced glutathione by hydrogen peroxide to oxidized glutathione per minute at pH=7.0, 25°C. Data are presented as mean with standard error. Dunnett's two-tailed t-test was used to test whether enzyme activity was different between livers of lake trout from Lake Ontario and Lake Superior. Means labelled with the same letter are not significantly different for comparisons within the same year and for the same sex (α=0.05).

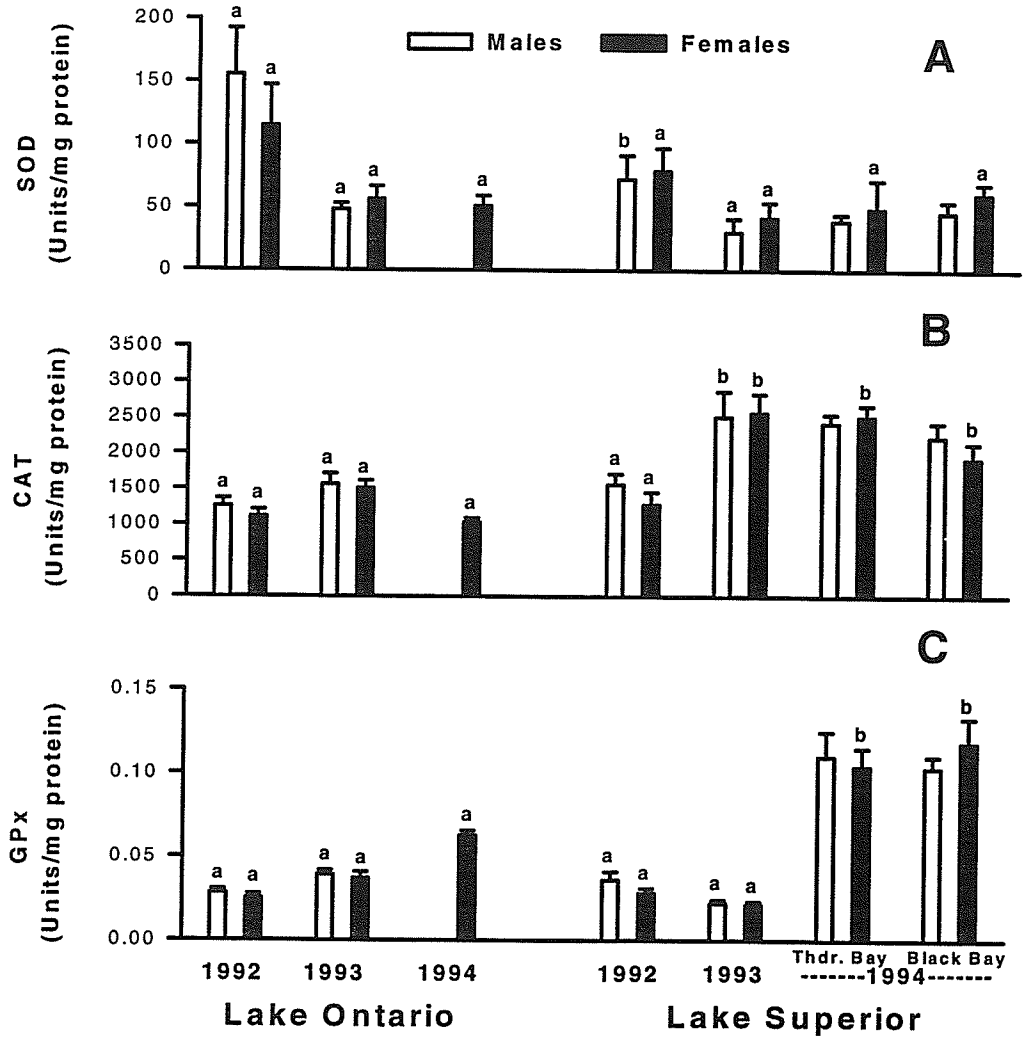

Males
Females
A
B
C
SOD
(Units/mg protein)
CAT
(Units/mg protein)
GPx
(Units/mg protein)
200
150
100
50
0
3500
3000
2500
2000
1500
1000
500
0.15
0.10
0.05
0.00
1992
1993
1994
1992
1993
Thdr. Bay
Black Bay
1994
Lake Ontario
Lake Superior

Figure 3.4: Concentrations of the non-enzymatic antioxidants tocopherol (panel A) and ascorbic acid (panel B) in liver of lake trout collected from Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior during 1992-94. Both variables are presented as µg per gm of wet liver weight. Data are presented as mean with standard error. Dunnett's one-tailed t-test was used to test whether tocopherol concentrations were lower in livers of lake trout from Lake Ontario than in livers of lake trout from Lake Superior. Dunnett's two-tailed t-test was used to test whether ascorbic acid concentrations were different between livers of lake trout from Lake Ontario and Lake Superior. For tocopherol, means from Lake Ontario labelled with the same letter as means from lake Superior are not significantly lower for comparisons within the same year and for the same sex. For ascorbic acid, means from Lake Ontario labelled with the same letter as means from Lake Superior are not significantly different for comparisons within the same year and for the same sex (α=0.05).

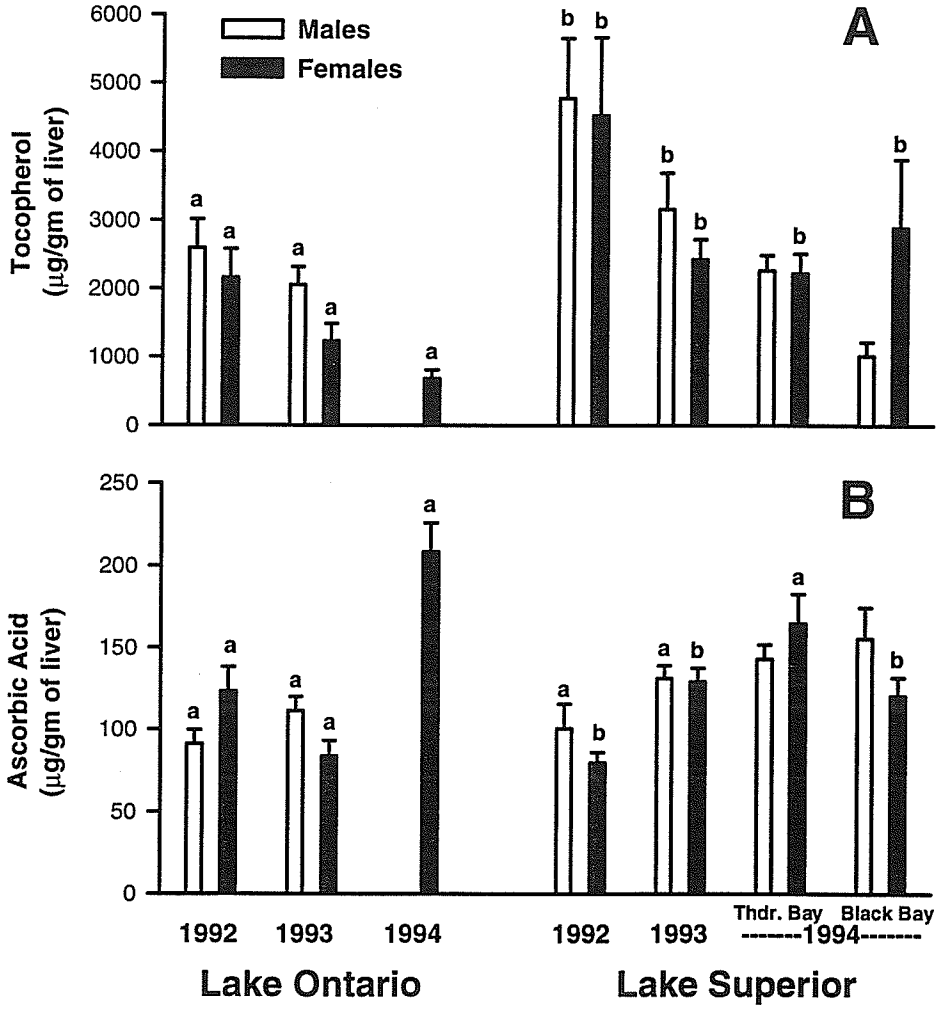

Males
Females
A
B
Tocopherol (µg/gm of liver)
Ascorbic Acid (µg/gm of liver)
6000
5000
4000
3000
2000
1000
0
250
200
150
100
50
0
1992
1993
1994
1992
1993
Thdr. Bay
Black Bay
1994
Lake Ontario
Lake Superior

Figure 3.5: Concentrations of the vitamin A compounds retinol (panel A), didehydroretinol (panel B) and retinyl palmitate (panel C) in liver of lake trout collected from Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior during 1992-94. Retinol and didehydroretinol are presented as ng per gm of wet liver weight and retinyl palmitate is presented as µg per gm of wet liver weight. Data are presented as mean with standard error. Dunnett's one-tailed t-test was used to test whether retinol and retinyl palmitate were lower, and didehydroretinol concentrations were higher, in livers of lake trout from Lake Ontario than in livers of lake trout from Lake Superior. Means from Lake Ontario labelled with the same letter as means from Lake Superior are not significantly lower (retinol and retinyl palmitate) or higher (didehydroretinol) for comparisons within the same year and for the same sex (α=0.05).

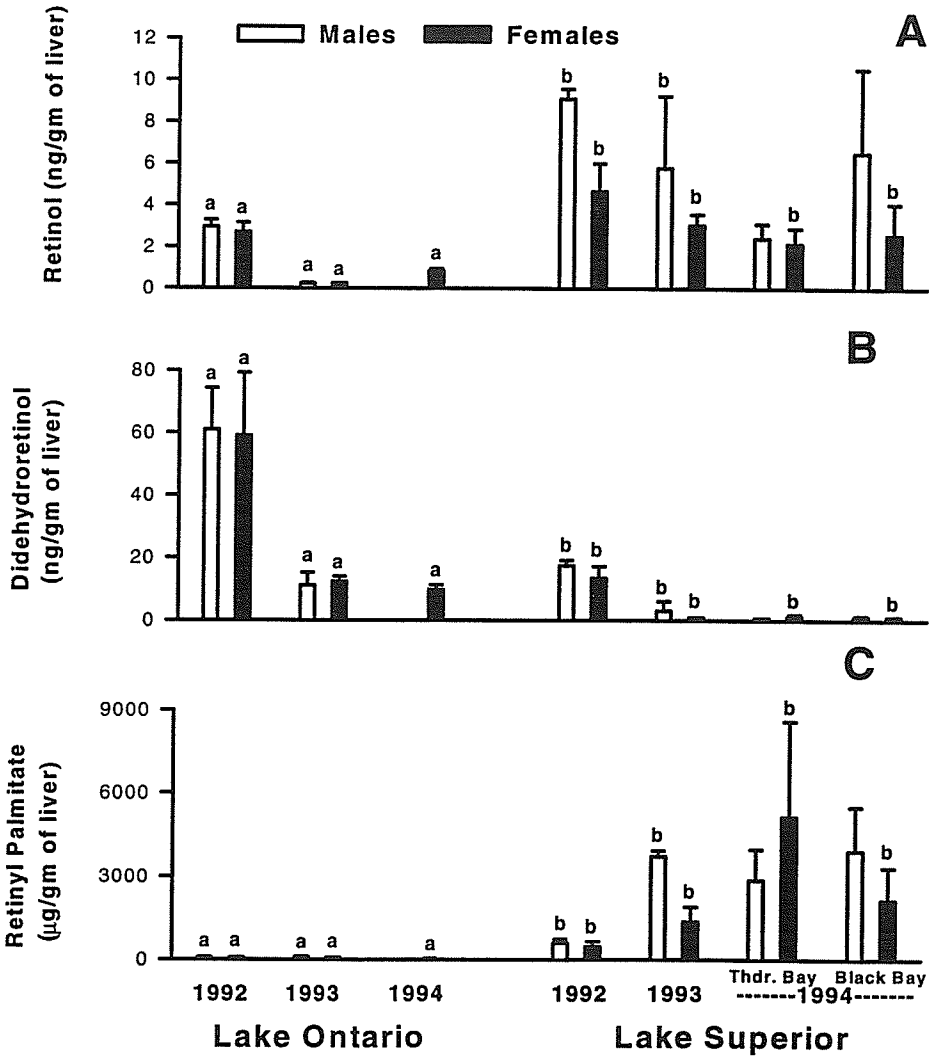
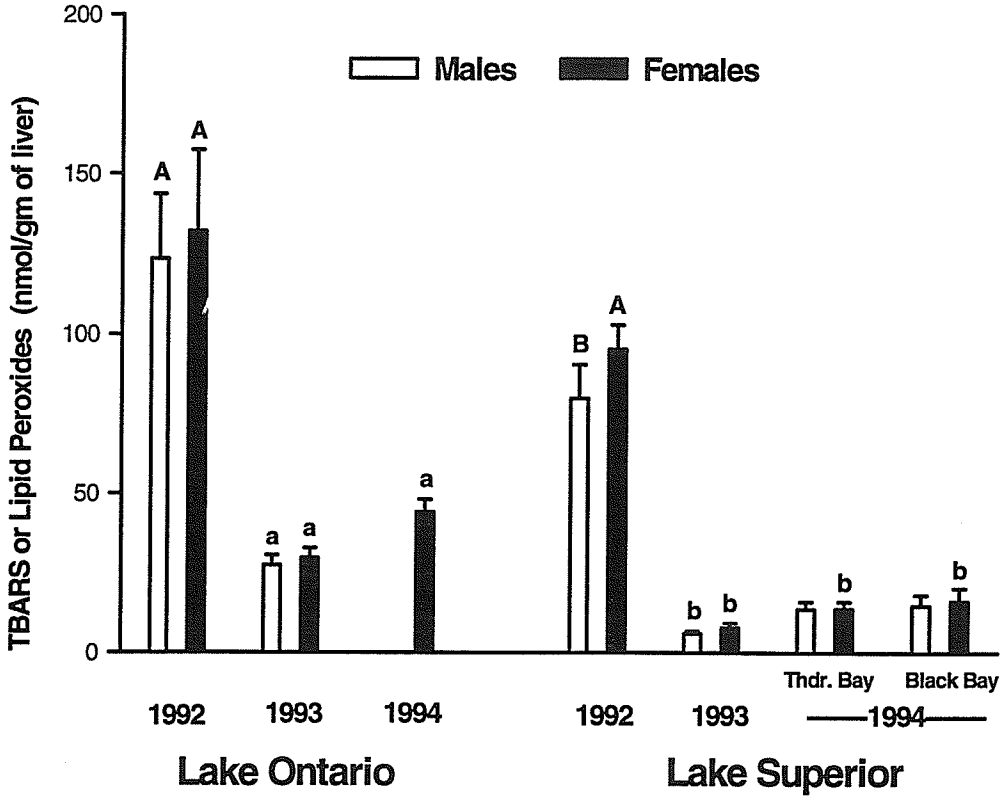

Males
Females
A
B
C
Retinol (ng/gm of liver)
Didehydroretinol (ng/gm of liver)
Retinyl Palmitate (µg/gm of liver)
1992
1993
1994
Thdr. Bay
Black Bay
1994
Lake Ontario
Lake Superior

Figure 3.6: Concentrations of TBARS, for 1992, or lipid peroxides, for 1993 and 1994, in liver of lake trout collected from Lake Ontario (n=30) and the Black Bay (n=10 males/7 females) and Thunder Bay (n= 13 males/7 females) areas of Lake Superior. Both variables are presented as nmol per gm of wet liver weight. Data are presented as mean with standard error. Dunnett's one-tailed t-test was used to test whether TBARS and lipid hydroperoxide concentrations were higher in livers of lake trout from Lake Ontario than in livers of lake trout from Lake Superior. Means from Lake Ontario labelled with the same letter as means from Lake Superior are not significantly higher for comparisons within the same year and for the same sex (α=0.05).


200
150
100
50
0
TBARS or Lipid Peroxides (nmol/gm of liver)
Males
Females
A
A
a
a
a
B
A
b
b
b
b
Thdr. Bay
Black Bay
1992
1993
1994
1992
1993
1994
Lake Ontario
Lake Superior

Chapter 4

Relationships Among Oxidative Stress, Antioxidant Vitamins and Early Mortality Syndrome of Lake Trout (*Salvelinus namaycush*) From Lake Ontario

Abstract

Virtually complete mortality of offspring from certain female lake trout in Lake Ontario appears just before swimup, and is known as early mortality syndrome (EMS). Adult lake trout from Lake Ontario also have high tissue concentrations of organic contaminants and elevated activities of hepatic Phase I (MFO) biotransformation enzymes. Previous examinations of lake trout with elevated Phase I activity have also identified increased indices of oxidative stress. Phase I activity, enzymatic (SOD, CAT, GPx) and non-enzymatic antioxidants (vitamins A, C and E), and oxidative membrane breakdown (lipid hydroperoxides) were quantified in livers of adult female lake trout from Lake Ontario and from a reference population in Lake Manitou in order to examine this relationship. Eggs from each of the females were reared in the laboratory and analyzed 6 times before the swimup stage for concentrations of the antioxidant vitamins A, C and E. Phase I capacity of the fry was assessed only at the swimup stage. Adults whose offspring had high EMS had lower content of vitamin E compared to the low EMS group from Lake Ontario. Hepatic lipid hydroperoxides in adults, indicative of oxidative membrane breakdown, were strongly correlated with the appearance of EMS in the offspring of the high EMS group. Vitamin concentrations were comparable in eggs from lake trout with and without EMS from Lake Ontario and reference lake

trout. Although there was no correlation between Phase I capacity or vitamin concentrations and the prevalence of EMS at any of the developmental stages, only survivors of the high EMS group could be analyzed at the later stages of development. Measurements of additional indices of oxidative stress, including enzymatic defenses and low molecular weight scavengers are required, particularly at developmental stages near the appearance of EMS.

Introduction

Attempts to re-establish self-sustaining lake trout populations in the lower Great Lakes through massive hatchery stocking efforts have been disappointing (Gilbertson 1992). In addition to other factors, the lack of recruitment is related to high mortality of lake trout fry at a specific time during their development. This occurs just before the swimup stage when the yolk sac has been almost completely resorbed, and has been referred to as early mortality syndrome (EMS) (Mac and Gilbertson 1990). EMS is distinct from blue-sac mortality previously described for lake trout from Lake Ontario (Symula et al. 1990) and is characterized by edema, a loss of equilibrium, abnormal swimming patterns, fry which lie on their sides on the bottom of the tank and hyperexcitability, culminating in death (Fitzsimmons et al. 1995, Fitzsimmons 1995). Previous reports indicate that up to 50% of female lake trout from Lake Ontario have offspring exhibiting EMS and that, among these, offspring mortality is near 100% (Mac 1990).

There is some evidence to suggest a link between organochlorine contaminants and EMS. For example, EMS is prevalent in the more contaminated lower Great Lakes, occurs at the end of yolk absorption when the body burden of organochlorines is greatest and is more common among

populations of the Great Lakes with induced mixed function oxidase (MFO) enzyme activity (Hodson et al. 1990, Mac et al. 1985). Finally, EMS closely resembles the fry mortality syndrome of salmonids from contaminated Swedish waters that is referred to as M74 (Bengtsson et al. 1994). However, direct linkages between EMS and specific contaminants or toxic mechanisms have not yet been adequately examined in fish from the Great Lakes (Mac and Gilbertson 1990).

Oxidative stress resulting in cellular and subcellular membrane breakdown has received increasing attention in fish as a toxic mechanism resulting from organochlorine exposure (Livingstone et al. 1993, Thomas and Wofford 1993). Specifically, coplanar organochlorines that interact with the Ah receptor and induce MFO enzymes have been shown to induce oxidative stress resulting in cellular and subcellular membrane breakdown in juvenile lake trout (Chapter 2). Indices of greater oxidative stress in the livers of adult lake trout from Lake Ontario compared to lake trout from less contaminated sites in Lake Superior, have also been presented (Palace et al. 1996b). There has been speculation connecting the lesions associated with both EMS and the toxicity of organochlorines, which are structurally similar to TCDD, to oxidative stress and lipid peroxidation (Spitsbergen et al. 1991). However, the specific relationships between oxidative stress, membrane damage and EMS exhibited by the offspring of female lake trout from Lake Ontario have not yet been

investigated. Given that edema resulting from membrane damage and increased permeability within the cardiovascular system are characteristics preceding the death of lake trout fry with EMS (Fitzsimmons et al. 1995), examination of this relationship is warranted.

To examine this relationship, female lake trout captured during 1994 from Lake Ontario, and from a less contaminated reference site in Lake Manitou, were assessed for several indices of oxidative stress. Liver concentrations of enzymatic and non-enzymatic antioxidants as well as lipid hydroperoxides, an indicator of oxidation in cellular and subcellular membranes (Ohishi et al. 1985), were included in this evaluation. Potential for the generation of oxyradicals by induced MFO biotransformation enzymes were assessed in both adult females lake trout and their offspring. In addition, eggs stripped from each corresponding female were fertilized, reared in the laboratory, and monitored throughout their development to the swimup stage for survival, the appearance of EMS, and concentrations of non-enzymatic antioxidant vitamins.

Materials and Methods

Collection of Adults and Gametes

Live broodstock lake trout were captured from a contaminated site in Lake Ontario (n=30), near Port Weller,

and from a less contaminated reference site in Lake Manitou (n=6) in October and November of 1994 (Fig. 4.1). All lake trout were captured during spawning, using small mesh trap nets set over gravel spawning substrate. Only ripe and running (ie. ovulated) live females, with no evidence of protein precipitation (which indicates over-ripening) in the eggs, were selected. The captured lake trout were all between 5 and 14 years of age according to otolith annuli determinations, and weighed 3,664 ± 197 gm from Lake Ontario and 2,430 ± 319 gm from Lake Manitou. After removal from the nets, fish were transported to shore in tanks aerated with fresh circulating lake water.

Fish were anesthetized in MS-222 (80 mg L^{-1}) rinsed in freshwater, blotted dry and weighed and measured. Eggs were manually expressed into a dry graduated glass container to determine total egg volume. The eggs were then transferred to dry glass jars and held, on ice, for transport to the laboratory. A glass pipette inserted into the urogenital opening was used to collect 1-2 ml of semen. Semen was also transported, on ice, in dry glass jars to the laboratory. Transport time was in all cases less than 6 hours.

Following collection of their eggs, adult female fish were sacrificed by a blow to the head. Liver tissue was removed, placed in sterile plastic bags, frozen between slabs of dry ice for transport to the laboratory and then stored at -100 ± 10°C until analysis. Phase I and Phase II enzyme

activities and non-enzymatic antioxidant vitamins A, E and C were determined as previously described in Chapter 3 by the method of Palace and Brown (1994).

Fertilization

Sperm from at least 6 males from the corresponding lake was pooled. Three subsamples of approximately 200 eggs from each individual female were fertilized with 100 µL of semen per 20 ml of eggs. The semen and eggs were gently mixed in plastic petri dishes, covered with water (9°C) for 15 minutes and then rinsed 3 times with fresh water. After rinsing, eggs were randomly placed in horizontal flow raceways and reared at 8°C in water which had greater than 80% oxygen saturation. A photoperiod consisting of 12 hours light:12 hours dark was used throughout egg development. The percentage of successful fertilization for eggs from each female was determined and eggs or fry were examined daily for mortality and developmental abnormalities. Fry exhibiting any signs of EMS (eg. loss of equilibrium, lying on their sides on the bottom of the tank, hyperexcitability) were removed daily.

Subsamples of the eggs or fry obtained at green egg (0 degree days), epiblastula (235 degree days), eyed (433 degree days), posthatch (600 degree days), modal hatch (750 degree days) and swimup (900 degree days) stages were placed in sterile vials, immediately frozen on dry ice and stored at -

100 ± 10°C until analysis. Degree days expresses the cumulative temperature of incubation as a product of time at a given temperature. For example a fish reared at 8°C for 10 days would have undergone 80 degree days (i.e., 8° X 10days = 80 degree days) of development. Vitamin A and E concentrations were determined using 10 eggs in the extraction and analysis methods previously described in Chapter 3 by the method of Palace and Brown (1994). Vitamin C content of the eggs was determined by homogenizing 5 eggs in 1 ml of DDW and then using an aliquot of this homogenate in the spectrophotometric method of Jagota and Dani (1982). A separate subsample of eggs or fry taken at corresponding developmental stages were fixed in 10% buffered formalin (pH=7.0). They were then embedded in paraffin, sectioned into 5 µm thick slices, stained and analyzed for cyp1A protein concentration by the immunohistochemical method described by Smolowitz et al. (1991).

Data Analysis

All data were analyzed using a completely randomized one way ANOVA with statistical significance set at $P<0.05$. Duncan's multiple range test was used to compare treatment means. Treatment means were deemed significantly different at the $\alpha=0.05$ level of confidence. Simple linear regression analysis was employed to examine correlations between the

percentage of EMS in embryos and biochemical indices of oxidative stress in adult and embryonic fish. Coefficients of determination and p-values are noted where applicable.

Results and Discussion

Reproductive Parameters and Oxidative Stress in Adult Lake Trout

Reproductive parameters and the biotransformation enzyme activities of adult female lake trout captured from Lake Ontario and Lake Manitou are summarized in Table 4.1. To allow comparisons based on the prevalence of EMS, the lake trout from Lake Ontario have been divided into two sub-populations: those whose offspring exhibited more than 50% mortality due to EMS and those with less than 10% mortality from EMS. Females with offspring that had >50% EMS comprised 27% of all adult female lake trout captured from Lake Ontario while females with offspring that had <10% EMS made up 57% of the studied population. Only 5 females (17% of females) were captured whose offspring showed between 10 and 50% EMS. Because this group represents a relatively small proportion of the total collection, and since there is a greater degree of error in separating true EMS from other losses in these offspring, data from this group have been excluded from the current discussion. None of the female lake trout captured at Lake Manitou had

offspring that exhibited EMS type mortality.

The percentage of female lake trout from Lake Ontario whose offspring exhibited EMS was similar to previous studies, which reported values between 20 and 50% (Casselman 1990, Fitzsimmons et al. 1995). Overall mortality of fry from pooled egg samples taken from Lake Ontario lake trout has also been measured as high as 50% (Mac 1990). The percentage of successful fertilization was not significantly different between the two sub-populations from Lake Ontario and the Lake Manitou population. While eggs from the >50% EMS group had a smaller diameter than the eggs from the <10% EMS Lake Ontario population and the lake trout from Lake Manitou, the smaller egg diameter did not correlate with the prevalence of EMS.

Although not different from each other, the two sub-populations of lake trout from Lake Ontario had significantly greater hepatic activities of Phase I or MFO biotransformation enzyme activity, measured as ethoxyresorufin-O-deethylase (EROD), than the lake trout from Lake Manitou (Table 4.1). EROD activities in liver of lake trout from Lake Ontario in this experiment were similar to activities previously found for lake trout from Lake Ontario (L.Lockhart and D.A. Metner, unpub. data, Dept. of Fisheries and Oceans, Winnipeg, MB). Induced MFO enzyme activity in livers of fish is a reliable indicator of exposure to planar organic contaminants that are structurally similar to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Melancon et al. 1988). Induced activity has also been linked to

production of eggs with reduced viability and survival and greater incidence of malformations (Spies and Rice 1988, Branchaud et al. 1995). It is therefore, not surprising that lake trout from Lake Ontario, a system known to be contaminated with a variety of organic compounds similar to TCDD (Baumann and Whittle 1988, Whittle et al. 1992), have greater hepatic MFO activity than lake trout from the proximate, but relatively uncontaminated, Lake Manitou system.

Activities of the phase II enzyme, glucuronyltransferase (UGT), were not significantly different between any of the three lake trout populations (Table 4.1). Following the initial biotransformation of contaminant molecules by MFO enzymes, Phase II enzymes conjugate these substrates with polar endogenous molecules so that the entire complex can be more readily excreted (Clarke et al. 1991). Exposure to organic contaminants that induce MFO enzyme activity can also induce activity of phase II enzymes. However, Phase II enzyme activity is often not as responsive to contaminant induction as MFO enzymes (Jimenez and Stegeman 1990), especially in fish (Ianelli et al. 1994). Similar UGT activities in livers of lake trout from Lake Ontario and Lake Manitou were, therefore, expected.

We have previously shown that fish exposed to planar organic contaminants have induced activities of Phase I enzymes that correlate with greater indices of oxidative stress in Chapter 2 and 3 (Palace et al. 1996a, Palace et al. 1996c).

These indices include alterations in antioxidant enzyme activities, depletion of non-enzymatic antioxidants and elevated concentrations of membrane breakdown byproducts. Concentrations of the non-enzymatic antioxidant vitamins A, C and E and activities of the enzymatic antioxidants SOD, CAT and GPx, in liver of the two sub-populations of lake trout from Lake Ontario, and from Lake Manitou, are given in Table 4.2. The mechanisms and importance of each of these antioxidants for protecting cells against oxidative damage have been previously discussed in Chapter 1 (Palace et al. 1996b, Palace and Klaverkamp 1993). Concentrations of hepatic lipid hydroperoxides, which are elevated by oxidative membrane breakdown (Ohishi et al. 1985), are also given in Table 4.2.

While liver concentrations of retinol were similar among all of the lake trout, didehydroretinol was more concentrated in livers of the two sub-populations of lake trout from Lake Ontario than in livers of lake trout from Lake Manitou. The greater prevalence of didehydroretinol in lake trout from Lake Ontario is probably due more to dietary differences, and specifically to a greater intake of astaxanthin, a provitamin form of retinol (Guillou et al. 1989), than to the presence of contaminants in Lake Ontario (Palace et al. 1996b). While vitamin A may be depleted because of its activity as an antioxidant (Ribera et al. 1991) or because of direct metabolism by Phase I and Phase II enzymes (Gilbert et al. 1995, Bank et al. 1989), it is also an important morphogen in

fish (Holder and Hill 1991). Increased metabolism, or additional use as an antioxidant, of the vitamin following exposure to contaminants could then affect the stores available to support early development. In fact, edema and cranial malformations induced in fry exposed to organochlorines (Walker et al. 1994, Walker et al. 1992, Spitsbergen et al. 1991), resemble those of retinol deficiency (Poston et al. 1977). Similar connections have been proposed for mammalian and avian (Gilbertson et al. 1991, Spear et al. 1992) model systems.

Retinol and didehydroretinol concentrations in the livers of adult female lake trout from this experiment were not correlated with the presence of EMS in their offspring. However, further examination of biotransformation potential of Lake Ontario lake trout is required, especially concerning retinoic acid, the most important morphogenic form of vitamin A (Chambon et al. 1991). Effects on the deposition of retinoids in oocytes also need to be examined, and are considered later, in the "Eggs and Larval Fish" discussion.

Vitamin C was significantly higher in the livers of both sub-populations of adult lake trout from Lake Ontario than from Lake Manitou (Table 4.2). Linear regression analysis revealed a strong relationship between these elevated vitamin C concentrations in the livers of female lake trout from Lake Ontario and the prevalence of EMS in their offspring (R^2 = 0.75, $p<0.005$). While antioxidant activity of ascorbic acid is widely accepted (Chatterjee and Nandi 1991), it may conversely

act as an oxidant even at physiological concentrations, when vitamin E concentrations are low (Staats and Colby 1989, Miller and Aust 1989).

Vitamin E concentrations in livers of lake trout from Lake Ontario whose offspring exhibit >50% EMS were significantly lower than those whose offspring had <10% EMS. While activities of the enzymatic antioxidants SOD and GPx were comparable in livers of all adult female lake trout, CAT activity was significantly lower in the livers of lake trout whose offspring had >50% EMS. Although CAT activity did not correlate with the presence of EMS, lower hepatic activity of the enzyme has been found in fish exposed to contaminants that induce oxidative stress (Matkovics et al. 1984). Lower activity of the enzyme would certainly afford less protection against oxidant generation by H_2O_2-dependent mechanisms in fish whose offspring had >50% EMS (Palace and Klaverkamp 1993).

The balance of CAT, vitamins C and E, as well as the overall balance of pro-oxidants versus antioxidants, could be factors in determining the oxidative membrane breakdown in livers of lake trout from Lake Ontario. The mean concentrations of lipid hydroperoxides, a measure of membrane breakdown, were not significantly different between the two sub-populations of lake trout from Lake Ontario. However, lipid hydroperoxides were correlated with the prevalence of EMS only in lake trout whose offspring exhibited >50% EMS from Lake Ontario (Fig. 4.2), suggesting that there may be a measure of predisposition

toward EMS in offspring of lake trout with greater lipid hydroperoxide concentrations.

High indices of oxidative stress in the adult lake trout from Lake Ontario that we reported in the previous chapter (Palace et al. 1996b) motivated the current examination of links between biotransformation enzyme activity, oxidative stress and EMS among the offspring in the current experiment. Furthermore, the strong correlations of EMS with membrane breakdown in high EMS lake trout from Lake Ontario indicate that an investigation of the ontogeny of Phase I activity and antioxidant defenses in eggs and larval stages is warranted.

Eggs and Larval Fish

It has been known for some time that the capacity for Phase I induction increases in salmonid embryos near the time of hatching (Binder and Stegeman 1983). Comparisons of the Phase I capacity of offspring from the current experiment were made only at the swimup stage of development (Table 4.3). At this stage, the embryo has full capacity for Phase I induction and has been exposed to the cumulative burden of maternally deposited contaminants that were present in the yolk before absorption (Walker et al. 1994). The immunohistochemical method for determining Phase I capacity is dependent on assignment of subjective scores based on the occurrence and intensity of staining, and has previously been well correlated with other

methods of analysis (Smolowitz et al. 1991). The subjective scores resulting from immunohistochemical analysis of Phase I proteins showed no significant differences between the lake trout embryos with and without EMS from Lake Ontario, and lake trout embryos from Lake Manitou (Table 4.3).

There was no significant difference in the amount of didehydroretinol per egg between the two sub-populations from Lake Ontario and the lake trout from Lake Manitou at any of sampled stages of embryonic development (Fig. 4.3, panel A). Similarly, there was no consistent differences in the content of the dominant pro-retinoid pigment, astaxanthin, in embryos from any of the lake trout populations (Fig. 4.3, panel B). Retinol concentrations in the green eggs of lake trout from Lake Ontario were initially 2.5 to 3 times lower than green eggs from Lake Manitou (data not shown), again, probably due to dietary differences among the adult lake trout from the two lakes. Once these eggs had reached the modal-hatch stage, retinol concentrations in both sub-populations from Lake Ontario fell below the detection limit. Concentrations of vitamins C (Fig. 4.4, panel A) and E (Fig. 4.4, panel B) were, with a few exceptions, not different between any of the populations at the 6 stages of embryonic development.

While we are not aware of any previous quantifications of retinol and didehydroretinol in fish eggs or larvae, concentrations of both of these parameters from lake trout in this experiment were near those found in ovaries of rainbow

trout (*Salmo gairdneri*) (Guillou et al. 1989). Astaxanthin, the predominant carotenoid pigment quantified in embryos of all lake trout from this study, was present in green eggs in concentrations (2.3 to 3.4 µg gm^{-1}) within the range that Ando and co-workers (1989, 1991) had previously reported (0.036 to 7.2 µg gm^{-1}) for a number of salmonids including the genus *Oncorhynchus*. Similarly, concentrations of vitamins C and E were comparable at 4 different stages of development to those previously reported for salmon eggs (Cowey et al. 1985). Dabrowski and Blom (1994) measured vitamin C concentrations in livers of adult female rainbow trout (*Cncorhynchus mykiss*), deposition of the vitamin in their eggs, and disappearance of the vitamin during development and obtained results that were all virtually identical to the same measurements for lake trout in this experiment.

Because of the high content of unsaturated fatty acids in eggs and increasing aerobic metabolism that generates oxidative radicals in developing embryos, and because antioxidant enzyme activity develops relatively late in embryonic development, early antioxidant protection by vitamins is essential (Cowey et al. 1985). However, similar vitamin A, C and E concentrations were maintained in lake trout embryos from Lake Ontario, with and without EMS, and lake trout from Lake Manitou, throughout their development. Maintenance of the vitamin A concentration deposited in eggs has also been shown in white suckers (*Catostomus commersoni*) even when their liver stores have been

depleted (Branchaud et al. 1995). An absence of any correlations between vitamin concentrations and the presence of EMS suggests that EMS is not related to greater oxidative stress resulting from vitamin deficiency in the embryo. It should be noted, however, that live embryos are required for analyses of vitamins. The group showing EMS would, therefore, have had its mortalities excluded from the analysis at the later stages of development, possibly concealing an effect. More extensive evaluation of developmental stages that are closer in embryonic development to the stage at which EMS appears are required before the relationship between oxidative stress and EMS can be clarified. Measurements of additional indices of oxidative stress in the embryo, including enzymatic defenses and low molecular weight scavengers, are also needed.

The lack of correlation between antioxidant vitamins measured in embryos of lake trout from this study and EMS is significant in light of other recent information. Specifically, strong correlations between M74, a mortality syndrome similar to EMS, and oxidative stress, measured as malonaldehyde concentrations, have been reported for salmon from Swedish waters (Pettersson 1995). Important differences in the biology of salmon and lake trout may explain the different relationships. For example, spawning activity in salmon is accompanied by a significant period of fasting which alone has been shown to increase indices of oxidative stress in mammalian systems (Pohjanvirta et al. 1990). Since lake trout do not

restrict feeding to the same extent during spawning, this factor would have less of an influence on oxidative stress parameters.

Summary and Conclusions

Analyses of several biochemical indicators in adult female lake trout from Lake Ontario suggests that increased oxidative stress in these fish may predispose their offspring to develop a characteristic early mortality syndrome (EMS). Specifically, we found a strong correlation between lipid hydroperoxides, an indicator of lipid membrane breakdown, and EMS in lake trout whose offspring exhibit >50% EMS. Deposition of antioxidant vitamins A, C and E into eggs by the females does not appear to be correlated with the development of EMS. The vitamins are depleted in lake trout embryos with and without EMS in a similar manner throughout their development. Additional analysis of the embryos, particularly at developmental stages near the manifestation of EMS, for indices of oxidative stress may clarify the relationships of oxidative stress and EMS in lake trout from Lake Ontario.

Table 4.1: Reproductive variables and biotransformation enzyme activities of adult lake trout from Lakes Ontario and Manitou. Data are expressed as mean with standard error. Means labelled with different letters are significantly different from each other ($p<0.05$).

Location	n	% of females per site	Mean % fertilization	Mean % EMS	Egg Diam. (mm)	EROD (nmol/min/mg of protein)	UGT (nmol/min/mg of protein)
L.Ont. >50% EMS	8	27	93.6 ± 1.1^{a}	81.6 ± 6.1^{a}	5.2 ± 0.1^{a}	0.52 ± 0.15^{a}	0.21 ± 0.06^{a}
L. Ont. <10% EMS	17	57	90.1 ± 2.0^{a}	1.6 ± 0.9^{b}	5.5 ± 0.1^{b}	0.66 ± 0.11^{a}	0.29 ± 0.05^{a}
L. Manitou	6	100	86.7 ± 3.0^{a}	0^{b}	5.5 ± 0.1^{b}	0.22 ± 0.03^{b}	0.24 ± 0.04^{a}

Table 4.2: Antioxidants and lipid hydroperoxide concentrations in liver of adult lake trout from Lakes Ontario and Manitou. Data are expressed as mean with standard error. Means labelled with different letters are significantly different from each other ($p<0.05$). [1] One unit of SOD inhibits autooxidation of epinephrine by 50% at pH=7.0, 25 °C. [2] One unit of CAT decomposes 1.0 µmol of H_2O_2 per minute at pH=7.0, 25 °C. [3] One unit of GPx catalyzes the oxidation of 1.0 µmol of reduced glutathione by H_2O_2 to oxidized glutathione per minute at at pH=7.0, 25 °C.

Location	Retinol (µg/gm of liver)	Didehydro-retinol (µg/gm of liver)	Vitamin C (µg/gm of liver)	Vitamin E (µg/gm of liver)	SOD (Units per mg liver protein)1	CAT (Units per mg liver protein)2	GPx (Units per mg liver protein)3	Lipid Hydroper. (nm/gm of liver)
L.Ont. >50% EMS	0.80 ± 0.17^{a}	11.5 ± 3.7^{a}	194.3 ± 24.6^{a}	418.7 ± 106.0^{a}	54.9 ± 13.1^{a}	864 ± 82^{a}	0.067 ± 0.004^{a}	40.9 ± 3.5^{a}
L. Ont. <10% EMS	0.90 ± 0.13^{a}	9.5 ± 1.8^{a}	225.2 ± 26.1^{a}	889.9 ± 191.2^{b}	50.2 ± 6.1^{a}	1150 ± 65^{b}	0.058 ± 0.003^{a}	39.3 ± 4.4^{a}
L. Manitou	0.81 ± 0.18^{a}	1.3 ± 0.3^{b}	124.7 ± 12.7^{b}	414.7 ± 75.5^{a}	48.3 ± 8.5^{a}	1415 ± 59^{b}	0.060 ± 0.002^{a}	26.5 ± 3.4^{b}

Table 4.3: Scores for immunohistochemical determination of Phase I protein in lake trout embryos. Data are expressed as mean with standard error. Means labelled with the same letter are not significantly different from each other ($p<0.05$)

Location	Scores for Immunohistochemical Phase I Protein Analysis
Lake Manitou	6.25 ± 1.67[a]
Lake Ont. >50% EMS	5.50 ± 1.02[a]
Lake Ont. <10% EMS	4.91 ± 0.75[a]

Figure 4.1: Sample locations for lake trout collections.

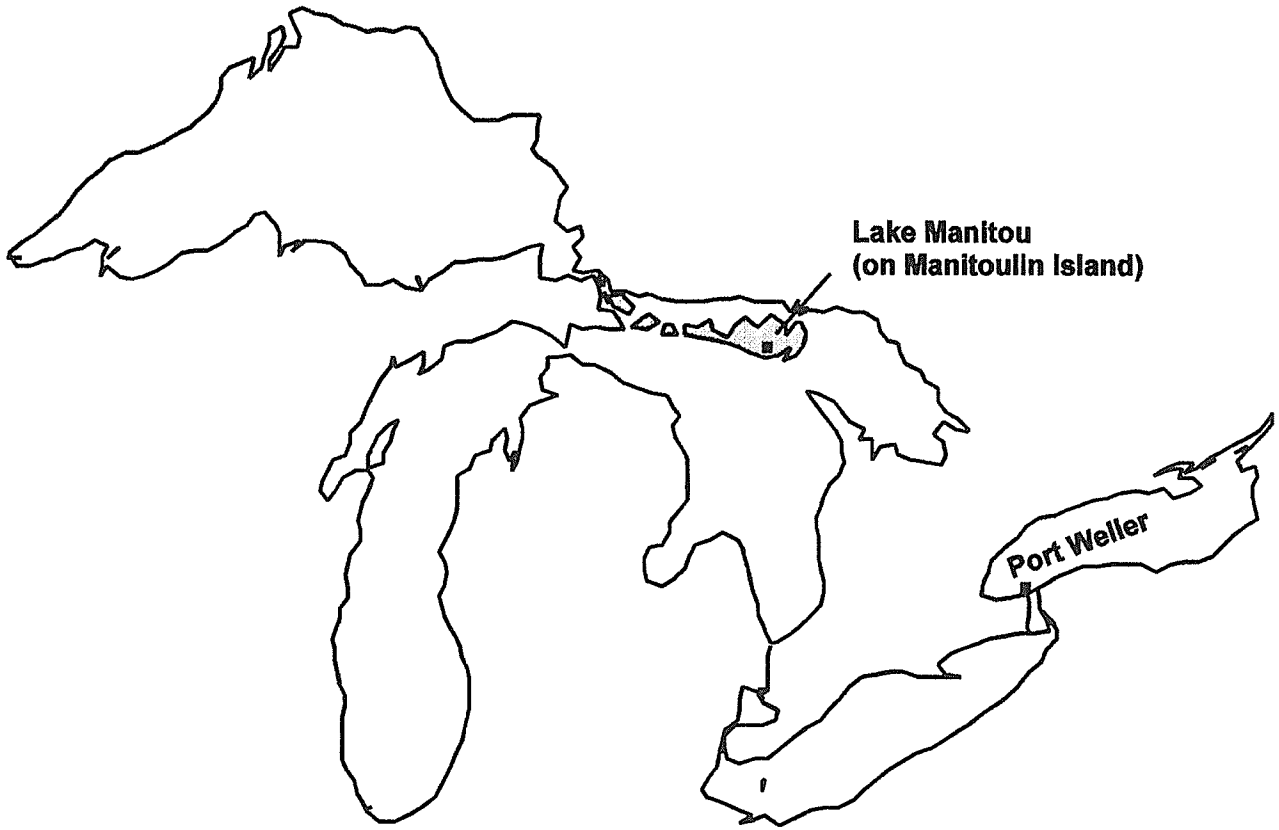

Lake Manitou
(on Manitoulin Island)
Port Weller

Figure 4.2: Linear regression plot, including 95% confidence intervals, of hepatic lipid hydroperoxides in adult female lake trout whose offspring exhibit >50% EMS against the actual percentage of EMS in their offspring.

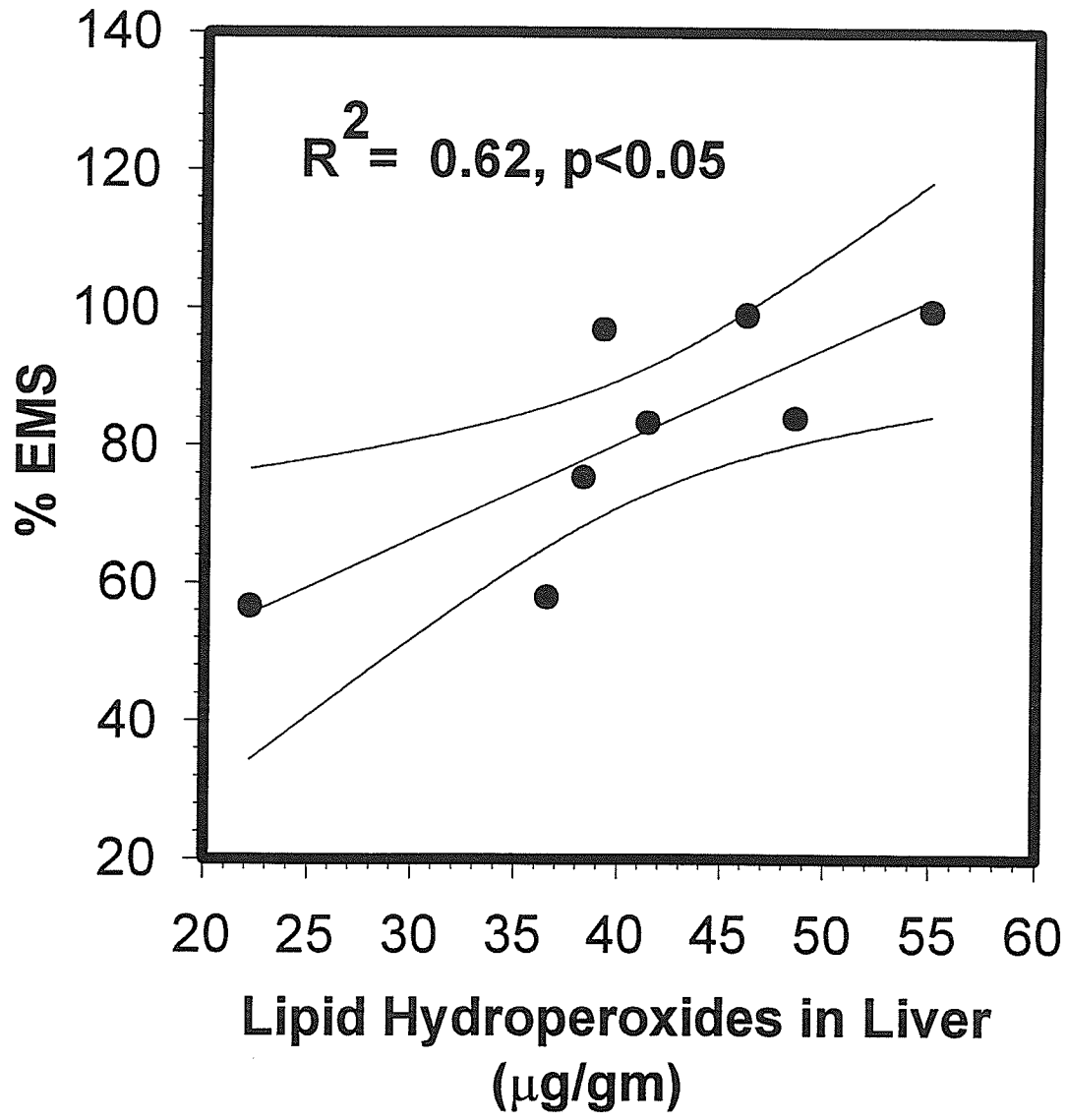

140
120
100
80
60
40
20
% EMS
R^2= 0.62, p<0.05
20 25 30 35 40 45 50 55 60
Lipid Hydroperoxides in Liver
(μg/gm)

Figure 4.3: Concentrations (µg gm^{-1} embryos) of didehydroretinol (panel A) and the proretinoid pigment, astaxanthin (panel B), in lake trout embryos with and without EMS from Lake Ontario and from Lake Manitou at six stages of embryonic development. Data are expressed as mean ± standard error (n=8,17 and 6 for >50% EMS population from Lake Ontario, <10% EMS population from Lake Ontario and Lake Manitou populations respectively). Means labelled with * are significantly different from the other two means for the same sample period, as determined by Duncan's multiple range test ($p<0.05$).

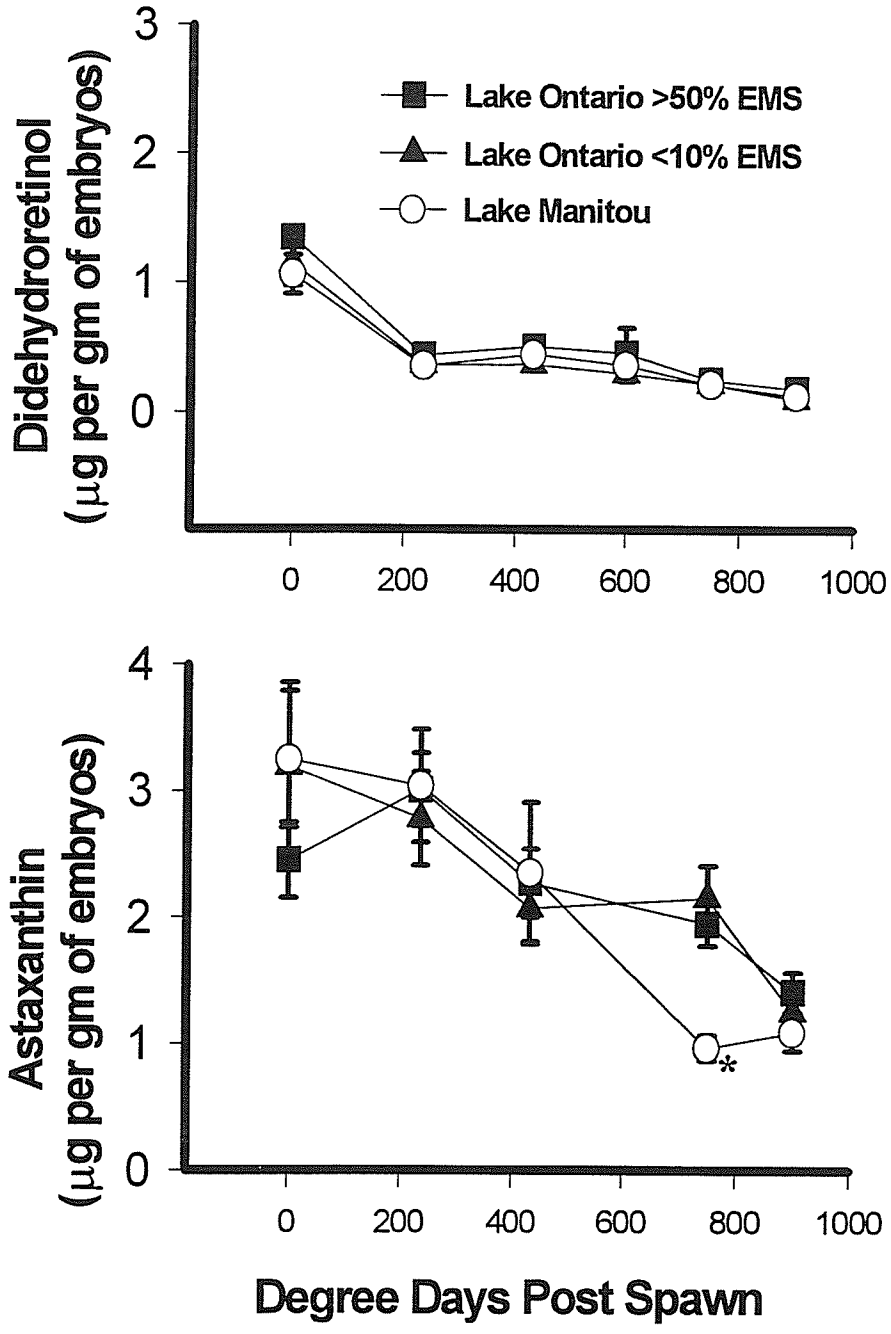
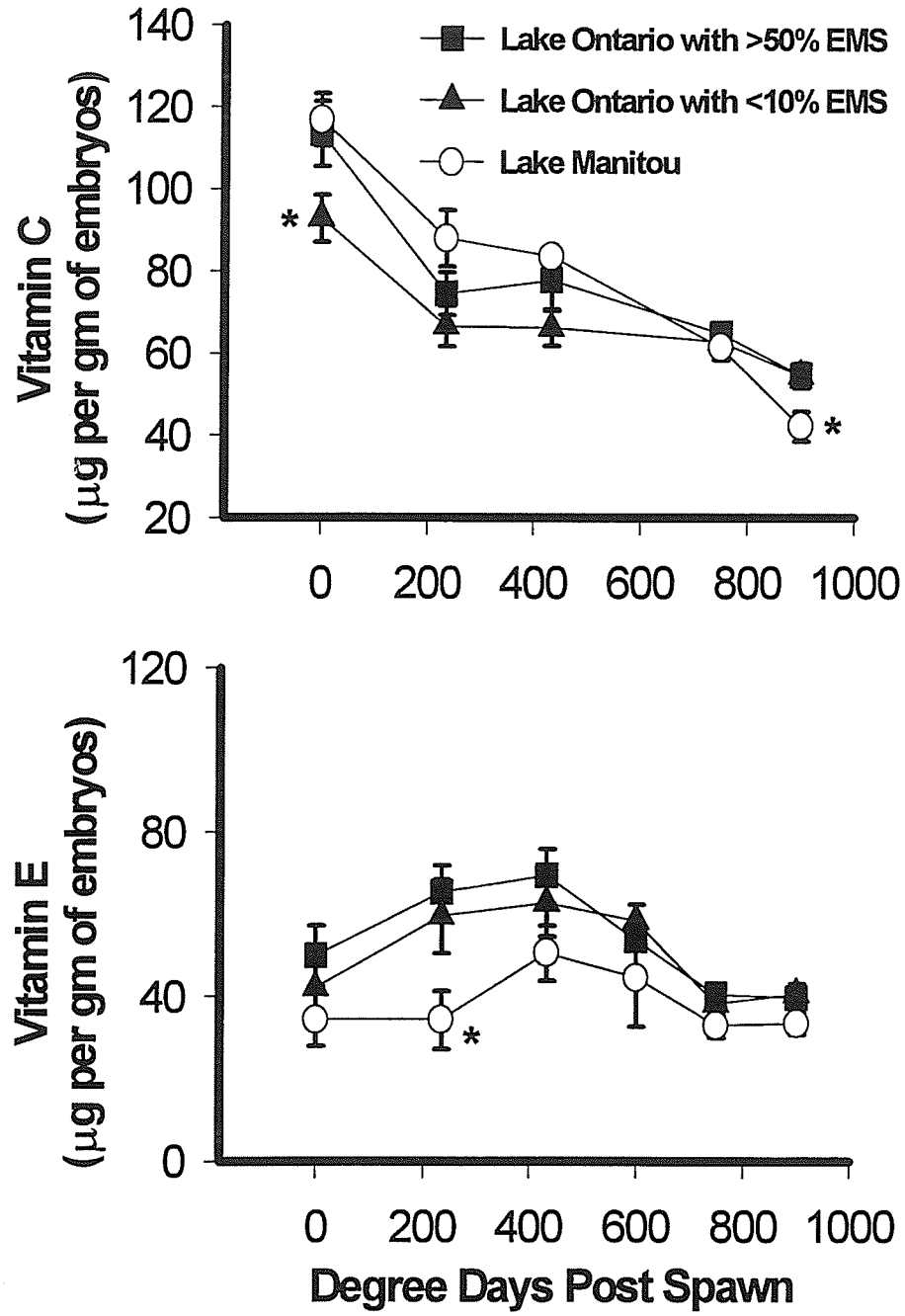

Didehydroretinol
(µg per gm of embryos)
3
2
1
0
Lake Ontario >50% EMS
Lake Ontario <10% EMS
Lake Manitou
0
200
400
600
800
1000
Astaxanthin
(µg per gm of embryos)
4
3
2
1
0
*
0
200
400
600
800
1000
Degree Days Post Spawn

Figure 4.4: Concentrations (µg gm^{-1} embryos) of vitamin C (panel A) and vitamin E (panel B) in lake trout embryos with and without EMS from Lake Ontario and from Lake Manitou at six stages of embryological development. Data are expressed as mean ± standard error (n=8,17 and 6 for >50% EMS population from Lake Ontario, <10% EMS population from Lake Ontario and Lake Manitou populations respectively). Means labelled with * are signifcantly different from the other two means for the same sample period, as determined by Duncan's multiple range test ($p<0.05$).


Lake Ontario with >50% EMS
Lake Ontario with <10% EMS
Lake Manitou
Vitamin C
(µg per gm of embryos)
140
120
100
80
60
40
20
0
200
400
600
800
1000
*
*
Vitamin E
(µg per gm of embryos)
120
80
40
0
*
Degree Days Post Spawn

Chapter 5

Insights into Retinoid Metabolism Provided by Recovery of ^{3}H-Retinol from Tissues of Lake Trout (*Salvelinus namaycush*) Pre-exposed to 3,3',4,4',5-Pentachlorobiphenyl (PCB 126)

Abstract

To provide insight into retinoid metabolism in PCB-exposed fish, juvenile lake trout were orally exposed to 0, 3, 10 or 30 µg PCB 126 kg^{-1} bodyweight for 12 weeks. Phase I (MFO) enzyme activity was induced by 60-, 177- and 450-fold, while Phase II enzyme activity was 2.3-, 2.5- and 5-fold higher in the 3,10 and 30 µg PCB kg^{-1} dose groups, respectively, versus the fish untreated with PCB. After 12 weeks, cannulae were inserted into the dorsal aorta. Two days later ^{3}H-retinol was injected as a bolus in charcoal-stripped plasma through the cannula. Blood samples were obtained 15 and 30 minutes and 1,3,6,15,30,50 and 75 hours after the injection. The radioactivity recovered from plasma was compared for each PCB dose group and was not significantly different for any of the treatments at any of the sample times. However, greater radioactivity recovered from bile after 75 hours indicates accelerated metabolism of ^{3}Hretinol with increasing PCB dose. Tissue distributions of aqueous and lipophilic soluble radioactivity indicates that direct metabolism of retinol by MFO and phase II conjugation enzymes is probably responsible for depletion of retinoids in PCB 126-exposed lake trout.

Introduction

Retinol and its derivative forms, collectively known as vitamin A, are essential for vision, maintenance of epithelial tissues, growth and reproduction (Zile 1992). Vitamin A stores have been shown to decline in tissues from birds (Spear et al. 1990), mammals (Hakansson et al. 1992) and fish (Ndayibagara et al. 1995, Palace and Brown 1994) exposed to organic contaminants that interact with the cellular Ah receptor. By interacting with the Ah receptor, planar organic contaminants induce the biotransformation enzyme system consisting of phase I or mixed function oxidases (MFO) and phase II or conjugation enzymes (Dillon et al. 1990, Jimenez and Stegeman 1990). While MFO and conjugation enzymes have shown utility as biomarkers for exposure to Ah-active contaminants, their linkage to fundamental mechanisms of toxicity have not been clearly established. However, induction of biotransformation enzymes may be directly linked to depleted tissue stores of vitamin A (Zile 1992).

Several mechanisms have been proposed to explain the depletion of vitamin A that appears concurrently with induction of biotransformation enzymes. First, certain retinoids may be directly metabolized by phase I and phase II enzymes, producing more polar vitamin A forms that are readily excreted (Gilbert et al. 1995). Secondly, organic contaminant metabolites, such as those arising from PCB metabolism, may disturb the binding

of retinol to the retinol-binding protein(RBP)-transthyretin complex. The liberated retinol could then be excreted in the urine due to increased filtration through the glomerular membrane (Brouwer and van den Berg 1986). Exposure to Ah-active compounds has also been correlated with declining vitamin A intake due to loss of appetite (Spear et al. 1994) and reduced capacity to store retinoids because of non-competitive inhibition of esterifying enzymes (Mercier et al. 1990). Finally, induction of MFO enzymes can increase proliferation of oxyradicals and oxidative stress in cellular and subcellular membranes (Palace et al. 1996a or Chapter 2, Lehtinen 1990). Vitamin A exhibits antioxidant activity (Roberfroid 1995) and, therefore, may be increasingly consumed in tissues of MFO-induced and oxidatively stressed organisms (Ribera et al. 1991).

To examine the mechanistic links among organic contaminant exposure, biotransformation enzyme induction, and vitamin A depletion, ^{3}H-retinol was injected directly into the circulation of juvenile lake trout which had been pre-exposed to 0,3,10 or 30 µg PCB 126 kg^{-1} of bodyweight for 12 weeks. Blood plasma and bile radioactivity recovery profiles, as well as tissue distributions of radioactivity and unlabelled retinoids, were examined for each PCB dose group. These measures, in addition to the Phase I and Phase II enzyme activities in liver tissue, allow an assessment of possible mechanisms for depletion of retinoid stores in PCB-exposed lake

trout.

Materials and Methods

Fish Holding

Juvenile lake trout (260 ± 14 gm) were acclimated to dechlorinated Winnipeg city tap water ($CaCo_3$ = 82.5 mg L^{-1}; conductivity = 165 µm hr cm^{-1}; pH = 7.8) in 130 L fiberglass tanks for 5 weeks (10 fish per tank). Each tank received at least 2 L of aerated and dechlorinated Winnipeg city tap water (11.5 - 13.1°C) per gram of fish per day. Fish were fed commercial trout chow pellets (Martin Feed Mills, Elmira, Ont.) at a ration of 1% of bodyweight every second day.

PCB Dosing

3,3',4,4',5-pentachlorobiphenyl (IUPAC congener 126) was purchased from Ultra Scientific (Kingstown, RI) and purified by reverse-phase HPLC to >99.5% according to capillary GC analysis. The PCB was dissolved in 2 ml of ethanol and then added to 9 ml of warmed 60 bloom gelatin (Sigma Chemical Co., St. Louis, Mo.) (Sijm et al. 1990). Fish were anesthetized until they lost equilibrium by immersion in physiological saline containing 0.38 mM MS222 neutralized to tank pH with

sodium hydroxide (Barton et al. 1980). A single oral dose, 1 ml per 250 gm bodyweight, of warmed gelatin/ethanol was delivered directly to the gut of each fish through flared polyethylene tubing (I.D. 1.57 mm) attached to a 16G needle and 3 ml syringe. Four groups of four fish each were randomly assigned to receive nominal dose concentrations of 0 (control), 3, 10 or 30 µg PCB 126 kg^{-1} bodyweight. These doses are near the range found in tissues of lake trout captured from contaminated sites in the Great Lakes (Smith et al. 1990). After dosing, fish were returned to tank water where they recovered equilibrium within 3 minutes. They were then maintained identically to the previous acclimation period for a further 12 weeks.

^{3}H-Retinol Dosing and Blood Sampling

Each fish was lightly anesthetized as before and secured on a surgical table. Aerated physiological saline or saline containing MS222 were alternately directed through the buccal cavity as required, to maintain only slight fin movement. A catheter (18 gauge) completely filled with physiological saline containing 25 Units heparin ml^{-1} was inserted into the aorta through the palate using a 20 gauge needle as described by Brown et al. (1986). A polyethylene tubing cannula (ID. 0.58 mm, O.D. 0.965 mm, total length approximately 100 cm) (Clay Adams, New Jersey), also filled with saline containing 25 U heparin ml^{-1} was fed through the catheter and into the aorta.

The cannula was then sutured in place on the palate and the catheter was removed. An unobstructed blood flow from the aorta through the cannula was confirmed by pulsatory blood flow at the distal end of the cannula. Following insertion, the end of the cannula was sealed with Critoseal plastic putty (Monoject Scientific, St. Louis, Mo.) and fish were returned to tank water where they recovered equilibrium within 5 minutes. During this part of the experiment one fish was housed in each 130 L tank. Tank volume, water depth and cannula length were such that blood could be withdrawn, and heparin-saline solution could be administered, through the cannula above the surface and without handling the fish.

Approximately 6 hours after insertion, the saline containing 25 U heparin ml^{-1} was withdrawn from the cannula using a 1 ml syringe and 16 gauge blunt needle. The volume of saline removed was recorded and replaced with the same volume with an additional 30 µl of saline containing 250 U ml^{-1} heparin delivered through the cannula with a sterile syringe and 16 gauge blunt needle. The extra 30 µl of saline with heparin was injected to inhibit clotting so that blood could be sampled through the cannula. Fish were allowed to acclimate for 2 days after cannula insertion. Unobstructed flow through the tubing was maintained during this period by injecting 10 µl of additional 250 U heparin ml^{-1} in saline daily.

After acclimation, saline from the tubing was withdrawn, and then a clean 1 ml syringe and 16 gauge blunt needle rinsed

with heparin solution (25 U ml^{-1}) were used to withdraw 100 µl of blood. ^{3}H-Retinol (Aldrich Chem. Co., Milwaukee, WI), with a purity of >98.5% based on HPLC quantification, was suspended in plasma that had been stripped of its endogenous retinol, but not the retinol-binding protein, by filtration through activated charcoal. More than 98% of the retinol is typically removed by this method (unpubl. observ.). A 50 µl bolus of this ^{3}H-retinol (5 µCi) in plasma solution was delivered through the cannula followed by the 100 µl of blood withdrawn earlier. The cannula was then refilled with 250 U heparin ml^{-1} of saline.

Blood samples (100 µl) were obtained 15 and 30 minutes and 1,3,6,15,30,50 and 75 hours after the ^{3}H-retinol injection. Following each sample the cannula was refilled with saline/heparin solution and the end was sealed. Blood samples were immediately centrifuged to obtain plasma, which was then frozen at -100 °C until analysis. Retinoid concentrations remain stable at this temperature for at least 5 years (Edmonds and Nierenberg 1993, Comstock et al. 1993).

Analyses

Following the last blood sample, fish were anesthetized in pH-neutralized MS222 until gill movement had ceased (approximately 5 minutes). Liver, kidney and bile were removed from the carcass and immediately frozen at -100 °C until analyzed. MFO activity as represented by ethoxyresorufin-O-

deethylase (EROD) and microsomal protein concentration were simultaneously determined using Kennedy and Trudeau's (1994) modification of the fluorometric microplate method of Kennedy et al. (1993). Phase II conjugation enzyme activity as glucuronyltransferase was measured by the method of Burchell and Whetherhill (1981). Retinoids were extracted by the previously described isocratic HPLC method (Palace and Brown 1994 or Appendix A). Detection in this method has been improved by including propionic acid (1%) in the mobile phase, extending elution times to 25 minutes and by monitoring four detection channels: 325 nm for detection of dehydroretinol, dehydroretinyl palmitate and the internal standard retinol acetate; 292 nm for tocopherol and the internal standard tocopherol acetate; fluorescence excitation at 330 nm and detection at 480 nm for retinol and retinyl palmitate; and 450 nm for astaxanthin, canthaxanthin and β-carotene. Total retinol and retinol derivatives recovered in plasma and bile after ^{3}H-retinol injection were determined, after extraction into polar (DDW) or non-polar solvents (dichloromethane), by liquid scintillation counting (LSC), corrected for background counts by subtracting values obtained from tissues of similarly treated unexposed fish. HPLC fractions of retinoids in liver and kidney were also collected and analyzed by LSC.

Statistics

All data were analyzed using a completely randomized one way ANOVA. To evaluate the effects of PCB dose on various response parameters, Duncan's multiple range test was used to compare treatment means. Treatment means were deemed significantly different at the α=0.05 level of confidence. Simple linear regression analysis parameters are noted where applicable.

Results and Discussion

Biotransformation Enzymes

Mixed function oxidase (MFO) enzyme activity measured as ethoxyresorufin-O-deethylase (EROD), was induced dose-dependently in the liver of lake trout 12 weeks after exposure to PCB 126 (Fig.5.1, panel A). MFO was induced above the activity of control fish by 60-, 177- and 450-fold in the 3,10 and 30 µg PCB kg^{-1} dose groups, respectively. While EROD activity is well correlated with other MFO enzyme activities, retinoids may be metabolized in mammals and fish by retinoid-specific MFO isozymes (Gilbert et al. 1995, Hakansson et al. 1992). Roberts et al. (1992) showed that retinol can be converted to a 4-hydroxy metabolite by P450 protein isolated from rabbit liver. Additionally, Gilbert et al. (1995)

identified elevated concentrations of hydroxy retinoic acid in livers of rainbow trout exposed to 3,3',4,4'-tetrachlorobiphenyl doses that induced EROD activity. Further HPLC work is required to detect biotransformed retinol so that retinol-specific MFO activities can be quantified in fish exposed to inducing contaminants.

Phase II conjugation enzyme activity, measured as glucuronyltransferase (UGT), was also induced dose dependently in the liver of lake trout 12 weeks after exposure to PCB 126 (Fig.5.1, panel B). Phase II induction was 2.3-, 2.5- and 5-fold over the activity of control fish in the 3, 10 and 30 μg kg^{-1} dose groups, respectively. Conjugation enzymes are generally less responsive to induction than MFO enzymes (Jimenez and Stegeman 1990). However, recent work using specific endogenous substrates has shown greater levels of induction than with traditional substrates, such as the p-nitrophenol used in this study (pers. commun., K. Finnson, University of Manitoba). Increased capacity for retinoic acid glucuronidation has recently been identified in rats treated with 3-methylcholanthrene, a potent inducer of biotransformation enzymes (Sass et al. 1994). Results from the current study also indicate that liver tissue can exhibit different glucuronidation capacity for closely related retinoids. In fact, the capacity for hydroxylation of retinol is much greater than for the same conversion of retinoic acid in rats (Leo and Liebler 1985). This means that retinoic acid

glucuronidation kinetics cannot simply be extended to retinol and that retinol-specific conjugation in fish requires further study.

Tissue Retinoids

Didehydroretinol was significantly depleted in liver of all PCB-exposed lake trout compared to the control group at the end of the experiment (Fig. 5.2, panel A). Similarly, retinol and retinyl palmitate concentrations were significantly lower in livers of lake trout from the two highest PCB dose groups. Liver retinoids appear to be depleted in a PCB-dose- dependent manner. However, the lower concentrations of both didehydroretinol and retinyl palmitate were more closely correlated with elevated MFO activities (R^2=0.699, $p<0.001$) than with PCB concentrations in the liver of PCB-dosed fish. The strong negative correlation of liver MFO and retinoid concentrations appears to support direct metabolism of retinoids by MFOs as a mechanism for tissue depletion of the vitamin. Additionally, concurrent depletion of liver didehydroretinol and retinol indicates that antioxidant activity of retinol, and oxidative conversion to didehydroretinol, is not an important mechanism in tissue retinoid depletion for fish with induced MFO enzyme activity.

Tissue retinoid stores may decline due to a third

mechanism in fish with induced MFO enzyme activity. Biotransformation by MFOs may produce PCB metabolites that interfere with retinol's binding to the RBP-transthyretin complex (Brouwer and van den Berg 1986). This would allow the vitamin to be depleted from plasma as it is increasingly filtered through the glomerular membrane. Elevated kidney retinoid concentrations have been reported in rats exposed to the MFO inducer TCDD, and these elevations may be related to a compensatory mechanism in which elevated kidney esterifying enzyme activity operates to recapture free retinol from the circulation (Jurek et al. 1990). Similar to results from the liver, however, concentrations of retinoids in kidney declined with exposure to PCB 126 in lake trout from this experiment (Fig. 5.2, panel B). Additionally, recoveries of circulating radioactivity in plasma were not different for any of the dose groups at any of the sample times ($p=0.05$) after ^{3}H-retinol injection (Fig. 5.3), indicating that there was no accelerated loss from the plasma of PCB-dosed fish.

Depleted retinyl ester concentrations in the liver of rats exposed to PCBs have also been correlated with non-competitive inhibition of esterifying enzyme activity (Mercier et al. 1990). The authors postulated that binding of a hydroxylated metabolite of tetrachlorobiphenyl was responsible for the lower enzyme activity. We have previously shown that lake trout have a limited capacity for metabolism of PCB 126, even after 30 weeks of exposure (Palace et al. 1996a or Chapter 2).

Therefore, the mechanism of retinoid depletion described by Mercier et al. (1990) for rats exposed to tetrachlorobiphenyl, is not likely responsible for retinoid depletion in lake trout exposed to pentachlorobiphenyl.

^{3}H-Retinol Recovery

Plasma concentrations of ^{3}H-radioactivity declined rapidly in the first hour after injection, and continued to fall between 1 and 30 hours after injection in all PCB dose groups (Fig. 5.3). Similar recoveries have been obtained in rats injected with ^{3}H-retinol (Green et al. 1993). Between 30 and 75 hours after injection, in our experiments, there was no significant decline in plasma radioactivity for any of the PCB dose groups. There was no significant difference in the radioactivity recovered from plasma in any of the PCB dose groups at each of the sample times. These similar radioactivity plasma recovery profiles between control and PCB dose groups indicate that PCB metabolites did not appear to interfere with retinol binding to RBP (Brouwer and van den Berg 1986) and that this is not an important mechanism to account for retinoid depletion in PCB 126 exposed lake trout.

Analysis of the unlabelled retinoid HPLC data from this experiment indicates that MFO biotransformation of retinoids to more readily excreted forms is responsible for retinoid depletion in lake trout with induced MFO activity. To support

this, lower tissue concentrations of ^{3}H-retinoids would also be expected in lake trout with induced MFO activity. Analysis of HPLC fractions containing either didehydroretinol and retinol or retinyl palmitate by LSC, indeed, showed lower ^{3}H-retinoid content in liver of PCB-exposed lake trout (Fig. 5.4, panel A). Furthermore, there was a significant negative correlation between MFO enzyme activity and ^{3}H-retinoid recovery in liver (R^2=0.57, $p<0.05$). Lower ^{3}H-retinoid concentrations were also detected in HPLC fractions isolated from the kidneys of PCB-exposed lake trout (Fig. 5.4, panel B).

Recoveries of the initial doses of radioactivity in plasma, liver and kidney compartments were, not surprisingly, lower than those previously reported for rats injected with ^{3}H-retinol (Green et al. 1993). In addition to species differences, the lower recoveries from this experiment likely reflect an important methodological difference from the Green experiments. While Green delivered ^{3}H-retinol to the circulation of rats circulation either bound in chylomicrons or bound to transthyretin, we delivered the ^{3}H-retinol to the fish as a suspension in charcoal stripped plasma. As Green noted for ^{3}H-retinol delivered to rats in chlyomicrons, a large portion of the suspended ^{3}H-retinol likely remained unbound in nonphysiological form and was, therefore, rapidly cleared from the circulation.

Biotransformation of ^{3}H-retinol by MFO and Phase II enzymes would be expected to produce polar metabolites that

could be found in bile, urine or feces. LSC analysis of bile from fish in this experiment found a greater percentage of the original dose of radioactivity in fish from the 2 highest PCB dose groups compared with the control group (Fig. 5.5). More than 90% of radioactivity in bile was recovered from the water soluble portion (data not shown). Elevated radioactivity in this fraction also correlated with induced liver MFO enzyme activity (R^2=0.513, $p<0.05$). Zile (1992) has recently reviewed evidence for increased excretion of retinoid metabolites in feces and urine of organochlorine-exposed organisms and has also provided new data confirming excretion of glucuronide-conjugated retinoids in the bile of rats exposed to polyhalogenated aromatic hydrocarbons (PHAH).

Summary and Conclusions

Lake trout exposed to PCB 126 had induced MFO and phase II biotransformation enzyme activity. Retinoid concentrations were depleted in tissues of lake trout exposed to PCB and were negatively correlated with MFO activity measured as EROD. Direct metabolism by MFO activity followed by Phase II conjugation appears to be the mechanism responsible for depletion of retinoids in PCB-exposed lake trout. Other mechanisms, including increased use of retinol as an antioxidant and disturbances of retinol transport and storage processes, were evaluated but do not appear to contribute in a

major way to retinoid depletion in pentachlorobiphenyl-exposed lake trout. Depletion related to antioxidant activity is unlikely since both retinol and the oxidized form, didehydroretinol, were depleted in PCB exposed lake trout. Similar recovery of radioactivity from plasma of all PCB dose groups also suggest that interference of retinol binding to transthyretin is not disturbed by pentachlorobiphenyl treatment in lake trout. Finally, since retinoids were depleted in kidney as well as liver tissue, elevation of the esterifying enzyme activity in kidney of PCB treated lake trout appears unlikely. Further analysis of retinoid-specific MFO and Phase II enzyme activities is required to quantify the potential for biotransformation of retinoids in fish exposed to organic contaminants.

Figure 5.1: Mixed function oxidase (MFO) enzyme activity (panel A), measured as ethoxyresorufin-O-deethylase (EROD), and Phase II enzyme activity (panel B), measured as glucuronyltransferase (UGT), in liver of control and PCB-dosed lake trout. Data are presented as mean ± standard error. Means labelled with letters are significantly greater than the control mean and means with different letters are significantly different from each other ($n=4$, $\alpha=0.05$).

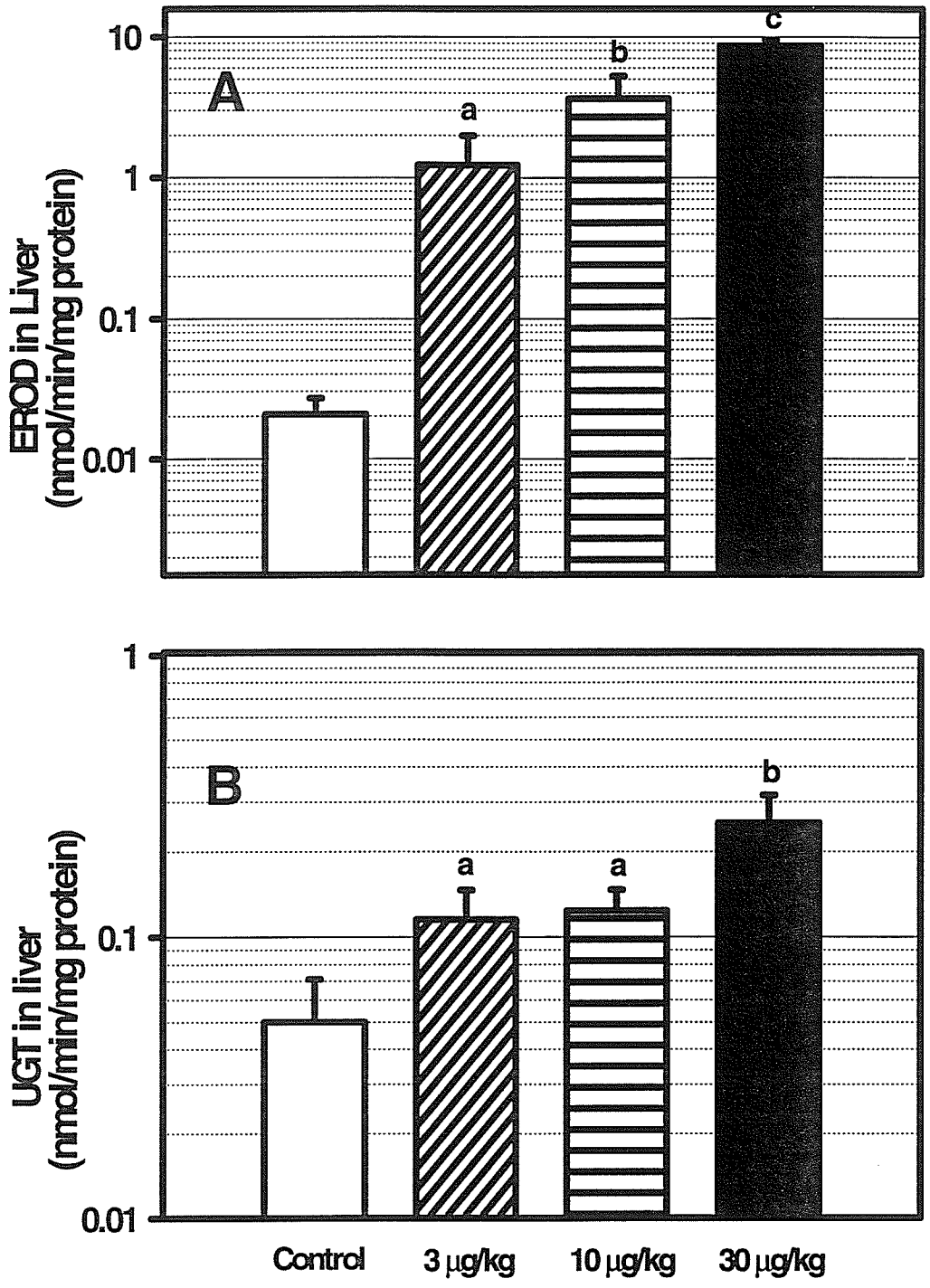

A
B
EROD in Liver
(nmol/min/mg protein)
UGT in liver
(nmol/min/mg protein)
10
1
0.1
0.01
a
b
c
Control
3 µg/kg
10 µg/kg
30 µg/kg

Figure 5.2: Didehydroretinol, retinol and retinyl palmitate concentrations in liver (panel A) and kidney (panel B) of control and PCB-dosed lake trout. Data are presented as mean ± standard error. Means labelled with letters are significantly lower than the control mean for the same parameter. For the same parameter, means labelled with different letters are significantly different from each other (n=4, α=0.05).

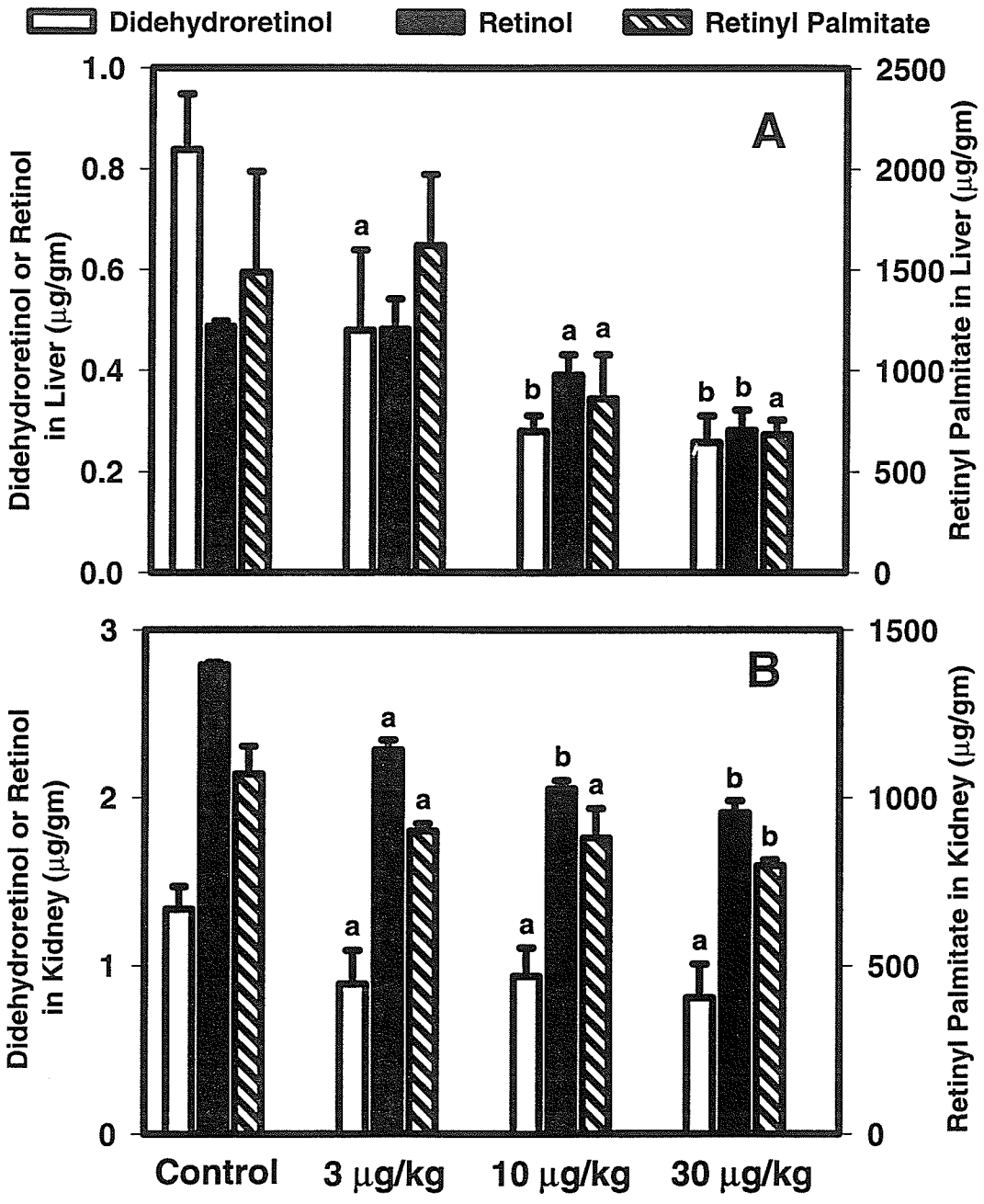

Didehydroretinol
Retinol
Retinyl Palmitate
A
Didehydroretinol or Retinol in Liver (μg/gm)
Retinyl Palmitate in Liver (μg/gm)
1.0
0.8
0.6
0.4
0.2
0.0
2500
2000
1500
1000
500
0
a
b
a a
b b a
B
Didehydroretinol or Retinol in Kidney (μg/gm)
Retinyl Palmitate in Kidney (μg/gm)
3
2
1
0
1500
1000
500
0
a
a a
a b a
a b b
Control
3 μg/kg
10 μg/kg
30 μg/kg

Figure 5.3: Percentage of the initial radioactivity dose recovered from plasma of control and PCB-dosed lake trout following a single bolus injection of ^{3}H-retinol. Data are presented as mean ± standard error (n=4, α =0.05).

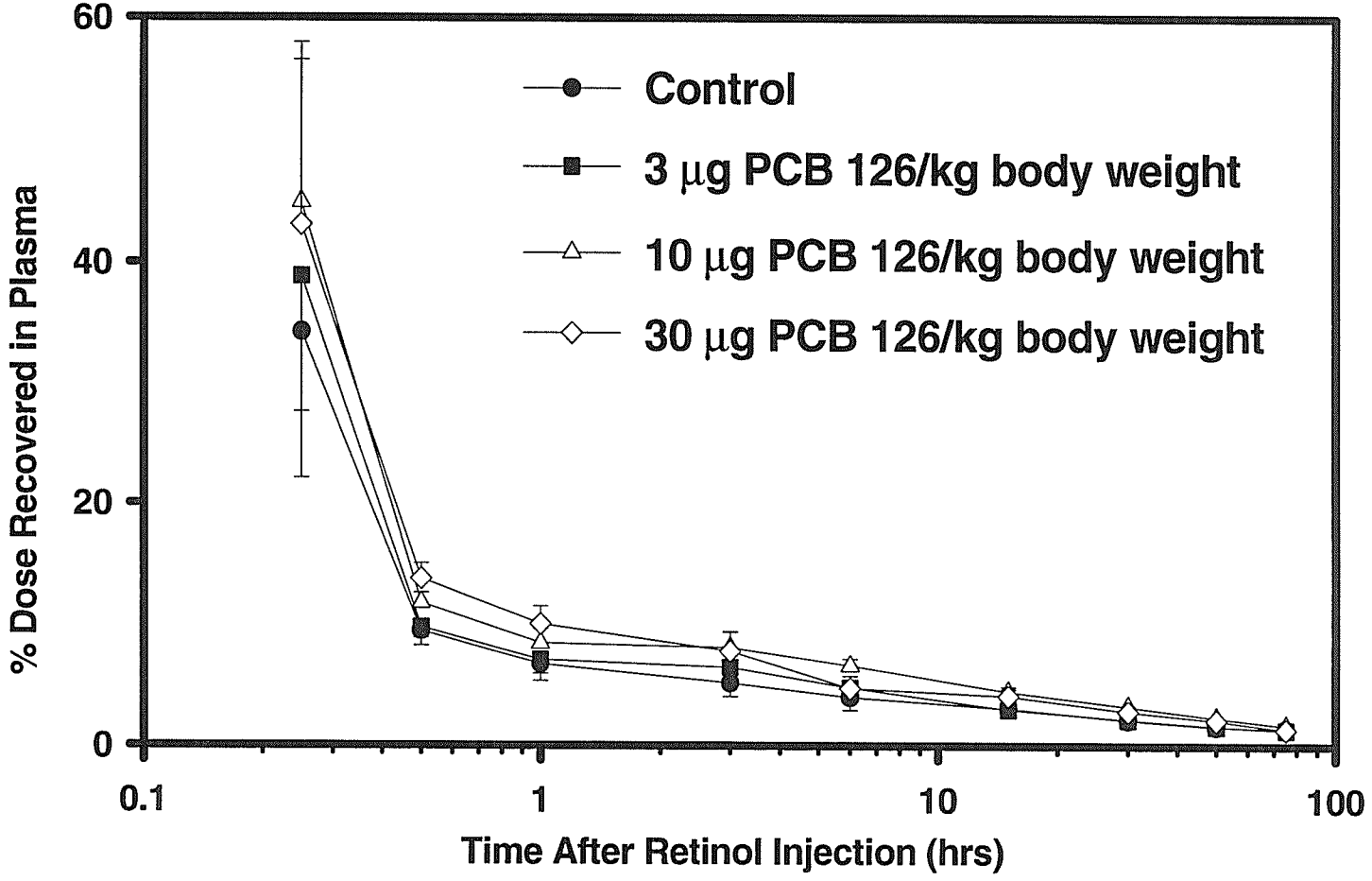

60
40
20
0
% Dose Recovered in Plasma
Control
3 μg PCB 126/kg body weight
10 μg PCB 126/kg body weight
30 μg PCB 126/kg body weight
0.1
1
10
100
Time After Retinol Injection (hrs)

Figure 5.4: Percentage of the initial radioactivity dose recovered in the combined didehydroretinol and retinol fraction and the retinyl palmitate fraction isolated by HPLC separation of liver (panel A) and kidney (panel B) homogenates from control and PCB-dosed lake trout following a single bolus injection of ^{3}H-retinol. Data are presented as mean ± standard error (n=4, α=0.05). Means labelled with letters are significantly lower than the control mean for the same radioactive fraction. For the same radioactive fraction, means labelled with different letters are significantly different from each other (n=4, α =0.05).

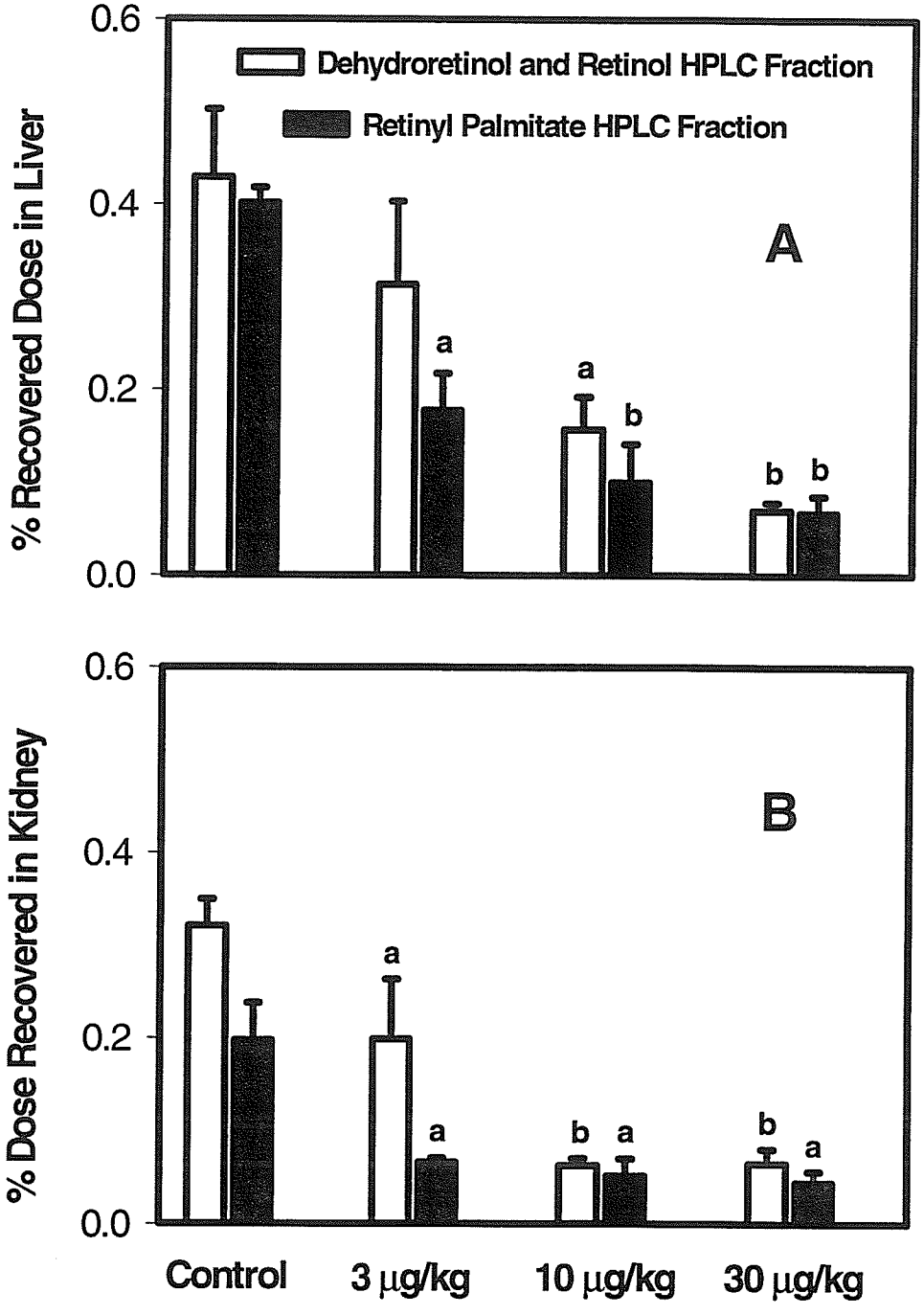
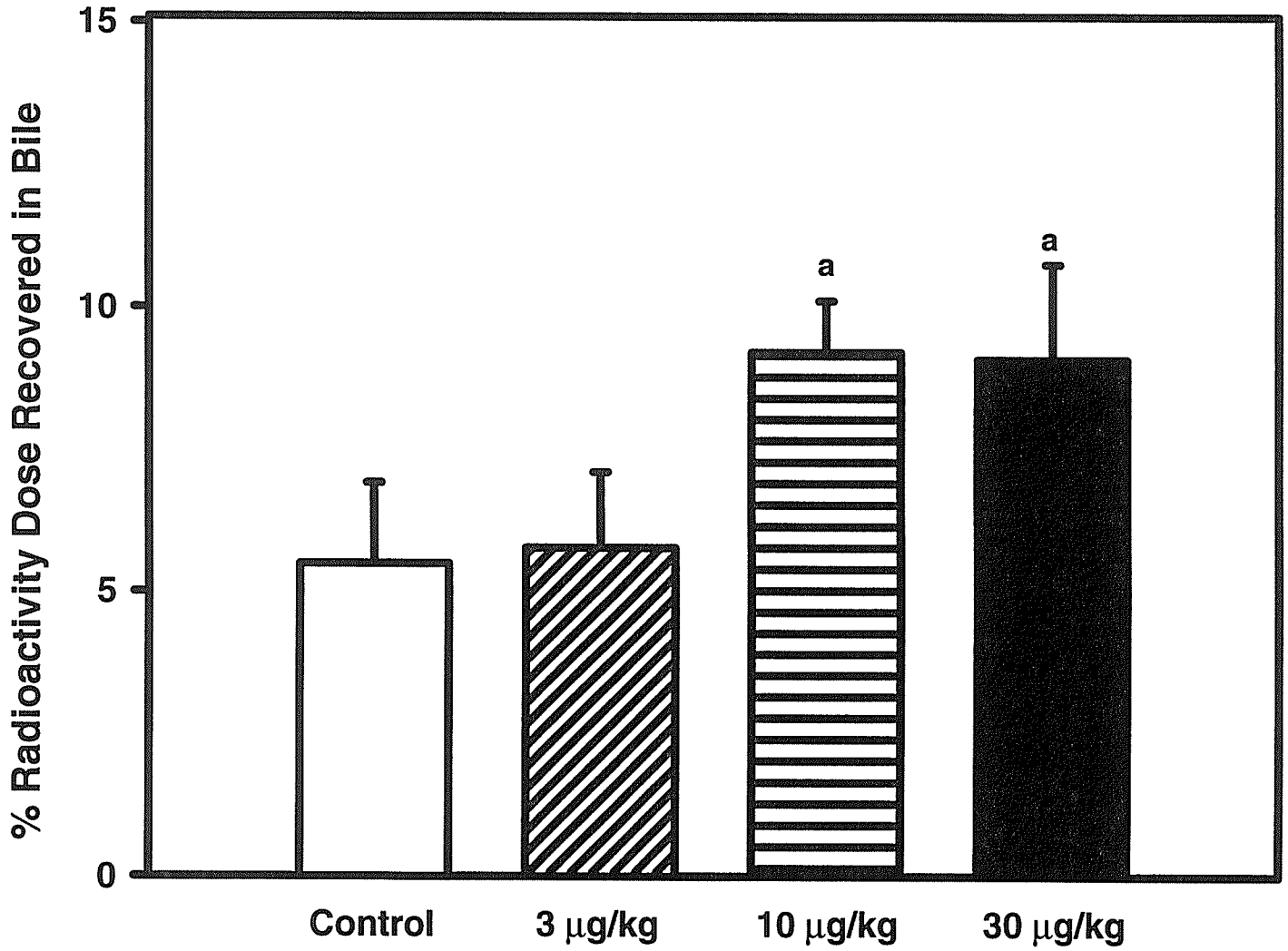

Dehydroretinol and Retinol HPLC Fraction
Retinyl Palmitate HPLC Fraction
% Recovered Dose in Liver
0.6
0.4
0.2
0.0
A
a
a
b
b
b
% Dose Recovered in Kidney
B
a
a
b
a
b
a
Control
3 µg/kg
10 µg/kg
30 µg/kg

Figure 5.5: Percentage of the initial radioactivity dose recovered in bile of control and PCB-dosed lake trout following a single bolus injection of ^{3}H-retinol. Data are presented as mean ± standard error (n=4, α=0.05). Means labelled with letters are significantly greater than the control mean. Means labelled with different letters are significantly different from each other (n=4, α =0.05).


% Radioactivity Dose Recovered in Bile
15
10
5
0
a
a
Control
3 μg/kg
10 μg/kg
30 μg/kg

Chapter 6

Oxidative Stress in Lake Sturgeon (*Acipenser fulvescens*) Orally Exposed to 2,3,7,8-Tetrachlorodibenzofuran

Abstract

Juvenile lake sturgeon were orally dosed with gelatin containing nominal concentrations of 0, 0.16 or 1.6 ng [^{3}H]2,3,7,8-tetrachlorodibenzofuran (TCDF) kg^{-1} fish weight. Liver, kidney and blood were collected 10 and 27 days after exposure. Phase I (mixed-function oxidase (MFO)) and Phase II (glucuronyltransferase) enzyme activities were determined in liver. Concentrations of non-enzymatic (tocopherol, retinoids) and enzymatic (superoxide dismutase, catalase, glutathione peroxidase) antioxidant parameters were also measured in liver and kidney. TCDF concentrations were elevated in liver and kidney of both dose groups at 10 and 27 days. Lower tissue concentrations of TCDF at 27 days ,compared with 10 day exposures, as well as the presence of polar metabolites in bile, may indicate rapid metabolism and clearance of the contaminant. MFO enzyme activity, measured as ethoxyresorufin-O-deethylase (EROD), was induced in liver with activity at the two sample periods reflecting the TCDF concentrations. Greater concentrations of hepatic lipid peroxides in both dose groups indicate that oxidative stress was produced by contaminant exposure and/or induced activity of metabolic enzymes .

Introduction

Polychlorinated dibenzofurans (PCDFs) are produced as byproducts during the production of halogenated aromatics and metal chlorides, from municipal waste incineration and in effluent from kraft pulp and paper mills using chlorine-bleaching processes (Safe 1990). PCDFs are lipophilic and generally resistant to breakdown; they have been identified in terrestrial and aquatic ecosystems and tend to bioaccumulate in food chains (Walker and Peterson 1991). Although there are 135 possible PCDF congeners (Rappe and Buser 1989), 2,3,7,8-tetrachlorodibenzofuran (TCDF) is one of the most prevalent and toxic congeners found in aquatic systems (Muir et al. 1992). TCDF has been found in fish tissues from all of the Great Lakes (Safe 1990) and some bottom feeding species captured near the discharge of chlorine-bleaching pulp mill effluent have tissue concentrations as high as 400 ng kg^{-1} (U.S.E.P.A 1991).

Concentrations of TCDF similar to those found in the Great Lakes have been shown to be toxic to fish, especially to early life stages (Walker and Peterson 1991). The toxicity of TCDF and similar compounds is thought to be mediated through interaction with the cellular Ah receptor which induces biotransformation systems including mixed-function oxidase (MFO) enzymes (Walker and Peterson 1991). Most fish have a second group of biotransformation enzymes referred to as

conjugation or phase II enzymes, which are also induced by exposure to TCDF and similar compounds (Clark et al. 1991). Both groups of enzymes are responsible for metabolising lipophilic chemicals, such as TCDF, to more readily excreted water-soluble forms (Goksoyr and Forlin 1992).

Lehtinen (1990) recently reviewed evidence that metabolism of TCDF and similar compounds by MFO and conjugation enzymes may also result in the proliferation of oxidative radicals. These radicals can deplete antioxidant defense molecules or induce the formation of lipid peroxides and breakdown of essential cellular and subcellular membranes (Gutteridge and Halliwell 1990). In fact, early life history stage mortality induced by compounds interacting with the Ah receptor is characterized by edema associated with increased membrane permeability and localized biotransformation enzyme activity (Walker and Peterson 1991).

Membrane breakdown due to the proliferation of radicals is usually preceded by depletion of cellular stores of antioxidants (Rodriguez-Ariza et al. 1993). Antioxidant molecules reduce membrane breakdown by quenching radicals before they can initiate oxidative breakdown reactions in cellular membranes (Winston and Di Giulio 1991). Previous work has established that the non-enzymatic antioxidants tocopherol (Vitamin E) and the retinoids (Vitamin A compounds) and the enzymatic antioxidants CAT, GPx and SOD are among the most sensitive indicators of radical proliferation (Palace and

Brown 1994, Ardelt et al. 1989). Since lake sturgeon are benthic fish, they are often exposed to high contaminant loads and have previously been shown to have lower concentrations of retinoids when captured from contaminated versus non-contaminated waters (Ndayibagira et al. 1995).

Sturgeon populations have declined steadily in North American and European freshwaters for at least 100 years (Birstein 1993). Because they are benthic organisms with high lipid content and a long age to sexual maturity, sturgeon have the potential to accumulate lipophilic contaminants that may contribute to their declining numbers (Rousseaux et al. 1995). In order to gain an understanding of the link between TCDF exposure, biotransformation enzyme activity and oxidative stress responses, juvenile lake sturgeon were exposed to low concentrations of TCDF (0, 0.16 or 1.6 ng kg^{-1} fish weight). Metabolic enzyme activities and oxidative stress parameters were examined in tissues 10 and 27 days after exposure. TCDF was chosen as the toxicant for examination in this chapter over the PCB 126 used in previous chapters because it is more readily metabolized (Muir et al. 1990) and may, therefore, increase the proliferation of oxyradicals through redox cycling of biotransformed TCDF (Winston and Di Giulio 1991).

Materials and Methods

Fish Holding

Lake sturgeon (*Acipenser fulvescens*) were received as eyed eggs from the Wild Rose Hatchery (Wild Rose, Wisconsin). The eggs were held and hatched at 15°C and sac-fry were then cultured at 15°C in 60 L fiberglass tanks with a flow rate of 1 L min^{-1} of dechlorinated Winnipeg city tap water. One day prior to the passing of the melanin plug from the hindgut, feeding of larvae was initiated with live brine shrimp hatched from brine shrimp eggs (Ocean Star International, USA) and the water temperature was raised to 20°C. Sixty days later the diet was switched to frozen adult brine shrimp (Hagen Feeds, Montreal) which was thawed for 5 minutes prior to presentation. Sturgeon were fed to satiation at each feeding. At 4 months of age the diet was again changed to ocean plankton (Murex, Vancouver) fed at a ration of 1% of body weight per day until the lake sturgeon were 20 months of age. During this period they were held at a temperature of 13 to 15°C. These 20-month-old lake sturgeon (214 ± 9 gm) were used for the TCDF exposure experiments during which they were fed ocean plankton at a ration of 1% of bodyweight per day. Throughout the exposure the sturgeon were maintained in tanks receiving at least 1 L of 11.5 to 13.1°C dechlorinated Winnipeg City tap water per gm of fish per day.

Chemicals

Unlabelled and 4,6 ^{3}H-labelled 2,3,7,8-tetrachlorodibenzofuran (18.9 Ci mmol^{-1}) were obtained from Wellington Laboratories (Guelph, Ont.) and Chemsyn Science Laboratories (Lenexa, KS), respectively. Both were purified to >98% by a previously described HPLC method (Muir et al. 1992). Purified unlabelled (90%) and labelled (10%) TCDF were suspended in HPLC grade ethanol and 60 bloom gelatin (2.2:11.5 v/v) (Sigma Chem. Co., St. Louis, Mo.) and warmed to 32°C in a water bath before dosing (Sijm et al. 1990).

Dosing

Fish were lightly anesthetized in water containing phenoxyethanol (0.4ml L^{-1}) until they lost equilibrium. They were then blotted dry and weighed. Fish were randomly assigned to the control group, receiving 1 ml of warmed ethanol/gelatin, or to TCDF dose groups, receiving 1 ml of TCDF in ethanol/gelatin solution per 250 gm of bodyweight to achieve dosages of 0.16 or 1.6 ng TCDF kg^{-1} of body weight. Solutions were delivered directly to the stomach through polyethylene tubing (I.D. 1.57 mm) attached to a 16 gauge needle mounted on a 3 ml syringe. Following oral dosing, fish were returned to the experimental holding tanks where they recovered equilibrium within 3 minutes.

Tissue Sampling

Ten and 27 days after exposure, 5 fish from each dose group were anesthetized for 3 minutes with the same dose of phenoxyethanol that had previously been used. Blood was obtained by caudal vein puncture with a preheparinized 20 gauge needle mounted on a 3 ml syringe and immediately centrifuged for 5 minutes at 3000 X g to obtain plasma which was frozen at -110°C until analysis. Tissues were dissected from the carcass, placed in sterile plastic bags and frozen immediately at -110°C until analysis.

Residue Analyses

TCDF and TCDF metabolite residues in tissues were calculated based on radioactivity detected by liquid scintillation counting (LSC) following homogenization and extraction with either toluene or distilled deionized water respectively (Millipore, Milli Q, Bedford, MA). Concentrations were corrected for background scintillation counts by subtracting counts from extracted tissues of control group fish. The parent TCDF compound from toluene tissue extracts was identified by a previously described HPLC method (Muir et al. 1992).

To confirm the presence of glucuronide conjugates of TCDF in bile that had been previously reported by Muir et al.

(1990), 200 µl aliquots of bile diluted with double distilled water were either extracted directly with dichloromethane or incubated with 1000 units ml^{-1} glucuronidase enzyme in 0.5 M phosphate buffer (pH=7.8) and then extracted with dichloromethane. Higher recoverable radioactivity from the glucuronidase treated aliquots was accepted as confirmation of the presence of glucuronide conjugates in bile.

Biochemical Analyses

Liver microsomes were prepared by homogenizing the tissue in ice cold buffer containing 0.15M KCl, 0.02M HEPES and 0.1mM EDTA (pH=7.5) with several passes of a Teflon pestle. The homogenate was centrifuged at 9,000 X g for 20 minutes at 4°C; supernatant was collected, and further centrifuged at 105,000 X g for 90 minutes at 4°C to obtain a microsomal pellet. Microsomes were resuspended in 0.1M phosphate buffer (pH=7.4) and this suspension was used directly in a microplate assay for EROD (MFO) activity using a 7620 Cambridge Technology (Watertown, MA) fluorescence detector (excitation filter 540 nm detection filter 585 nm) at 21°C (Eggens and Galgani 1992). Briefly, 25 µl microsome suspension was pipetted into wells containing 255 µl of 2uM 7-ethoxyresorufin (7-ER) in 0.1M phosphate buffer (pH=7.8). The reaction was initiated by adding 25 µl of 2mM NADPH in 0.1M phosphate buffer (pH=7.8) to each well. Three replicates were analyzed for each sample.

Fluorescence development was linear for at least 7 minutes. Microsomal protein content was determined using Peterson's (1977) modification of the Lowry method.

Microsomes for glucuronyltransferase conjugating activity were prepared as above except that 0.01M sodium phosphate 0.15M KCl homogenization buffer (pH=7.4) and a 0.1M sodium phosphate resuspension buffer (pH=7.4) were used. Glucuronyltransferase activity was also determined at room temperature using p-nitrophenol as a substrate in a spectrophotometric assay (Burchell and Whetherhill 1981). Microsomal SOD and crude homogenate CAT and GPx activities were assayed by previously described spectrophotometric assays (Sun and Zigman 1978, Rabie et al. 1972, Tappel 1978). Tocopherol and retinoids were quantified in liver, kidney and plasma using a recently developed reversed-phase HPLC technique (Palace and Brown 1994 or Appendix A) while lipid peroxides were determined in liver using the LPO-FF Determiner commercial kit (Kamiya Biomedical Co., Thousand Oaks, CA). This kit utilizes a derivative of methylene blue that specifically reacts with lipid peroxides in order to quantify membrane breakdown (Ohishi et al. 1985).

Data Analysis

Statistical analyses employed a completely randomized, one-way analysis of variance. Duncan's multiple range test was

used to compare treatment means at the α=0.05 level of probability. Linear regression analysis was used to assess correlations between parameters with statistical significance accepted at the α=0.05 level of confidence.

Results and Discussion

TCDF Accumulation and Disposition

TCDF concentrations in liver, kidney, bile and muscle, based on LSC of toluene extracts, were elevated in both dose groups at both sample times, with the high dose group assimilating the greater concentration of TCDF (Fig. 6.1, panel A). Previous work using rainbow trout demonstrated that more than 98% of toluene-extracted radiolabel from tissues is TCDF parent compound and that more than 98% of the parent compound is recoverable with toluene extraction (Muir et al. 1992). Similarly, HPLC analysis of toluene-extracted sturgeon tissues from this experiment indicated that more than 98% of the extracted radioactivity could be attributed to the parent compound. Because unextractable radioactivity was not determined in this experiment, the efficiency of TCDF recovery from sturgeon tissues using toluene extraction was not quantified. Muir et al. (1992), however, reported low values (1 to 8%) for this fraction in TCDF-exposed rainbow trout.

Although information regarding the organochlorine content

of feral sturgeon tissues is sparse, previous work indicates that sturgeon tend to accumulate these lipophilic contaminants more than other species because of their benthic habitat, high tissue lipid content, long age to sexual maturity and association with contaminated sediments (Rousseaux et al. 1995). Recent analysis of fish from Canadian freshwaters receiving pulp mill effluent reported TCDF concentrations in the carcass of benthic suckers (*Catostomus sp.*) up to 435 ng kg^{-1} (Whittle et al. 1993) while in pelagic fishes, concentrations range from 11 to 640 ng kg^{-1} (Whittle et al. 1993, DeVault et al. 1989). Clearly, the exposure doses (0.16 and 1.6 ng kg^{-1}) and accumulated concentrations in tissues (0.1 to 100 ng kg^{-1}) from this experiment are environmentally relevant for North American freshwater systems.

Lower TCDF was found in liver, kidney and bile of the high dose group 27 days after exposure than at 10 days after exposure (Fig. 6.1, panel A). In the low dose group, only liver concentrations were lower in the 27 day post-exposure group compared with the 10 day post-exposure fish. TCDF concentrations in muscle were elevated in both dose groups at 27 days compared to the 10 day samples. Calculation of total muscle contaminant load was extrapolated from subsample extraction and was based on 59% of total sturgeon body weight being attributed to muscle (T.A. Dick, University of Manitoba, unpubl. observ. for similar sized lake sturgeon). The difference in muscle contaminant load between sample times

indicates a re-distribution from splanchnic tissues to muscle tissue during the period of the experiment. While Muir et al. (1992) and Maslanka et al. (1992) reported that carcass, including muscle and bone, was the main compartment (62 to 74%) of TCDF disposition for rainbow trout exposed to TCDF , we recovered the major component of the inital dose from liver (53 ± 7%) followed by muscle (39 ± 4%), viscera (5 ± 1%), intestine (2 ± 1%), kidney (1 ± 0.6%) and bile (2 ± 0.7%) at the 27 day sample time (data not shown).

In addition to re-distribution to muscle tissue, the percentage of total TCDF dose recovered from all tissues at 27 days (65 ± 13%) was lower than at 10 days (102 ± 14%) indicating that TCDF metabolism may have occurred between the two times. Incubating aqueous bile solutions with glucuronidase enzyme increased dichloromethane recoverable radioactivity from bile by 24 to 63% compared to untreated aqueous bile solutions (data not shown). This shows that glucuronide conjugation accounts for a significant portion of TCDF metabolism in exposed sturgeon. Similar results have been previously reported for TCDF-exposed juvenile rainbow trout (*Onchorhynchus mykiss*) (Muir et al. 1992, Maslanka et al. 1992).

TCDF metabolites, assayed through liquid scintillation counting of distilled deionized water extracts, were elevated in liver, bile and muscle of low dose fish and in liver, kidney, bile and muscle of high dose fish over those of the

control fish (Fig. 6.1, panel B). MFO and Phase II enzymes are likely responsible for producing these polar extractable metabolites of TCDF. As stated earlier, work with other fish species has identified glucuronide conjugates as the predominant metabolites in TCDF-exposed fish (Muir et al. 1992, Maslanka et al. 1992).

Biotransformation Enzymes

The MFO enzyme system catalyzes the introduction of an oxygen atom into lipophilic substrates during the first phase of steroid, fatty acid or xenobiotic metabolism (Andersson and Forlin 1992). Mixed-function oxidase enzyme activity, measured as EROD was significantly elevated in both dose groups after 10 days but only in the high dose group 27 days after exposure (Fig. 6.2, panel A). Simple linear regression analysis indicated that liver EROD was closely correlated with liver concentrations of TCDF for both dose groups at both sample times (R^2 0.68-0.90, $p<0.005$). Because of the low TCDF doses used in this experiment, EROD activity was induced to a maximum activity of only 2-fold over that of the control group. This represents a modest induction compared to other species that often have 10- to 40-fold induction at organochlorine contaminated versus reference sites (Hodson et al. 1991). However, EROD activities and levels of induction from this experiment are similar to those reported for

sturgeon captured in the field and at similarly contaminated versus less contaminated sites (Rousseaux et al. 1995, Arinc and Sen 1993).

Although a dose response trend was evident, activities of the Phase II enzyme glucuronyltransferase were not significantly different between any of the treatment groups (Fig. 6.2, panel B). These results were not surprising, given the moderate MFO induction for sturgeon in this experiment and generally less responsive nature of Phase II enzymes to induction by contaminants in other fish species (Clark et al. 1991). An earlier study using sturgeon also reported less responsiveness to induction by glucuronyltransferase and glutathione transferase compared to the phase I enzymes arylhydrocarbon hydroxylase (AHH) and ethoxycoumarin deethylase (ECOD) (Perdu-Durand and Cravedi 1991). In fact, glucuronyltransferase activity was induced to a maximum of only 1.5-fold, even after exposure to an intraperitoneal dose of 100 mg kg^{-1} of the potent inducer β-napthoflavone. This relative inability of sturgeon to metabolize organic contaminants to even more polar compounds after phase I transformation could allow oxidatively active metabolites to accumulate. This may confer a greater degree of sensitivity to the toxicity of these contaminants upon sturgeon, and thereby have ecological significance.

Enzymatic Antioxidants

Catalase (Figure 6.3, panel A) and glutathione peroxidase (panel B) activities were not significantly different in liver or kidney between any of the treatment groups at both sample times. Superoxide dismutase (panel C) activity was significantly higher in liver of TCDF-exposed fish after 10 days but not after 27 days of exposure. SOD, CAT and GPx all contribute to protection of lipid membranes by scavenging oxygen radicals that initiate peroxidation (Winston and Di Giulio 1991). All three of these enzymes have also been identified as indicators of exposure to contaminants that induce MFO activity and the accompanying oxidative stress (Pohjanvirta et al. 1990). However, previous work in our laboratory has established that SOD is the most responsive of the three enzymes in freshwater fish and that elevated activity above that of controls often corresponds to other indicators of oxidative stress (Palace and Klaverkamp 1993).

Non-enzymatic Antioxidants

Lower retinoid stores have previously been reported in salmonids exposed to PCB that induced MFO activity (Palace and Brown 1994, Ndayibagira et al. 1995). Similar data have also been presented for sturgeon collected at organochlorine-contaminated versus non-contaminated sites (Ndayibagira et al.

1995). The mechanism of retinoid depletion in organochlorine-exposed fish has yet to be clarified. Catabolism by induced MFO and Phase II enzymes (Zile 1992), impaired binding within the transthyretin protein complex (Brouwer and van den Berg 1986), increased use of retinol as an antioxidant (Palace and Brown 1994) or lower dietary uptake due to loss of appetite (Spear et al. 1994) are possible reasons for retinoid depletion in organochlorine- exposed fish. Results from our experiment, however, indicate that short term exposure to low TCDF concentrations had no significant effect on retinol, dehydroretinol or retinyl palmitate concentrations in liver of sturgeon (Fig. 6.4). There were also no significant differences in plasma retinol or dehydroretinol between any of the treatment groups (data not shown).

Tocopherol concentrations were lower in liver of the high dose group after 27 days of exposure and in kidney of both dose groups after 27 days (Fig. 6.5). Tocopherol is used primarily as an antioxidant molecule (Burton and Traber 1990). Therefore, tissue stores would be expected to decline in the dose groups if TCDF exposure produced oxidative stress. These lower tissue stores may also reflect an early exposure phase transport of tocopherol from liver and kidney stores to other oxidatively stressed tissues. Marginally higher plasma tocopherol in the low dose group and significantly higher plasma tocopherol in the high dose group after 10 days of exposure is evidence for such a transport.

While alterations in tissue antioxidant concentrations may signal oxidative stress from contaminant exposure, specific oxidative stress effects on lipid membrane integrity can be quantified by examining membrane breakdown products. Whereas thiobarbituric reactive substances (TBARS), primarily malonaldehyde, have been used extensively in this area, this method has been criticized for non-specificity and because it amplifies the oxidative process within lipid membranes (Gutteridge and Halliwell 1990). Therefore, we specifically measured lipid peroxides to assess membrane breakdown in this experiment. Even at low TCDF exposures used in this experiment, lipid peroxides were higher in liver of both dose groups after 10 days of exposure and remained elevated in the high dose group after 27 days of exposure (Fig. 6.6). Simple linear regression analysis revealed a relationship between EROD activity in the liver and the concentration of lipid hydroperoxides (R^2=0.52, $p<0.001$).

Exposure to radiation can also induce lipid peroxidation and deplete tissue antioxidants (Greenstock 1993). However, injection solutions for the two dose groups in this experiment contained relatively low concentrations of ^{3}H-TCDF, with no significant correlation between lipid hydroperoxides and the concentration of ^{3}H-TCDF in liver tissue. Therefore, differences in the membrane damage between dose groups in this experiment cannot be attributed to radiation mediated oxidation.

Induction of the MFO biotransformation system can result in proliferation of oxyradicals that initiate lipid peroxidation (Rodriguez-Ariza et al. 1993). These radicals may arise from the Fenton reaction when intracellular iron is mobilized by MFO metabolites (Smith and De Matteis 1990) or they may be generated by redox cycling of biotransformed organic molecules (Winston and Di Giulio 1991). Regardless of the origin, increased proliferation of oxygen radicals can result in lipid peroxidation and associated erythrocyte lysis, membrane-bound enzyme inhibition, altered ionic balance and the creation of potentially mutagenic and carcinogenic by-products (Mohammadpour et al. 1988). Results from this experiment directly demonstrate greater oxidative breakdown of lipid membranes in sturgeon exposed to TCDF dosages that induce MFO enzyme activity.

Summary and Conclusions

We have demonstrated that sturgeon exposed to low concentrations of TCDF have significantly induced mixed-function oxidase enzyme (EROD) activity. Additionally, despite a lack of significant induction of phase II enzyme glucuronyltransferase enzyme activity, polar metabolites suspected to be glucuronide conjugates were recovered by aqueous extraction of bile. Lipid peroxides, tocopherol and

SOD measures in liver appear to be sensitive indicators of exposure to TCDF and indicate that TCDF-treated sturgeon were subject to greater oxidative stress than untreated fish. Additional studies are required to evaluate the relevance of MFO induction and oxidative stress as factors contributing to the decline of sturgeon populations at contaminated sites.

Figure 6.1: Panel A: TCDF concentrations, based on liquid scintillation counting of toluene extracts, in liver, kidney, bile and muscle from control and TCDF-dosed sturgeon. Panel B: TCDF metabolite concentrations, based on liquid scintillation counting of distilled water extracts of liver, kidney, bile and muscle from control and TCDF-dosed sturgeon. Data are expressed (mean with standard error) as ng of TCDF or TCDF equivalents per gm of tissue or ml of bile. All dose group data are significantly different from control group. Statistical differences for data from the same tissue are denoted with different letters (n=5, α=0.05).

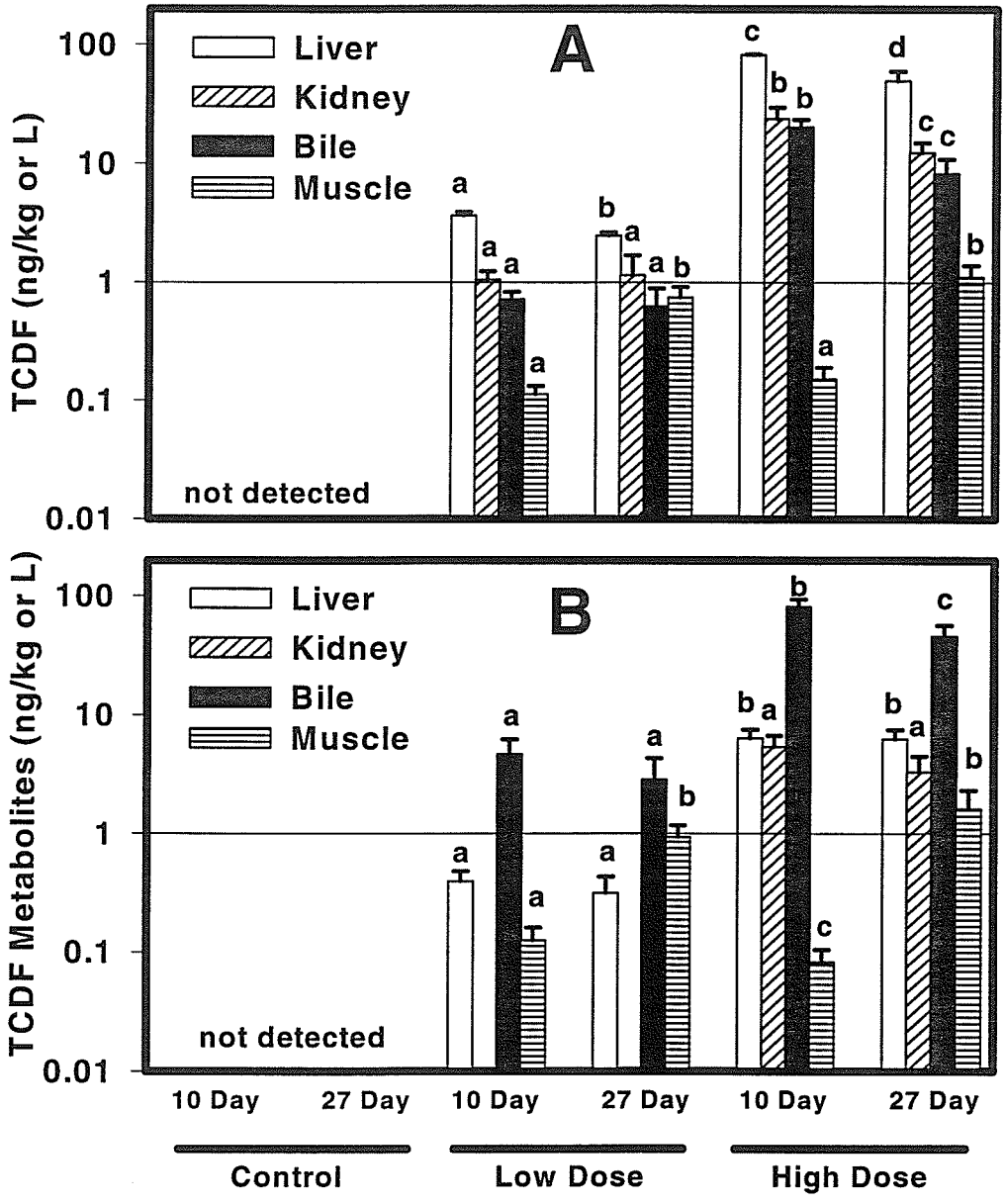

A
Liver
Kidney
Bile
Muscle
TCDF (ng/kg or L)
100
10
1
0.1
0.01
not detected
B
TCDF Metabolites (ng/kg or L)
10 Day
27 Day
Control
Low Dose
High Dose

Figure 6.2: Mixed-function oxidase activity, measured as EROD (panel A), and glucuronyltransferase enzyme activity (panel B) in liver of control and TCDF-dosed sturgeon . Data are expressed (mean with standard error) as pmol of ethoxyresorufin deethylated per minute per mg of microsomal protein or nmol of p-nitrophenol conjugated per minute per mg of microsomal protein respectively. Statistical differences between treatments are denoted with different letters (n=5, α=0.05).

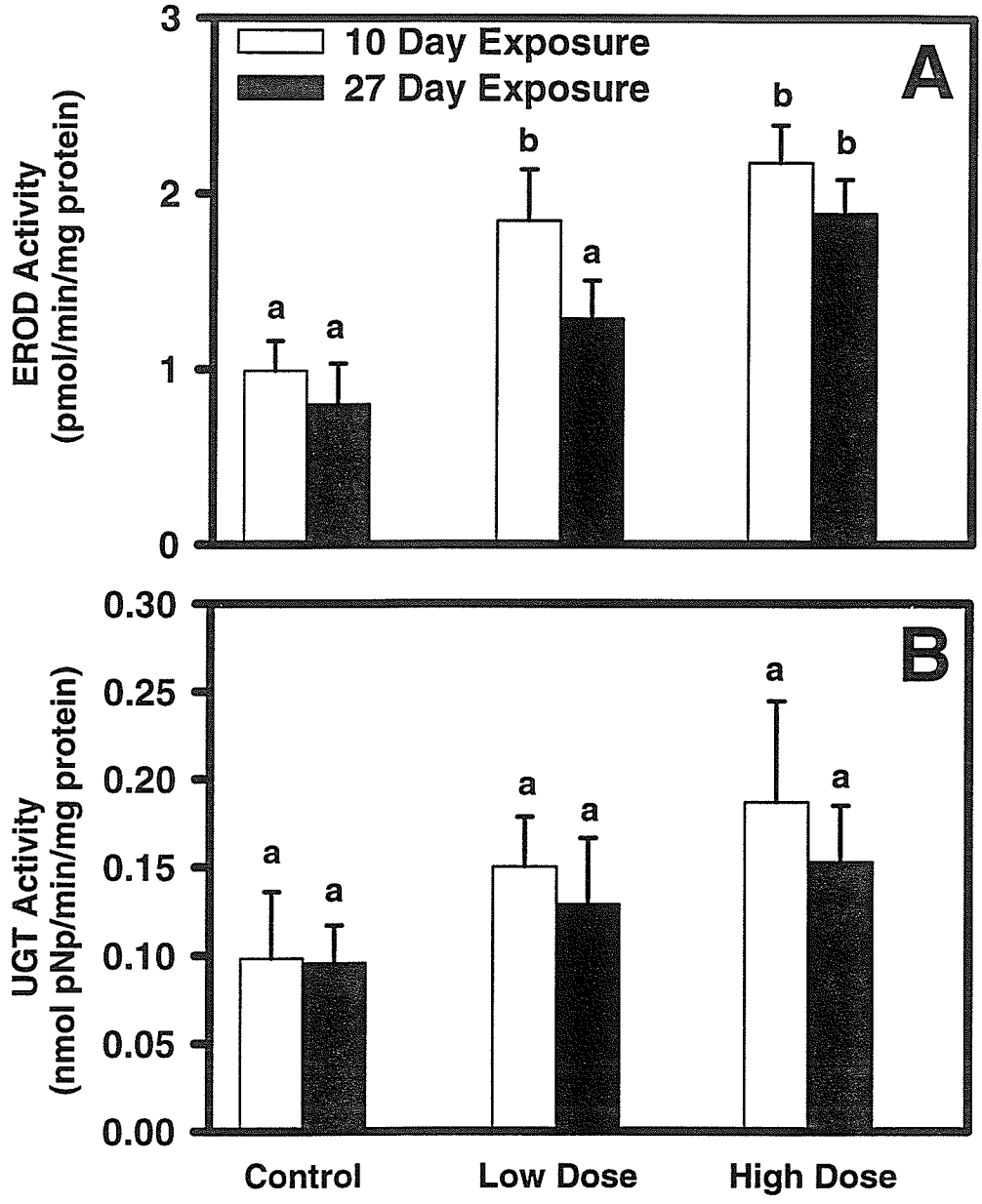

10 Day Exposure
27 Day Exposure
A
B
EROD Activity
(pmol/min/mg protein)
UGT Activity
(nmol pNp/min/mg protein)
3
2
1
0
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a
b
a
b
b
a
a
a
a
a
a
Control
Low Dose
High Dose

Figure 6.3: CAT (panel A), GPx (panel B) and SOD (panel C) in liver and kidney of control and TCDF-dosed sturgeon. Data are expressed (mean with standard error) as Units of activity per gm of wet liver or kidney weight. One unit of CAT decomposes 1.0 µmol of hydrogen peroxide at pH=7.0, 25°C. One unit of GPx catalyzes the oxidation of 1.0 mmol of reduced glutathione by hydrogen peroxide to oxidized glutathione per minute at pH=7.0, 25°C. One unit of SOD inhibits autooxidation of epinephrine by 50% at pH=7.0, 25°C. Statistical differences for data from the same tissue are denoted with different letters (n=5, α=0.05).

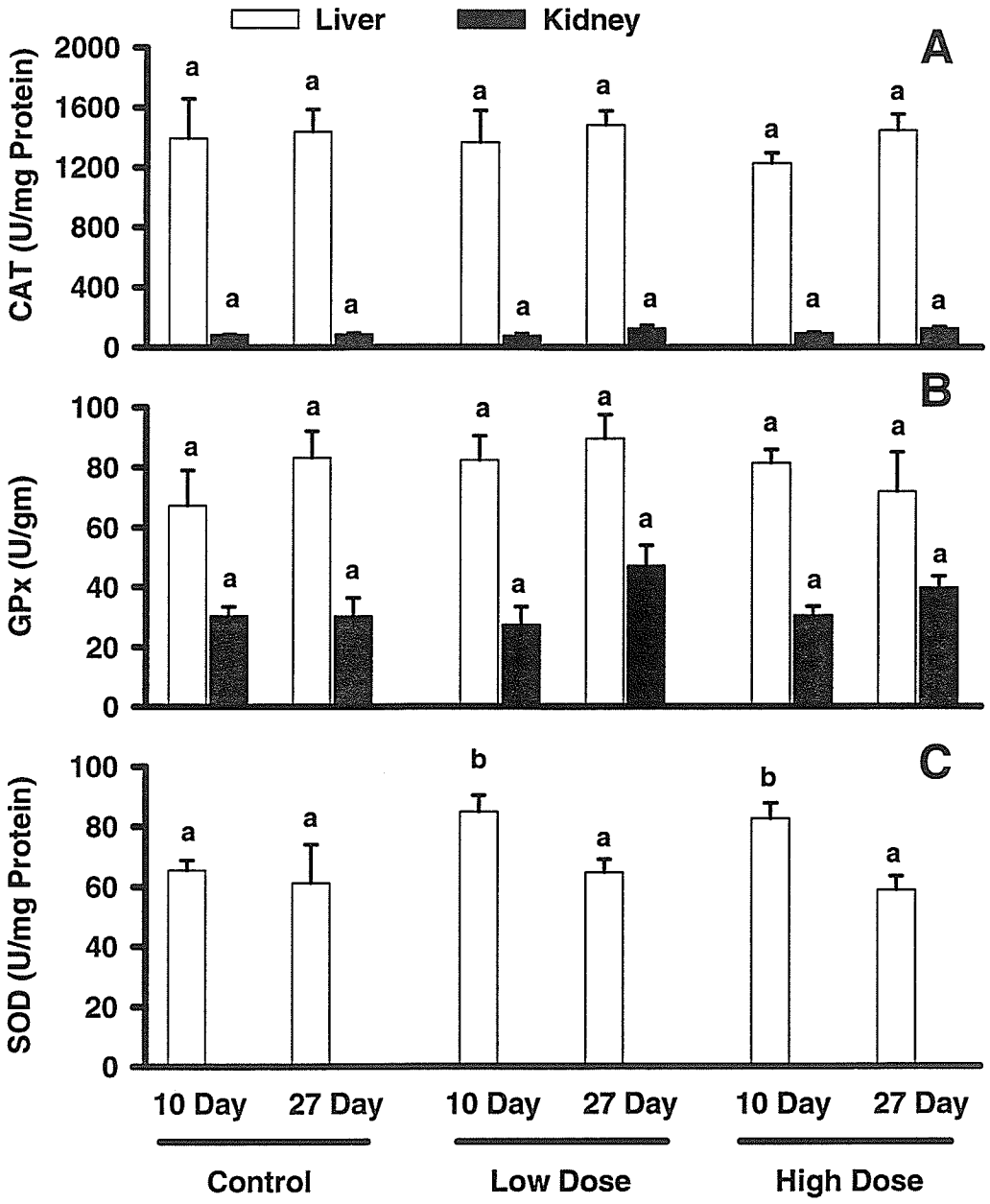

Liver
Kidney
A
B
C
CAT (U/mg Protein)
GPx (U/gm)
SOD (U/mg Protein)
2000
1600
1200
800
400
0
100
80
60
40
20
0
a
b
10 Day
27 Day
Control
Low Dose
High Dose

Figure 6.4: Retinol, didehydroretinol and retinyl palmitate in liver of control and TCDF-dosed sturgeon. Data are expressed (mean with standard error) as µg retinoid per gm of wet liver weight. Statistical differnces for data of the same parameter are denoted with different letters (n=5, α=0.05).

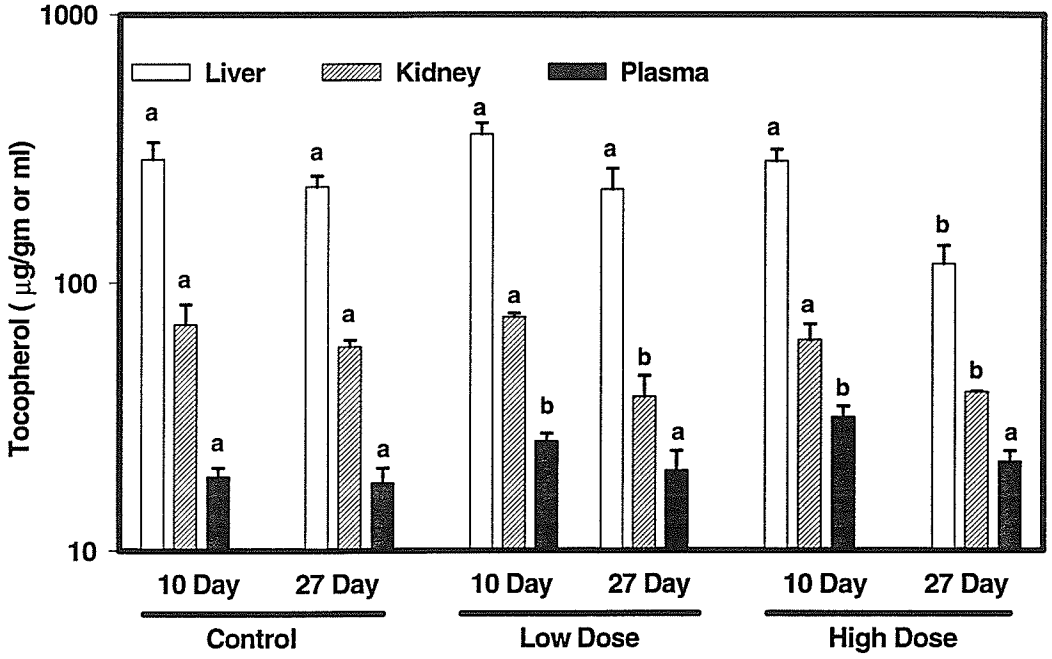

1000
100
10
Tocopherol (μg/gm or ml)
Liver
Kidney
Plasma
a
a
a
a
a
a
a
a
b
a
b
b
a
b
b
b
a
10 Day
27 Day
10 Day
27 Day
10 Day
27 Day
Control
Low Dose
High Dose

Figure 6.5: Tocopherol in liver, kidney and plasma of control and TCDF-dosed sturgeon. Data are expressed (mean with standard error) as µg of tocopherol per gm of wet tissue weight or ml of plasma. Statistical differences for data from the same tissue are denoted with different letters (n=5, α=0.05).

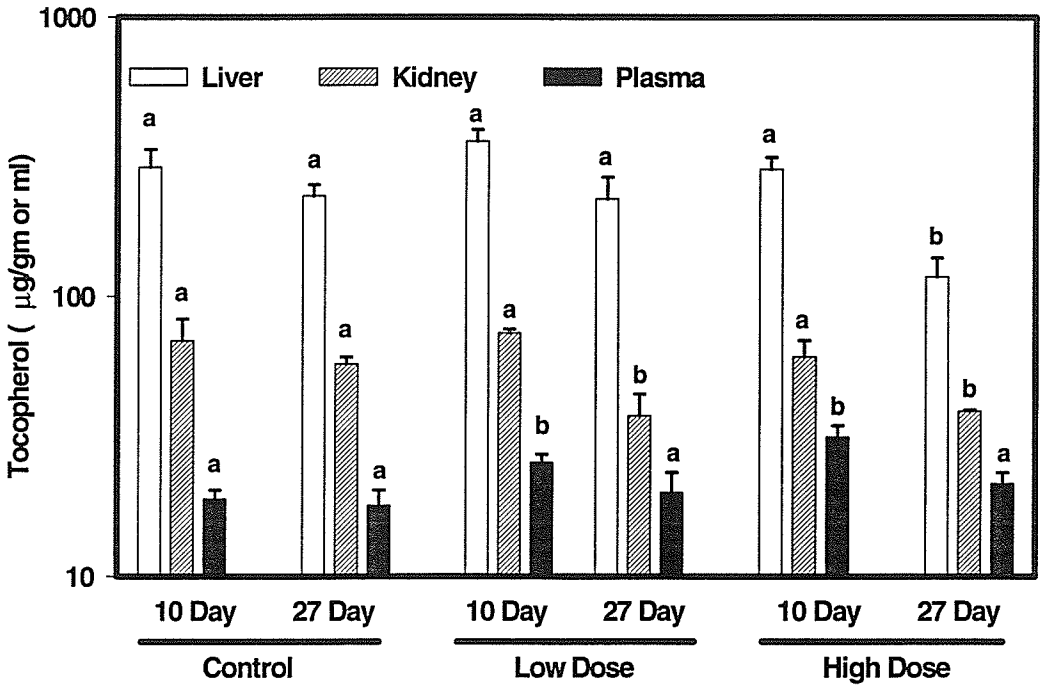
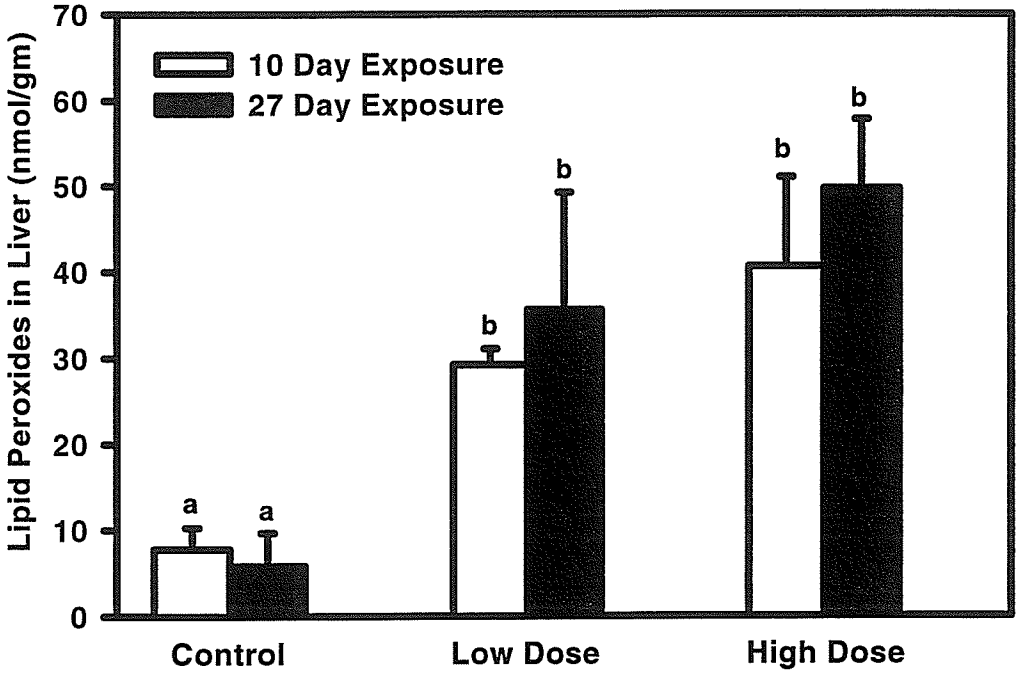

1000
100
10
Tocopherol (µg/gm or ml)
Liver
Kidney
Plasma
a
a
a
a
a
a
a
b
a
a
b
b
a
b
a
b
b
a
10 Day
27 Day
10 Day
27 Day
10 Day
27 Day
Control
Low Dose
High Dose

Figure 6.6: Lipid peroxides in liver of control and TCDF-dosed sturgeon. Data are expressed (mean with standard error) as nmol of lipid peroxides per gm of wet liver weight. Statistical differences between treatments are denoted with different letters (n=5, α=0.05).


Lipid Peroxides in Liver (nmol/gm)
70
60
50
40
30
20
10
0
10 Day Exposure
27 Day Exposure
a
a
b
b
b
b
Control
Low Dose
High Dose

Chapter 7

General Conclusions

Measuring the relative activity of phase I or mixed function oxidase (MFO) enzymes in tissues of test organisms has become an established method for detecting exposure to planar organic contaminants (Jimenez and Stegeman 1990, Payne et al. 1987, Luxon et al. 1987). Over the last decade more than 1,500 studies which included quantifications of phase I activity in aquatic organisms have appeared in the primary scientific literature. Despite the popularity of phase I measurements for establishing exposure of test organisms to organic contaminants, there are relatively few studies which attempt to link induction of phase I enzymes with specific mechanisms of toxicity.

Strong correlations between oxidative stress, measured by membrane breakdown products in the TBARS and lipid hydroperoxide assays, and induction of phase I enzymes were established in Chapters 2 and 6. Similarly, elevated activities of phase I enzymes and oxidative stress indices (lower tocopherol, elevated lipid hydroperoxides) were observed in tissues of lake trout collected from Lake Ontario, the most contaminated of the Great Lakes. We have clearly established that the cellular toxicity of planar organic contaminants is related to the elevated concentration of oxyradical intermediates mediated by induced phase I enzymes in fish tissues.

Indicators of membrane breakdown, including TBARS and lipid hydroperoxides, are relevant measurements for

determining the overall effect of increased oxyradical production (Uchiyama and Mihara 1978, Ohishi et al. 1985). Activities of enzymatic antioxidants and concentrations of non-enzymatic antioxidants can also provide useful information regarding the ability of an organism or specific tissue to withstand increased radical formation before the occurrence of membrane breakdown (Winston and DiGiulio 1991).

Lake trout from Lakes Ontario and Superior have different activities of the three antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). Different activities of these enzymes would afford varying levels of protection against increasing oxyradical production in these two populations of lake trout. However, no significant correlations between the altered activity in any of the three enzymes and induction of phase I activity were found in any of the laboratory or field experiments described in chapters 2 to 6. Variable responses to contaminants by these three enzymes have been reported in fish (Livingstone et al. 1993, Mather-Mihaich and Di Giulio 1991, Otto et al. 1994, Rodriguez-Ariza et al. 1993, Roberts et al. 1987). Winston and Di Giulio (1991) reviewed the relevant literature and concluded that the evidence for altered activities as indicators of contaminant exposure and oxidative stress was inconclusive. Based on data from Chapters 2 to 6, we must also conclude that alterations to the activities of the three antioxidant enzymes are not reliable indicators of oxidative

stress resulting from exposure to phase I inducers. However, it is likely that their measurement will continue to be a portion of any assessment of oxidative stress, because the enzymes are known to consume potentially toxic oxyradicals (Palace and Klaverkamp 1993).

Non-enzymatic antioxidants, including vitamins A, E and C, are also important factors contributing to the ability of an organism to defend against oxidative stress (Palozza and Krinsky 1991, Thomas 1987, Rousseau et al. 1992, Packer 1991). All three antioxidants were considered in our laboratory and field experiments. Data from chapters 2 and 3 indicated that vitamin E depletion was one of the most sensitive indicators of oxidative stress arising from exposure to contaminants that induce the phase I biotransformation system. Lower concentrations of tocopherol in tissues of lake trout from Lake Ontario, compared with lake trout from Lake Superior, appear to be a factor contributing to the higher oxidative stress of the Lake Ontario population.

Data from experiments in Chapters 2 and 3 appear to support the hypothesis that retinol was depleted in organisms exposed to organic contaminants because of its increased utilization as an antioxidant (Ribera et al. 1991, Wolf 1984). More specifically, hepatic retinol concentrations were depleted in lake trout exposed to PCB 126 in the laboratory and lake trout from Lake Ontario had lower tissue stores of retinol compared with lake trout from the less contaminated

Lake Superior. However, several mechanisms other than antioxidant action that had been proposed to account for the depletion of retinol in organochlorine-exposed organisms could not be dismissed based on data from the first two experiments.

By injecting radiolabelled retinol directly into the circulation of lake trout that had been preexposed to PCB 126, we were able to evaluate the proposed mechanisms of retinoid depletion. Recovery of both oxidized and reduced retinol was lower in PCB-dosed fish compared to control fish. Therefore, increased utilization of retinol as an antioxidant, with conversion to the oxidized form, is likely not the mechanism of retinoid depletion in fish with induced phase I enzymes. Instead, recoveries of unlabelled and labelled retinoids and correlations with hepatic phase I activity indicated that retinol is likely depleted due to direct metabolism by phase I enzymes. Vitamin A is essential for growth and differentiation and for maintaining epithelial cell layers (Zile 1992, Ndayibagara et al. 1995). Therefore, the induction of phase I enzymes in organochlorine-exposed fish is not only relevant to toxicity generated by increasing oxidative stress but also to potential toxicity arising from vitamin A depletion.

Additional research is required to determine if induced activity of phase I enzymes is also capable of increasingly metabolizing retinoic acid, a derivative of retinol (Horst et al. 1995). Retinoic acid is the most important form of vitamin

A for interacting with growth receptors. Gradients of retinoic acid within developing tissues are partly responsible for providing positional information by differentially promoting transcription (Tzimas et al. 1994, Holder and Hill 1991). If phase I activity is capable of altering the concentrations of retinoic acid in fish, as reported in mammals (Bank et al. 1989, Roberts et al. 1992), then implications for the embryonic development of phase I-induced organisms need to be examined.

The development of offspring from lake trout with induced phase I enzyme activity was appraised in chapter 4. Early mortality syndrome (EMS), characterized by mortality at a specific point of embryonic development and seen in the offspring of female lake trout from Lake Ontario (Fitzsimmons 1995, Fitzsimmons et al. 1995), correlates with hepatic concentrations of lipid hydroperoxides, a measure of oxidative stress. However, we cannot conclude that there is a direct relationship between EMS and oxidative stress, since this correlation holds only for those females whose offspring show greater than 50% mortality. It is possible that elevated oxidative stress in adult females may predispose their offspring to exhibit high EMS.

The relationships between EMS and the phase I capacity and vitamin antioxidant concentrations in lake trout embryos from Lake Ontario and a reference site (Lake Manitou) were also considered in chapter 4. There were no apparent

relationships between the percentage of EMS among the offspring and their phase I capacity, measured at the swimup stage. Additionally, antioxidant vitamin concentrations were similar between lake trout from Lake Ontario with and without EMS and lake trout from Lake Manitou at all of the 6 development stages considered. While these results suggest that EMS is unrelated to oxidative stress in embryonic lake trout, it should be noted that assays for phase I protein and antioxidant vitamins require that tissue specimens be frozen immediately. Dead embryos would have been necessarily excluded from analyses. Therefore, selection of embryos for analyses was biased toward survivors, especially among lake trout from Lake Ontario whose offspring exhibited >50% EMS and most noticeably at the critical later stages of development, when EMS appears. Additional analysis of the embryos, particularly at developmental stages near the manifestation of EMS, for indices of oxidative stress may clarify the relationships of oxidative stress and EMS in lake trout from Lake Ontario.

The relevance and applicability of relationships between phase I induction and oxidative stress that were established in experiments from chapters 2 to 5 were evaluated in a different fish species in chapter 6. Lake sturgeon (*Acipenser fulvescens*) were chosen as an additional test species for several reasons including their benthic lifestyle, high lipid content and a long age to sexual maturity (Birstein 1993). All of these factors contribute to sturgeons' great potential for

accumulating lipophilic contaminants and for being susceptible to their adverse effects (Rousseaux et al. 1995).

One of the contaminants found ubiquitously in tissues of fish from the Great Lakes is 2,3,7,8-tetrachlorodibenzofuran (TCDF) (Safe 1990). TCDF is a coplanar molecule, similar to PCB 126, and has been established as a potent inducer of phase I enzymes in fish (Walker and Peterson 1991). As with lake trout exposed to PCB 126, exposure of sturgeon to low doses of TCDF resulted in induced phase I enzyme activity which was correlated with increased breakdown of lipid membranes in liver tissue. Hepatic tocopherol concentrations also declined, providing additional evidence that TCDF-treated sturgeon were subject to greater oxidative stress.

Because they represent an important second step in detoxification of substrates that have already undergone phase I transformation , Phase II enzyme activity was monitored in lake trout tissues from experiments of Chapters 2 to 5. While phase II enzymes do not necessarily increase oxidative stress through their catalytic activity, the balance of their activity relative to phase I activity may contribute to an effect. Results from the sturgeon experiment emphasized this later point.

Similarly to earlier examinations of the biotransformation capacity of sturgeon (Perdu-Durand and Cravedi 1991), we found that sturgeon exposed to TCDF had low induction of phase II enzyme activity relative to the increase

of phase I activity. This relative inability of sturgeon to metabolize organic contaminants to even more polar compounds after phase I transformation could allow oxidatively active metabolites to accumulate, conferring a greater degree of sensitivity to the toxicity of these contaminants upon sturgeon. These results emphasized the importance of considering both phase I and phase II capacity when assessing the susceptibility of fish species to oxidative stress related to organochlorine exposure.

Appendix A

HPLC Determination of Tocopherol, Retinol, Dehydroretinol and Retinyl Palmitate in Tissues of Lake Trout (*Salvelinus namaycush*) Exposed to Coplanar 3,3',4,4',5-Pentachlorobiphenyl

Abstract

Tocopherol, retinol, dehydroretinol and retinyl palmitate were measured by reverse-phase HPLC in liver, kidney and plasma of lake trout exposed to orally administered coplanar 3,3',4,4',5-pentachlorobiphenyl. Tocopherol concentrations were unaffected after 8 weeks. Liver retinol, dehyroretinol and retinyl palmitate concentrations were lower while kidney retinyl palmitate was elevated in PCB exposed groups. Tissue retinoid concentrations provide sensitive indicators of coplanar PCB exposure in fish.

Introduction

A variety of important physiological processes depend upon adequate levels of tocopherol (Vitamin E) and retinol (Vitamin A). Tocopherol is important because of its activity as an antioxidant in protecting cellular and subcellular membranes (Burton and Traber 1990). Retinol deficiency in fish is associated with loss of vision, edema, depigmentation and impaired growth (Halver 1982). Both vitamins are essential dietary elements and have received increasing attention as indicators of exposure to a variety of environmental contaminants. Chemicals that bind with the aryl hydrocarbon (Ah) receptor induce biotransformation enzyme activities that in turn can increase oxidative stress (Lehtinen 1990) leading to a reduction of tissue stores of tocopherol (Packer 1991). Vitamin A concentrations in kidney, liver and serum of rats are altered by exposure to organic pollutants, particularly chemicals (eg. coplanar-PCBs) that interact with the Ah receptor (Chen et al. 1992).

Fish liver and liver oils represent rich sources of retinoids and tocopherol (Kutsky 1973). Retinol and retinyl palmitate have been identified as bioindicators of exposure to organic contaminants in mammals and birds (Chen et al. 1992, Spear et al. 1990, Fox 1993). However, information about retinoid stores in fishes exposed to organic contaminants is limited (Spear et al. 1992). Recently, Arnaud et al. (1991)

described a fast effective reverse-phase HPLC method for measuring both retinol and tocopherol. Although this method was developed for mammalian serum samples, we present results showing its utility for quantifying tocopherol, retinol, dehydroretinol and retinyl palmitate in liver, kidney and plasma of lake trout (*Salvelinus namaycush*). Moreover, similar methodology was recently used to quantify retinoids in serum, eyes and eggs of rainbow trout (*Salmo gairdneri*) (Guillou et al. 1989). We also present preliminary data examining the effect of a single oral dose of 3,3',4,4',5-pentachlorobiphenyl (PCB congener 126) on tocopherol, retinol, dehydroretinol and retinyl palmitate in liver, kidney and plasma of lake trout 8 weeks after ingestion. In this study, the PCB doses produced muscle concentrations similar to those previously reported in fish from the Great Lakes and other freshwater systems (Niimi and Oliver 1989, Hong et al. 1992).

Materials and Methods

Juvenile lake trout (247 ± 44 gm) were acclimated for 4 weeks in 140 L fibreglass tanks receiving at least 2 L/gm fish/day of dechlorinated Winnipeg city tap water (12 ± 2°C, [$CaCO_3$]= 82.5 mg/L, pH = 7.8). Fish were held on a 12 hour light:12 hour dark photoperiod and maintained with a diet of commercial trout chow at 1% of bodyweight every second day.

[^{14}C] PCB 126 (specific activity = 8.7 x 10^{11} Bq/mol) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in ethanol and warmed gelatin (Sijm et al. 1990). Fish were treated with a single oral dose of gelatin/ethanol at 2 ml/kg containing nominal concentrations of 0 (control), 1.2 (low dose) or 40 (high dose) µg [^{14}C] PCB 126/kg fish weight. Capillary gas-chromatography analysis of the PCB 126 showed a purity of >99.5%. Extractable radioactivity was determined after 8 weeks by homogenizing freeze-dried muscle in toluene and assaying portions of this extract by liquid scintillation counting. Following gavage (oral delivery of the contaminant dose directly to the gut through small diameter tubing), fish were returned to the same tanks. After 8 weeks, fish were anesthetized for 5 minutes with tricaine methanesulphonate neutralized to tank pH with ammonium hydroxide. Blood was collected from the caudal vessels with pre-heparinized 3 ml syringes and immediately centrifuged to obtain plasma. Tissues were dissected and put into sterile plastic bags and immediately frozen with the plasma at -120°C until analyzed. We used distilled deionized (Milli Q, Millipore Inc.) water as a homogenizing media for tissues. To precipitate proteins after homogenization, 200 µl of HPLC grade ethanol was added to 200 µl of tissue homogenate or plasma. Plasma, liver and kidney samples were extracted with 500 µl of (3:2, v/v) ethyl acetate/hexane. Residues from the ethyl acetate/hexane extracts were redissolved in mobile phase

and injected (20 µl) onto a 3 µm bead size Adsorbosphere HS C_{18} column, 4.6 mm i.d., 150 mm length, with attached 10 mm Adsorbosphere guard column (Alltech Associates Inc.). The HPLC system consisted of two model 302 solvent pumps, a model 231 sample injector, a model 704 system controller, a model 620 data module and a model 116 dual channel UV absorbance detector. The detector was set at 292nm for tocopherol and tocopherol acetate detection, and at 325nm for retinol and retinyl palmitate detection (Gilson Medical Electronics). The column was maintained by a thermostat at 26°C and samples and standards were eluted isocratically with acetonitrile/methanol/water (70:20:10, v/v/v) delivered at a flow rate of 1.0 ml/min. Standard retinol, retinyl palmitate, tocopherol and tocopherol acetate were purchased from Sigma Chemical Co. (St. Louis, Mo.) and 3,4-dehyroretinol was a generous gift from Dr. H. Keller (F. Hoffmann-La Roche Ltd., Basle, Switzerland). As with previous work (Edmonds and Nierenberg 1993), there was no loss of these compounds after tissue storage at -110 ± 1°C for 30 days.

Reproducibility of the method was evaluated by 10 measurements of tocopherol and retinoids in the same tissue. Tocopherol acetate was used as an internal standard, with the recovery of a known spike (575 ng) used to correct for the efficiency of each extraction. Recovery efficiencies were determined by spiking known amounts of tocopherol (875 - 4375 ng) and retinoids (8 - 400 ng). Varying amounts of tissue (2.5

- 50 mg) or plasma (50 - 200 µl) were analyzed to examine the correlation between the amount of tissue and the concentration of tocopherol and retinoids.

Data were analyzed using ANOVA. Pairwise comparisons were conducted with Fisher's least significant difference and Duncan's tests. A probability level 0.05 was considered significant.

Results and Discussion

Extracted standards eluted with characteristic retention times (Fig. App.1A) that did not differ between preparations. Figure App.1 also shows typical elution profiles from extracted lake trout liver (Fig. App.1B), kidney (Fig. App.1C) and plasma (Fig. App.1D). While both liver and kidney contained substantial amounts of tocopherol, retinol, dehydroretinol and retinyl palmitate, levels of retinyl palmitate and dehydroretinol were not routinely detectable in plasma.

A dilution series of liver tissue ranging from 2.5 mg to 50 mg of tissue in 200 µl of homogenate showed linearity of detection for tocopherol, retinol, dehydroretinol and retinyl palmitate with R^2 values of 0.997, 0.997, 0.996 and 0.933, respectively. Similar results were obtained when 50 to 200 µl volumes of plasma were used. Routinely, 10 mg of both liver

and kidney in 200 µl of homogenate or 200 µl of plasma were extracted. This allowed reproducible results while using the least practical amount of tissue. When tissue or plasma samples were spiked with known amounts of tocopherol or retinoids, recovery of the spikes was 93.0 to 94.8% and 87.7 to 98.5%, respectively. Tocopherol acetate, used as an internal standard, corrected for differences in sample extraction efficiencies for liver (77.3 ± 3.9%), kidney (75.1 ± 4.5%) and plasma (68.4 ± 1.0%). The precision of the method was evaluated by repeated measures of a single tissue sample. Ten separate extractions of 10 mg of tissue led to percent standard error measures of 1.14% for tocopherol and 2.18 - 4.21% for the retinoids, respectively. Similar results (2.4 - 5.1%) were obtained with other tissue amounts (2 - 100 mg).

Eight weeks after the oral dose, muscle PCB 126 concentrations of 0.622 ± 0.136 µg/kg and 12.69 ± 0.896 were found in the low and high dose groups respectively. These concentrations are environmentally relevant because similar concentrations of Ah-inducing chemicals have been reported in Great Lakes fish (Niimi and Oliver 1989) and in fish from other freshwater systems (Hong et al. 1992).

Concentrations of tocopherol in liver, kidney and plasma were similar to those we previously reported in rainbow trout using a different analysis technique (Palace et al. 1993) and to those reported in other fish (Cowey et al. 1983, Hardie et al. 1990). Moreover, retinol, dehydroretinol and retinyl

palmitate concentrations were near concentrations previously reported in fish (Spear et al. 1992, Guillou et al. 1989, Scheidt et al. 1986).

There were no differences between treatments in tocopherol concentrations in plasma or liver. In kidney, fish exposed to the low PCB dose showed a slight but significantly higher tocopherol concentration than control or high dosed groups (Fig. App.2A). These results are surprising given that previous work has shown an increase of oxidative stress in rats exposed to Ah-inducing compounds similar to PCB 126 (Stohs et al. 1990) and that tocopherol stores can decline when exposed to oxidative radicals (Pallozza and Krinsky 1991). Exposure to PCB 126 for 8 weeks may be insufficient to produce oxidative stress capable of depleting tocopherol stores in lake trout.

Retinol and dehydroretinol concentrations in liver of PCB exposed fish were lower than in livers of fish treated with gelatin containing no PCB (Fig. App. 2 B and C). Also, the high dose fish had more severely depressed retinol than the low dose group. Although the high dose group appeared to have higher kidney retinol stores, there were no significant differences in either retinol or dehydroretinol concentrations. In plasma, the high dose group had lower plasma retinol than control and low dose fish.

The data from the high dose group of fish in this study (Fig. App. 2C) appear to support recent mammalian studies

(Hakannsson et al. 1991, Jurek et al. 1990) showing that lower retinoid concentrations in the liver are concurrent with a decline of plasma retinol but higher kidney retinyl palmitate. High retinoids in the kidney may be the result of altered esterifying enzymes and may represent an attempt to limit vitamin A loss (Jurek et al. 1990) and minimize potential vision, growth and metabolic disturbances.

There is a growing body of evidence showing that liver retinol stores decline with exposure to dioxin-like contaminants in mammals (Chen et al. 1992, Jurek et al. 1990, Mercier et al. 1990] and birds (Spear and Moon 1986). Lower liver retinol stores are often associated with contaminants that bind to the Ah receptor (Chen et al. 1992) and induce mixed function oxidase (MFO) enzyme activity. Induction of the phase I MFO enzyme activities and the phase II conjugating enzyme UDP-glucuronyltransferase are thought to account directly for increased metabolism of retinol in the liver (Vanden Bossche and Willemsens 1991, Zile 1992). Conversely, Brouwer and van den Berg (1986) reported altered retinoid concentrations in mice exposed to PCBs doses that did not induce MFO activity. They suggest that metabolites of PCB molecules may interfere with the binding of transthyretin to retinol-binding protein (RBP). Unbound RBP may be filtered through the glomerular membrane causing a loss of RBP and retinol. Because the liver can rapidly convert retinyl palmitate to retinol (Brouwer and van den Berg 1986), these

mechanisms may explain the concurrent decline of retinyl palmitate and retinol observed in lake trout liver in this study.

Summary and Conclusions

We have shown that the method of Arnaud et al. (1991) for simultaneously measuring tocopherol and retinol can effectively quantify tocopherol, retinol dehydroretinol and retinyl palmitate in plasma, liver and kidney tissues of freshwater fish. Although their dynamic mechanisms require examination, retinoids are promising bioindicators of exposure to PCB congener 126.

Figure App.1. HPLC chromatograms for: A) a standard containing 185 ng dehydroretinol (dR), 80 ng retinol (R), 80 ng retinol acetate (RA), 875 ng tocopherol (T), 570 ng tocopherol acetate (TA) and 400 ng retinyl palmitate (RP), B) liver C) kidney and D) plasma of lake trout. The detector was set at 325 nm from 0 - 4 minutes, 292 nm from 4.01 - 7 minutes and 325 nm from 7.01 - 12 minutes for each chromatogram. Full scale deflections for chromatograms A, B, C and D were 95, 43.3, 664.2 and 674.6 mV, respectively.

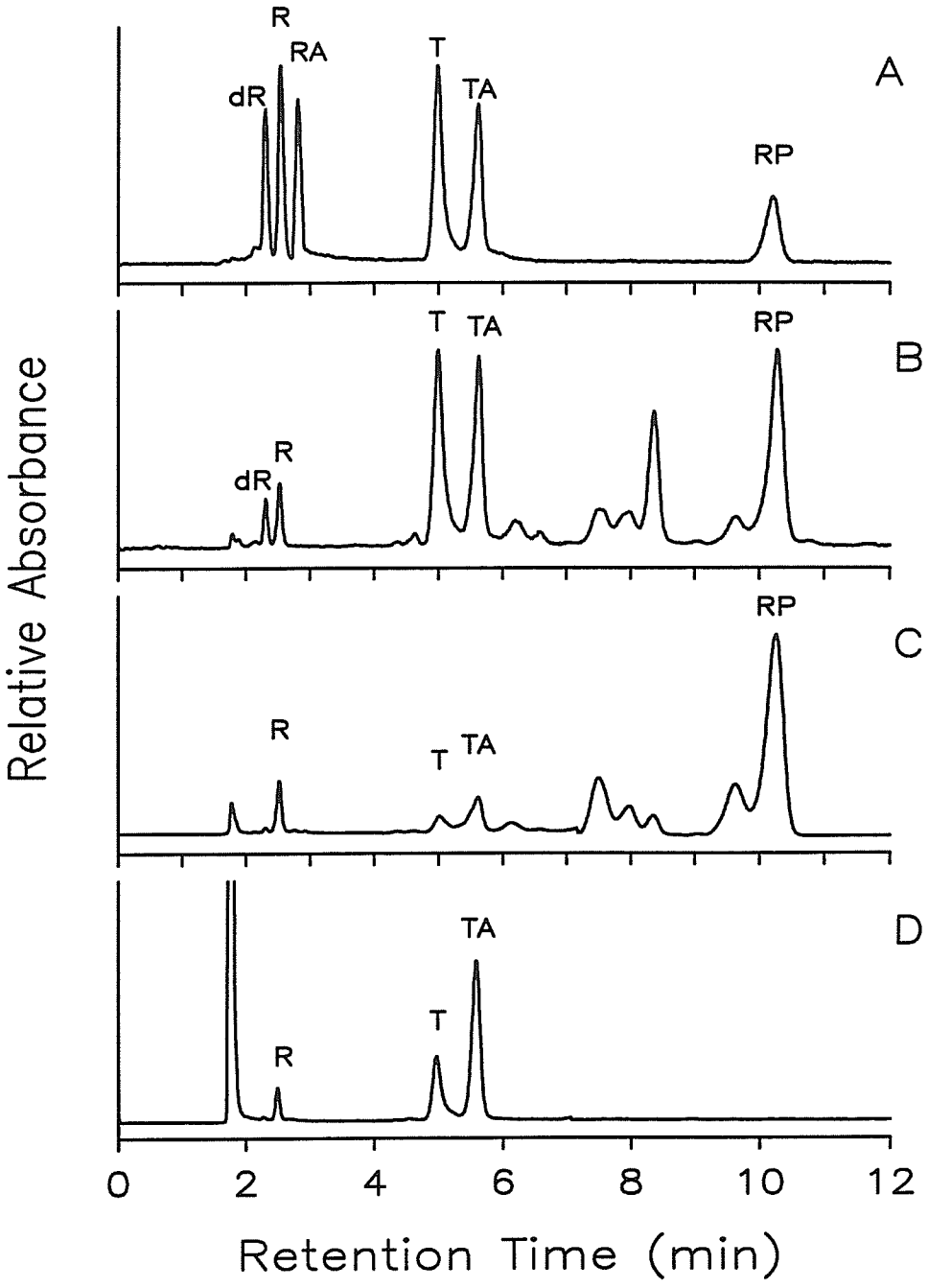

R
RA
dR
T
TA
RP
A
T
TA
RP
B
dR
R
RP
C
R
T
TA
TA
D
T
R
0
2
4
6
8
10
12
Relative Absorbance
Retention Time (min)

Figure App.2. Concentrations of A) Tocopherol in liver, kidney and plasma, B) retinol in liver (filled bars), kidney (open bars) and plasma (cross-hatch bars), C) dehydroretinol in liver and kidney and D) retinyl palmitate in liver and kidney of lake trout exposed to 0 (control), 1.2 (low dose) or 40 (high dose) µg PCB 126/kg fish weight. Statistical differences between treatment means are noted by * (n = 5, p = 0.05).

Tocopherol in liver (µg/gm)
Retinol in liver and kidney (µg/gm)
Dehydroretinol in liver (µg/gm)
Retinyl palmitate in liver (µg/gm)
A
B
C
D
Control
Low Dose
High Dose
Tocopherol in kidney or plasma (µg/gm or µg/ml)
Retinol in plasma (ng/ml)
Dehydroretinol in kidney (µg/gm)
Retinyl palmitate in kidney (µg/gm)

Literature Cited

Addison, R.F. and D.E. Willis. 1982. Variation of hepatic ethoxycoumarin-o-deethylase activity with body weight and other factors in brook trout (*Salvelinus fontinalis*). *Can. J. Fish. Aquat. Sci.* 39:924-926.

Aebi, H. 1974. Catalase. In *Methods of Enzymatic Analysis.* H.U. Bergmayer [Ed]. Academic Press, N.Y. pp.673-684.

Al-Bayati, Z.A.F., W.J. Murray and S.J. Stohs. 1987. 2,3,7,8-tetrachlorodibenzo-p-dioxin induced lipid peroxidation in hepatic and extrahepatic tissues of male and female rats. *Arch. Environ. Contam. Toxicol.* 16:159-166.

Andersson, T. and L. Forlin. 1992. Regulation of the cytochrome P450 enzyme system in fish. *Aquat. Toxicol.* 24:1-20.

Andersson, T., L. Forlin, J. Hardig and A. Larsson. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft mill effluents. *Can. J. Fish. Aquat. Sci.* 45:1525-1536.

Ando, S. and M. Hatano. 1991. Distribution of carotenoids in the eggs from four species of salmonids. *Comp. Biochem. Physiol.* 99B:341-344.

Ando, S., K. Osada, M. Hatano and M. Saneyoshi. 1989. Comparison of carotenoids in muscle and ovary from four genera of salmonid fishes. *Comp. Biochem. Physiol.* 93B:503-508.

Antoine, B., J.A. Boutin and G. Siest. 1988. Investigation of UDP-glucuronosyltransferase isoenzymes: restricted-specificity in liver microsomes. In *Molecular Aspects of Glucuronidation.* G. Siest, J. Magdalou, B. Burchell [Eds]. John Libbey Eurotext Ltd., London.pp79-324.

Ardelt, B.K., J.L. Borowitz and G.E. Isom. 1989. Brain lipid peroxidation and antioxidant protection mechanisms following acute cyanide intoxication. *Toxicol.* 56:147-154.

Arinc, E. and A. Sen. 1993. Characterization of cytochrome P-450 dependent mixed-function oxidase system of gilthead seabream (*Sparus aurata; Sparidae*) liver. *Comp. Biochem. Physiol.* 104B:133-139.

Arnaud, J., I. Fortis, S. Blachier and A. Favier. 1991. Simultaneous determination of retinol, ∝-tocopherol and ß-carotene in serum by isocratic high-performance liquid chromatography. *J. Chromatog.* 572:103-116.

Babaich, H., M.R. Palace and A. Stern. 1993. Oxidative stress in fish cells: *in vitro* studies. *Arch. Environ. Contam. Toxicol.* 24:173-178.

Bagchi, M. and S.J. Stohs. 1992. *In vitro* induction of reaction oxygen species by 2,3,7,8-tetrachlorobibenzo-p-dioxin and other halogenated cyclic hydrocarbons in rat peritoneal macrophages, and hepatic mitochondria and microsomes. *Chemosphere.* 25:905-909.

Bank, P.A., K.L. Salyers and M. Zile. 1989. Effect of tetrachlorodibenzo-p-dioxin (TCDD) on the glucuronidation of retinoic acid in the rat. *Biochim. Biophy. Acta.* 993:1-6.

Barton, B.A., R.E. Peter and C.R. Paulencu. 1980. Plasma cortisol levels of fingerling rainbow trout (*Salmo gairndneri*) at rest, and subjected to handling, confinement, transport and stocking. *Can. J. Fish. Aquat. Sci.* 37:805-811.

Baumann, P.C. and D.M. Whittle. 1988. The status of selected organics in the Laurentian Great Lakes: an overview of DDT, PCBs, dioxins, furans and aromatic hydrocarbons. *Aquat. Toxicol.* 11:241-257.

Bengtsson, B.E., A. Bergman, I. Brandt, C. Hill, N. Johansson, A. Sodergren and J. Thulin. 1994. Reproductive disturbances in Baltic fish. *Swedish Environmental Protection Agency Report #4319.* 30pp.

Binder, R.L. and J.J. Stegeman. 1983. Basal levels and induction of hepatic aryl hydrocarbon hydroxylase activity during the embryonic period of development in brook trout. *Biochem. Pharmacol.* 32:1324-1327.

Birstein, V.J. 1993. Sturgeons and paddlefishes: threatened fishes in need of conservation. *Conserv. Biol.*7:773-787.

Boon, J.P., J.M. Everaarts, M.T.J. Hillebrand, M.L. Eggens, J.Pijnenburg and A. Goksoyr. 1992. Changes in levels of hepatic biotransformation enzymes and haemoglobin levels in female plaice (*Pleuronectes platessa*) after oral administration of a technical polychlorinated biphenyl mixture (Clophen A40). *Sci. Tot. Environ.* 114:113-133.

Borgmann, U. and D. M. Whittle. 1991. Contaminant concentration in Lake Ontario lake trout (*Salvelinus namaycush*): 1977 to 1988. *J. Great Lakes Res.* 17:368-381.

Branchaud, A., A. Gendron, R. Fortin, P.D. Anderson and P.A. Spear. 1995. Vitamin A stores, teratogenesis and EROD activity in white sucker, *Catostomus commersoni*, from Riviere des Prairies near Montreal and a reference site. *Can. J. Fish. Aquat. Sci.* 52:1703-1713.

Bremner, I. and J.H. Beattie. 1990. Metallothionein and the trace minerals. *Ann. Rev. Nutr.* 10:63-83.

Bremner, I. 1987. Nutritional and physiological signifcance of metallothionein. *Experentia Suppl.* 52:81-107.

Brouwer, A. and K.J. van den Berg. 1986. Binding of a metabolite of 3,3',4,4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxin. *Toxicol. Appl. Pharmacol.* 85:301-312.

Brown, S.B., J.G. Eales and T.J. Hara. 1986. A protocol for estimation of cortisol plasma clearance in acid-exposed rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrin.* 62:493-502.

Burchell, B. and P. Whetherhill. 1981. 4-Nitrophenol UDP-glucuronyltransferase (Rat Liver). *Meth. Enzymol.* 77:169-177.

Burton, G.W. and M.G. Traber. 1990. Vitamin E: antioxidant activity, biokinetics and bioavailability. *Ann. Rev. Nutr.* 10:357-382.

Burton, G.W. and K.U. Ingold. 1984. β-carotene: an unusual type of lipid antioxidant. *Science*. 224:569-580.

Canada, A.T. and E.J. Calabrese. 1989. Superoxide dismutase:its role in xenobiotic detoxification. *Pharmc. Ther.* 44:285-295.

Casillas, E. and W. Ames. 1986. Hepatotoxic effects of CCl_4 on English Sole (*Parophrys vetulus*): possible indicators of liver dysfunction. *Comp. Biochem. Physiol.* 84C:397-400.

Casselman, J.M. 1990. Research Project: Lake trout rehabilitation studies. In *Lake Ontaro Fisheries Unit, Annual Report, LOA 91.1 (Chapter 4)*, Ontario Ministry of Natural Resources, pp.1-12.

Chambon, P., A. Zelent, M. Petkovich, C. Mendelsohn, P. Leroy, A. Krust, P. Kastner and N. Brand. 1991. The family of retinoic acid nuclear receptors. In *Retinoids: 10 Years On.* J.H. Saurat [Ed], S. Karger Publishers, Basel, Switzerland. pp.10-27.

Chatterjee, I.B. and A. Nandi. 1991. Ascorbic acid: a scavenger of oxyradicals. *Ind. J. Biochem. Biophys.* 28:233-236.

Chen, L.C., I. Berberian, B. Koch, M. Mercier, V. Azais-Braesco, H.P. Glauert, C.K. Chow and L.W. Robertson. 1992. Polychlorinated and polybrominated biphenyl congeners and retinoid levels in rat tissues: structure-activity relationships. *Toxicol. Appl. Pharmacol.* 114:47-55.

Clarke, D.J., S.G. George and B. Burchell. 1991. Glucuronidation in fish. *Aquat. Toxicol.* 20:35-56.

Comstock, G.W., A.J. Alberg and K. Helzlsouer. 1993. Reported effects of long-term freezer storage on concentrations of retinol, β-carotene and α-tocopherol in serum or plasma summarized. *Clin. Chem.* 39:1075-1078.

Cowey, C.B., J.G. Bell, D. Knox, A. Fraser and A. Youngson. 1985. Lipids and antioxidant systems in developing eggs of salmon (*Salmo salar*). *Lipids* 20:567-572.

Cowey, C.B., J.W. Adron and A. Youngson. 1983. The vitamin E requirement of rainbow trout (*Salmo gairdneri*) given diets containing polyunsaturated fatty acids derived from fish oil. *Aquaculture.* 30:85-93.

Curtis, G.L. 1990. Recovery of an offshore lake trout (*Salvelinus namaycush*) population in eastern Lake Superior. *J. Great Lakes Res.* 16:279-287.

Dabrowski, K. and J.H. Blom. 1994. Ascorbic acid deposition in rainbow trout (*Oncorhynchus mykiss*) eggs and survival of embryos. *Comp. Biochem. Physiol.* 108A:129-135.

Dabrowski, K. 1990. Gulunolactone oxidase is missing in teleost fish. *Biol. Chem. Hoppe-Seyler.* 371:207-214.

De Vault, D.D., W. Dunn, P.A. Bergqvist, K. Wiberg and C. Rappe. 1989. Polychlorinated dibenzofurans and polychlorinated dibenzo-p-dioxins in Great Lakes fish: a baseline and interlake comparison. *Environ. Toxicol. Chem.* 8:1013-1022.

Dillon, T.M., W.H. Benson, R.A. Stackhouse and A.M. Crider. 1990. Effects of selected PCB congeners on survival, growth and reproduction in *Daphnia magna*. *Environ. Toxicol. Chem.* 9:1317-1326.

Edmonds, B.K. and D.W. Nierenberg. 1993. Serum concentrations of retinol, d-α-tocopherol and β-carotene: effects of storage at -70°C for five years. *J. Chromatog.* 614:169-174.

Edwards, A.J., R.F. Addison, D.E. Willis and K.W. Renton. 1988. Seasonal variation of hepatic mixed function oxidases in winter flounder (*Pseudopleuronectes americanus*).*Mar. Environ. Res.* 26:299-309.

Edwards, C.J., R.A. Ryder and T.R. Marshall. 1990. Using lake trout as a surrogate of ecosystem health for oligotrophic waters of the Great Lakes. *J. Great Lakes Res.* 16:591-608.

Eggens, M.L. and F. Galgani. 1992. Ethoxyresorufin-O-deethylase (EROD) activity in flatfish: fast determination with a fluorescence plate-reader. *Mar. Environ. Res.* 33:213-221.

Elskus, A.A. and J.J. Stegeman. 1989. Further consideration of phenobarbital effects on cytochrome P-450 activity in killifish, *Fundulus heteroclitus*. *Comp. Biochem. Physiol.* 92C:223-230.

Eschenroder, R. 1990. History and current status of lake trout reproduction in the Great Lakes In *Proceedings of the Roundtable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commission. Windsor, Ontario.

Esterbauer, H., R.J. Schaur and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Rad. Biol. Med.* 11:81-128.

Falandysz, J., S. Tanabe and R. Tatsukawa. 1994. Most toxic and highly bioaccumulative PCB congeners in cod-liver oil of Baltic origin processed in Poland during the 1970s and 1980s, their TEQ-values and possible intake. *Sci. Tot. Environ.* 145:207-212.

Fitzsimmons, J.D., S. Huestis and B. Williston. 1995. Occurrence of a swim-up syndrome in Lake Ontario lake trout in relation to contaminants and cultural practices. *J. Great Lakes Res.* 21(suppl.1):277-285.

Fitzsimmons, J.D. 1995. The effect of B-vitamins on a swim-up syndrome in Lake Ontario lake trout. *J. Great Lakes Res.* 21(suppl. 1):286-289.

Fitzsimmons, J.D. 1990. Yellow perch predation on lake trout eggs in Keuka Lake, New York. *J. Great Lakes Res.* 16:130-132.

Forlin, L. and T. Andersson. 1985. Storage conditions of rainbow trout liver cytochrome P-450 and conjugating enzymes. *Comp. Biochem. Physiol.* 80B:569-572.

Forlin, L. and T. Hansson. 1982. Effects of oestradiol-17β and hypophysectomy on hepatic mixed function oxidases in rainbow trout. *J. Endocrin.* 95:245-252.

Forlin, L. and C. Haux. 1990. Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. *J. Endocrin.* 124:207-213.

Forlin, L. and C. Haux. 1985. Increased excretion in the bile of 17β-[^{3}H]estradiol-derived radioactivity in rainbow trout treated with β-napthoflavone. *Aquat. Toxicol.* 6:197-208.

Fox, G.A. 1993. What biomarkers told us about the effects of contaminants on the health of Great Lakes wildlife. *J. Great Lakes Res.* 20:212-216.

Frei, B., L. England and B.N. Ames. 1989. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci.* 86:6377-6381.

Frimith, J. 1990. Lake Ontario coho salmon. In *Roundtable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commission. Windsor, Ontario.

Futterman, S. and J.S. Andrews. 1964. The composition of liver vitamin A ester and the synthesis of vitamin A ester by liver microsomes. *J. Biol. Chem.* 239:4077-4080.

Gabryelak, T., M. Piatrowska, W. Leyko and G. Peres. 1983. Seasonal variation in the activities of peroxide metabolism enzymes in erythrocytes of freshwater fish. *Comp. Biochem. Physiol.* 75C:383-385.

Geetha, A., J. Catherine and C.S. Shyamamla Devi. 1989. Effect of α-tocopherol on the microsomal lipid peroxidation induced by doxorubicin: influence of ascorbic acid. *Indian J. Physiol. Pharmacol.* 33:53-58.

Giesy, J.P., J. Newsted and D.L. Garling. 1986. Relationships between chlorinated hydrocarbon concentrations and rearing mortality of chinook salmon (*Oncorhynchus tshawytscha*) eggs from Lake Michigan. *J. Great Lakes Res.* 12:82-98.

Gilbert, N.L., M.J. Cloutier and P.A. Spear. 1995. Retinoic acid hydroxylation in rainbow trout (*Onchorhynchus mykiss*) and the effect of a coplanar PCB, 3,3',4,4'-tetrachlorobiphenyl. *Aquat. Toxicol.* 32:177-187.

Gilbertson, M. 1992. PCB and dioxin research and implications for fisheries research and resource management. *Fisheries.* 17:26-27.

Gilbertson, M., T. Kubiak, J. Ludwig and G. Fox. 1991. Great Lakes embryo mortality, edema and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. *J. Toxicol. Environ. Health.* 33:455-520.

Goksoyr, A. and L. Forlin. 1992. The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquat. Toxicol.*22:287-312.

Goksoyr, A. and H.E. Larsen. 1991. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): I basal properties and induction of P450 1A1 in liver of immature and mature fish. *Fish Physiol. Biochem.* 9:339-349.

Goksoyr, A., H.E. Larsen and A.M. Husoy. 1991. Application of a cytochrome P-450 IAI-ELISA in environmental monitoring and toxicological testing of fish. *Comp. Biochem. Physiol.* 100C:157-160.

Gonzalez Flecha, B.S., M. Repetto, P. Evelson and A. Boveris. 1991. Inhibition of microsomal lipid peroxidation by α-tocopherol and α-tocopherol acetate. *Xenobiotica*. 21:1013-1022.

Green, M.H., J. Balmer Green, T. Berg, K.R. Norum and R. Blumhoff. 1993. Vitamin A metabolism in rat liver: a kinetic model. *Am. J. Physiol.* 264:G509-G521.

Greenstock, C.L. 1993. Radiation and ageing: free radical damage, biological response and possible antioxidant intervention. *Med. Hypoth.* 41:473-482.

Gregus, Z., J.B. Watkins, T.N. Thompson, M.J. Harvey, K. Rozman and C.D. Klassen. 1983. Hepatic phase I and phase II biotransformations in quail and trout: comparison to other species commonly used in toxicity testing. *Toxicol. Appl. Pharmacol.* 67:430-441.

Guengerich, P.F. 1990. Enzymatic oxidation of xenobiotic chemicals. *Crit. Rev. Biochem Mol. Biol.* 25:97-153.

Guillou, A., G. Choubert, T. Storebakken, J. de la Noue and S. Kaushik. 1989. Bioconversion pathway of astaxanthin into retinol2 in mature rainbow trout (*Salmo gairdneri* Rich.). *Comp. Biochem. Physiol.* 94B:481-485.

Günzler, W.A. and L. Flohé. 1986. Glutathione peroxidase. In *CRC Handbook of Methods for Oxygen Radical research.* R.A. Greenwald [Ed]. CRC Press, Boca Raton, FL. pp.285-290.

Gutteridge, J.M.C. and B. Halliwell. 1990. The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biochem. Sci.* 15:129-135.

Hakansson, H., E. Manzoor and U. Ahlborg. 1992. Efects of technical preparations and fractions therof on vitamin A levels in mink (*Mustela vison*). *Ambio.* 21:588-590.

Hakansson, H., E. Manzoor and U.G. Ahlborg. 1991. Interaction between vitamin A and single oral doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the TCDD-induced toxicity and on the vitamin A status in the rat. *J. Nutr. Sci. Vitaminol.* 37:239-255.

Halliwell, B., J.R. Hoult and D.R. Blake. 1988. Oxidants, inflammation and antiinflammatory drugs. *FASEB J.* 2:2867-2873.

Halver, J.E. 1982. The vitamins required for cultivated salmonids. *Comp. Biochem. Physiol.* 73B:43-50.

Hardie, L.J., T.C. Fletcher and C.J. Secombes. 1990. The effect of vitamin E on the immune response of the Atlantic Salmon (*Salmo salar* L.). *Aquaculture.* 87:1-13.

Hartman, W.L. 1988. Historical changes in the major fish resources of the Great Lakes. In *Toxic Contaminants and Ecosystem Health: A Great Lakes Focus.* M.S. Evans [Ed]. John Wiley and Sons, New York. pp. 103-131.

Haux, C. and L. Forlin. 1988. Biochemical methods for detecting effects of contaminants on fish. *Ambio.* 17:376-380.

Helder, T. 1981. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on early life stages of rainbow trout (*Salmo gairdneri* Richardson). *Toxicology.* 19:101-112.

Hodson, P.V., P.J. Kloepper-Sams, K.R. Munkittrick, W.L. Lockhart, D.A. Metner, P.L. Luxon, I.R. Smith, M.M. Gagnon, M. Servos and J.F. Payne. 1991. Protocols for Measuring Mixed Function Oxygenases of Fish liver. *Canadian Technical Report of Fisheries and Aquatic Sciences #1829.* 51pp.

Hodson, P.V., H. Ralph, L. Luxon, B. Gray and M. McWhirter. 1990. Mixed function oxidase (MFO) activity of Great Lakes fish. pp.38-39. In *Proceedings of the Roundtable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commission, Windsor, Ont. Sept 24-25.

Hodson, P.V., K. Ralph, L. Luxon, B. Gray and M. McWhirter. 1989. Mixed function oxidase (MFO) activity of Great Lakes fish. Abstract P182, In *Proceedings of the 10th Annual Meeting of the Society of Environmental Toxicology and Chemistry.*, p. 236.

Holder, N. and J. Hill. 1991. Retinoic acid modifies development of the midbrain-hindbrain border and affects cranial ganglion formation in zebrafish embryos. *Development.* 113:1159-1170.

Hong, C.S., B. Bush and J. Xiao. 1992. Coplanar PCBs in fish and mussels from marine and estuarine waters of New York State. *Ecotox. Environ. Saf.*23:118-131.

Horst, R.L., T.A. Reinhardt, J.P. Goff, B.J. Nonnecke, V.K. Gambhir, P.D. Fiorella and J.L. Napoli. 1995. Identification of 9-cis,13-cis retinoic acid as a major circulating retinoid in plasma. *Biochemistry.* 34:1203-1209.

Horton, A.A. and S. Fairhurst. 1987. Lipid peroxidation and mechanisms of toxicity. *CRC Crit. Rev. Toxicol.*18::27-49.

Iannelli, M.A., I. Marcucci and L. Vittozzi. 1994. Xenobiotic-metabolizing enzyme systems in test fish. V. Comparative studies of liver microsomal glucuronyltransferases. *Ecotox. Environ. Saf.* 28:172-180.

Ichinose, T., M.G. Miller and T. Shibamoto. 1994. Determination of free malonaldehyde formed in liver microsomes upon CCl_4 oxidation. *J. Appl. Toxicol.* 14:453-455.

International Joint Commission. 1990. *Proceedings of the Roundtable on Contaminant-Caused Reproductive Problems in Salmonids.* M. Mac and M. Gilbertson. [Eds]. Windsor, Ontario. Sept. 24-25. 45pp.

Jagota, S.K. and H.M. Dani. 1982. A new colorimetric technique for the estimation of vitamin C using Folin Phenol reagent. *Analyt. Biochem.* 127:178-182.

Janz, D.M., T.L. Metcalfe and C.D. Metcalfe. 1992. Relative concentrations of cytochrome P450-active organochlorine compounds in liver and muscle of rainbow trout from Lake Ontario. *J. Great Lakes Res.* 18:759-765.

Janz, D.M. and C.D. Metcalfe. 1991. Relative induction of aryl hydrocarbon hydroxylase by 2,3,7,8-TCDD and two coplanar PCBs in rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* 10:917-923.

Jimenez, B.D. and J.J. Stegeman. 1990. Detoxification enzymes as indicators of environmental stress on fish. *Amer. Fish. Soc. Sympos.* 8:67-79.

Jimenez, B.D. and L.S. Burtis. 1989. Influence of environmental variables on the hepatic mixed-function oxidase system in bluegill sunfish, *Lepomis machrochirus*. *Comp. Biochem. Physiol.* 93C:11-21.

Jagota, S.K. and H.M. Dani. 1982. A new colorimetric technique for the estimation of vitamin C using Folin phenol reagent. *Analyt. Biochem.* 127:178-182.

Jones, K.C. 1988. Determination of polychlorinated biphenyls in human foodstuffs and tissues: suggestions for a selective congener analytical approach. *Sci. Tot. Environ.* 68:141-159.

Jurek, M.A., R.H. Powers, L.G. Gilbert and S.D. Aust. 1990. The effect of TCDD on acylCoA:retinol acyltransferase activity and vitamin A accumulation in the kidney of male Sprague-Dawley rats. *J. Biochem. Toxicol.* 5:155-160.

Kagan, V.E., E.A. Serbinova and L. Packer. 1990 Recycling and antioxidant activity of tocopherol homologs of different hydrocarbon chain lengths in liver microsomes. *Arch. Biochem. Biophys.* 282:221-225.

Kägi, J.H.R. and A. Schäeffer. 1988. Biochemistry of metallothionein. *Biochemistry.* 27:8509-8515.

Kappus, H. 1986. Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In H. Sies [Ed] *Oxidative Stress.* Academic Press, London. pp273-310.

Kennedy, S.W., A. Lorenzen, C.A. James and B.T. Collins. 1993. Ethoxyresorufin-O-deethylase and porphyrin analysis in chicken embryo hepatocyte cultures with a fluorescence multiwell plate reader. *Analyt. Biochem.* 211:102-112.

Kennedy, S.W. and S. Trudeau. 1994. Adaptation of the simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader to wildlife samples. *Society of Environmental Toxicology and Chemistry, 15th Annual Meeting Abstract,* Denver, CO. Oct.30-Nov.3, p.179.

Kohlmüller, D. and W. Kochen. 1993. Is n-pentane an index of lipid peroxidation in humans and animals? a methodological reevaluation. *Analyt. Biochem.* 210:268-276.

Kornbrust, D.J. and R.D. Mavis. 1980. Relative susceptibility of microsomes from lung, heart, liver, kidney, brain and testes to lipid peroxidation: correlation with vitamin E content. *Lipids* 15:315-322.

Koul, A., K.L. Khanduja, I.B. Koul, M.P. Gupta, S. Majid and R.R. Sharma. 1989. Effect of ascorbic acid on antioxidant defense systems and lipid peroxidation in guinea pig. *J. Clin. Biochem. Nutr.* 6:21-27.

Kutsky, R.J. 1973. *Handbook of Vitamins and Hormones.* Van Nostrand Reinhold, New York, New York. 278pp.

Larsen, H.E., M. Celander and A. Goksoyr. 1992. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activites and isozyme patterns during an annual reproductive cycle. *Fish Physiol. Biochem.* 10:291-301.

Larsson, A., T. Andersson, L. Forlin and J. Hardig. 1988. Physiological disturbances in fish exposed to bleached kraft mill effluents. *Wat. Sci. Tech.* 20:67-70.

Leatherland, J.F. 1993. Field observations on reproductive and developmental dysfunction in introduced and native salmonids from the Great Lakes. *J. Great Lakes Res.* 19:737-751.

Leatherland, J.F. and R.A. Sonstegard. 1982. Bioaccumulation of organochlorines by yearling coho salmon (*Oncorhynchus kisutch* Walbaum) fed diets containing Great Lakes' coho salmon, and the pathophysiological responses of the recipients. *Comp. Biochem. Physiol.* 72C:91-99.

Leedle, R.A. and S.D. Aust. 1990. The effect of glutathione on the vitamin E requirement for inhibition of liver microsomal lipid peroxidation. *Lipids.* 25:241-245.

Lehtinen, K.J. 1990. Mixed function oxygenase enzyme responses and physiological disorders in fish exposed to kraft pulp mill effluents: a hypothetical model. *Ambio* 19:259-265.

Lemaire, P., A. Matthews, L. Forlin and D.R. Livingstone. 1994. Stimulation of oxyradical production of hepatic microsomes of flounder (*Platichthyes flesus*) and perch *(Perca fluviatilis*) by model and pollutant xenobiotics. *Arch. Environ. Contam. Toxicol.* 26:191-200.

Leo, M.A. and C.S. Liebler. 1985. New pathway for retinol metabolism in liver microsomes. *J. Biol. Chem.* 260:5228-5231.

Liebler, D.C. 1993. The role of metabolism in the antioxidant function of vitamin E. *Crit. Rev. Toxicol.* 23:147-169.

Liebler, D.C., D.S. Kling and D.J. Reed. 1986. Antioxidant protection of phospholipid bilayers by alpha-tocopherol. Control of alpha-tocopherol status and lipid peroxidation by ascorbic acid and glutathione. *J. Biol. Chem.* 261:12114-12119.

Liehr, J.G. and D. Roy. 1990. Free radical generation by redox cycling of estrogens. *Free Rad. Biol. Med.* 8:415-423.

Livingstone, D.R., P. Lemaire, A. Matthews, L. Peters, D. Bucke and R.J. Law. 1993. Pro-oxidant, antioxidant and 7-ethoxyresorufin O-deethylase (EROD) activity responses in liver of dab (*Limanda limanda*) exposed to sediment contaminated with hydrocarbons and other chemicals. *Mar. Poll. Bull.* 26:602-606.

Luxon, P.L., P.V. Hodson and U. Borgmann. 1987. Hepatic aryl hydrocarbon hydroxylase activity of lake trout (*Salvelinus namaycush*) as an indicator of organic pollution. *Environ. Toxicol. Chem.* 6:649-657.

Mac, M.J., T.R. Schwartz, C.C. Edsall and A.M. Frank. 1993. Polychlorinated biphenyls in Great Lakes lake trout and their eggs: relations to survival and congener composition 1979-1988. *J. Great Lakes Res.* 19:752-765.

Mac, M.J. 1990. Lake trout egg qualtiy in Lakes Michigan and Ontario. In *Proceedings of the Roundtable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commission, Windsor, ON, Sept.14-15. pp.5-7.

Mac, M. and M. Gilbertson. 1990. Executive summary. In *Proceedings of the Rountable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commision. Windsor, ON, Sept. 14-15. pp.1-2.

Mac, M.J., C.C. Edsall and J.G. Seelye. 1985. Survival of lake trout and fry reared in water from the upper Great Lakes. *J. Great Lakes Res.* 11:520-529.

Madenjian, C.P., S.R. Carpenter and P.S. Rand. 1994. Why are PCB concentrations of salmonine individuals from the same lake so highly variable?. *Can. J. Fish. Aquat. Sci.* 51:800-807.

Maellaro, E., A.F. Casini, B. Del-Bello and M. Comporti. 1990. Lipid peroxidation and anitoxidant systems in the liver injury produced by glutathione depleting agents. *Biochem. Pharmacol.* 39:1513-1521.

Malins, D.C. and G.K. Ostrander. 1991. Perspectives in aquatic toxicology. *Annu. Rev. Pharmacol. Toxicol.* 31:371-399.

Mantyla, E. and M. Ahotupa. 1993. Polychlorinated biphenyls and napthalenes: long-lasting induction of oxidative stress in the rat. *Chemosphere.* 27:383-390.

Marquenski, S. 1990. Drop-out syndrome in steelhead fry in Wisconsin. In *Proceedings of the Rountable on Contaminant-Caused Reproductive Problems in Salmonids.* International Joint Commission. Windsor, Ontario.

Marsden, J.E., C.C. Krueger and C.P. Schneider. 1988. Evidence of natural reproduction by stocked lake trout in Lake Ontario. *J. Great Lakes Res.* 14:3-8.

Maslanka, R., A. R. Steward, J. Pangrekar, S. Kumar and H.C. Sikka. 1992. Disposition and metabolism of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in rainbow trout. *Mar. Environ. Res.* 34:255-259.

Mather-Mihaich, E. and R.T. Di Giulio. 1991. Oxidant, mixed function oxidase and peroxisomal responses in channel catfish exposed to a bleached kraft mill effluent. *Arch. Environ. Contam. Toxicol.* 20:391-397.

Mather-Mihaich, E. and R.T. Di Giulio. 1986. Antioxidant enzyme activities and malondialdehyde, glutathione and methemoglobin concentrations in channel catfish exposed to DEF and n-butyl mercaptan. *Comp. Biochem. Physiol.* 85C:427-432.

Matkovics, B., L. Szabo, S.I. Varga, K. Barabas, G. Berencsi and J. Nemcsok. 1984. Effects of herbicide on the peroxide metabolism enzymes and lipid peroxidation in carp fish (*Hypothalmichthys molitrix*). *Acta Biol. Hung.* 35:91-96.

McCord, J.M. and I. Fridovich. 1988. Superoxide dismutase: the first twenty years. *Free Rad. Biol. Med.* 5:363-369.

McFarland, V.A. and J.U. Clarke. 1989. Environmental occurrence, abundance and potential toxicity of polychlorinated biphenyl congeners: considerations for a congener-specific analysis. *Environ. Hlth. Perspec.* 81:225-239.

McMaster, M.E., G.J. Van der Kraak, C.B. Portt, K.R. Munkittrick, P.K. Sibley, I.R. Smith and D.G. Dixon. 1991. Changes in hepatic mixed-function oxygenase (MFO) activity, plasma steroid levels and age at maturity of a white sucker (*Catostomus commersoni*) population exposed to bleached kraft pulp mill effluent. *Aquat. Toxicol.* 21:199-218.

Melancon, M.J., R.L. Binder and J.J. Lech. 1988. Environmental induction of monooxygenase activity in fish. In *Toxic Contaminants and Ecosystem Health: A Great Lakes Focus.* John Wiley and Sons, New York, NY. pp.215-236.

Melchiorri, D., R.J. Reiter, A.M. Attia, M. Hara, A. Burgos and G. Nistico. 1994. Potent protective effect of melatonin on *in vivo* paraquat-induced oxidative damage in rats. *Life Sci.* 56:83-89.

Menzer, R.E. and J.O. Nelson. 1986. Water and soil pollutants. In *Toxicology.* C.D. Klassen, M.O. Amdur and J. Doull [Eds]. Macmillan Publishing Company, New York. 974pp.

Mercier, M., G. Pascal and V. Azais-Braesco. 1990. Retinyl esterhydrolase and vitamin A status in rats treated with 3,3',4,4'-tetrachlorobiphenyl. *Biochim. Biophys. Acta.* 1047:70-76.

Metcalfe, C.D., V.W. Cairns and J.D. Fitzsimmons. 1988. Experimental induction of liver tumours in rainbow trout (*Salmo gairdneri*) by contaminated sediment from Hamilton Harbour, Ontario. *Can. J. Fish. Aquat. Sci.* 45:2161-2167.

Michel, X., D. Ribera, S. Decoudu and J.F. Narbonne. 1993. Comparison of *in vitro* benzo[a]pyrene metabolism and mutagenesis in mussel, sea bass and rat. *Mar. Environ. Res.* 35:211-212.

Miller, D.M. and S.D. Aust. 1989. Studies of ascorbate-dependent, iron-catalyzed lipid peroxidation. *Arch. Biochem. Biophys.* 271:113-119.

Miller, M.A., N.M. Kassulke and M.D. Walkowski. 1993. Organochlorine concentrations in Laurentian Great Lakes salmonines: implications for fisheries management. *Arch. Environ. Contam. Toxicol.* 25:212-219.

Miller, M.A., C.P. Madenjian and R.G. Masnado. 1992. Patterns of organochlorine contamination in lake trout from Wisconsin waters of the Great Lakes. *J. Great Lakes Res.* 18:742-754.

Mills, E.M., J.H. Leach, J.T. Carlton and C.L. Secor. 1993. Exotic species in the Great Lakes: a history of biotic crises and anthropogenic introductions. *J. Great Lakes Res.* 19:1-54.

Mohammadpour, H., W.J. Murray and S.J. Stohs. 1988. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced lipid peroxidation in genetically responsive and non-responsive mice. *Arch. Environ. Contam. Toxicol.* 17:645-650.

Morrison, P.F., J.F. Leatherland and R.A. Sonstegard. 1985. Proximate composition and organochlorine and heavy metal contamination of eggs from Lake Ontario, Lake Erie and Lake Michigan coho salmon (*Oncorhynchus kisutch* Walbaum) in relation to egg survival. *Aquat. Toxicol.* 6:73-86.

Muir, D.C.G., A.L. Yarechewski, D.A. Metner and W.L. Lockhart. 1992. Dietary 2,3,7,8-tetrachlorodibenzofuran in rainbow trout: accumulation, disposition and hepatic mixed-function oxidase enzyme induction. *Toxicol. Appl. Pharmacol.* 117:65-74.

Muir, D.C.G., C.A. Ford, N.P. Grift, D.A. Metner and W.L. Lockhart. 1990. Geographic variation of chlorinated hydrocarbons in burbot (*Lota lota*) from remote lakes and rivers in Canada. *Arch. Environ. Contam. Toxicol.* 19:530-542.

Munkittrick, K.R., M.R. van den Heuvel, D.A. Metner, W.L. Lockhart and J.J. Stegeman. 1993. Interlaboratory comparison and optimization of hepatic microsomal ethoxyresorufin 0-deethylase activity in white sucker (*Catostomus commersoni*) exposed to bleached kraft pulp mill effluent. *Environ. Toxicol. Chem.* 12:1273-1282.

Munkittrick, K.R., G.J. Van der Kraak, M.E. McMaster and C.B. Portt. 1992. Response of hepatic MFO activity and plasma sex steroids to secondary treatment of bleached kraft pulp mill effluent and mill shutdown. *Environ. Toxicol. Chem.* 11:1427-1439.

Munkittrick, K.R., C.B. Portt, G.J. Van der Kraak, I.R. Smith and D. Rokosh. 1991. Impact of bleached kraft mill effluent on population charachteristics, liver MFO activity and serum steroid levels of a Lake Superior white sucker (*Catostomus commersoni*) population. *Can. J. Fish. Aquat. Sci.* 48:1371-1380.

Ndayibagira, A., M.J. Cloutier, P.D. Anderson and P.A. Spear. 1995. Effects of 3,3',4,4'-tetrachlorobiphenyl on the dynamics of vitamin A in brook trout (*Salvelinus fontinalis*) and intestinal retinoid concentrations in lake sturgeon (*Acipenser fulvescens*). *Can. J. Fish. Aquat. Sci.* 52:512-520.

Neff, J.M. 1985. Use of biochemical measurements to detect pollutant mediated damage to fish. In R.D. Cardwell, R. Purdy and R.C. Bahner [Eds]. *Aquatic toxicology and hazard assessment:seventh symposium.* American Society for Testing and Materials, Philadelphia, PA.

Nester, R.T. and T.P. Poe. 1984. First evidence of successful natural reproduction of planted lake trout in Lake Huron. *North Amer. J. Fish. Mngmt.* 4:126-128.

Niimi, A.J. and B.G. Oliver. 1989. Assessment of relative toxicity of chlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls in Lake Ontario salmonids to mammalian systems using toxic equivalent factors. *Chemosphere.* 18:1413-1423.

Niimi, A.J. 1990. Review of biochemical methods and other indicators to assess fish health in aquatic ecosystems containing toxic chemicals. *J. Great Lakes Res.* 16:529-541.

Niimi, A.J. 1983. Biological and toxicological effects of environmental contaminants in fish and their eggs. *Can. J. Fish. Aquat. Sci.* 40:306-312.

Ohishi, N., H. Ohkawa, A. Miike, T. Tatano and K. Yagi. 1985. A new assay for lipid peroxides using a methylene blue derivative. *Biochem. Internat.* 10:205-211.

Otto, D.M.E., P. Lindstrom-Seppa and C.K. Sen. 1994. Cytochrome P450-dependent enzymes and oxidant-mediated responses in rainbow trout exposed to contaminated sediments. *Ecotox. Environ. Saf.* 27:265-280.

Packer, L. 1991. Protective role of vitamin E in biological systems. *Am. J. Clin. Nutr.* 53:1050s-1055s.

Palace, V.P., J.F. Klaverkamp, W.L. Lockhart, D.A. Metner, D.C.G. Muir and S.B. Brown. 1996a. Oxidative stress in lake trout (*Salvelinus namaycush*) exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB 126). *Environ. Toxicol. Chem.* 15(6):955-960.

Palace, V.P., C.L. Baron and J.F. Klaverkamp. 1996b. Relationships between phase I and Phase II enzymatic activities and oxidative stress in adult lake trout (*Salvelinus namaycush*) from Lake Ontario and Lake Superior. *J. Great Lakes Res.* (Submitted).

Palace, V.P., T.A. Dick, S.B. Brown, C.L. Baron and J.F. Klaverkamp. 1996c. Oxidative Stress in Lake Sturgeon (*Acipenser fulvescens*) Orally Exposed to 2,3,7,8-tetrachlorodibenzofuran. Aquat. Toxicol. (In press).

Palace, V.P. and S.B. Brown. 1994. HPLC determination of tocopherol, retinol, dehydroretinol and retinyl palmitate in tissues of lake char (*Salvelinus namaycush*) exposed to coplanar 3,3',4,4',5-pentachlorobiphenyl. *Environ. Toxicol. Chem.* 13(3):473-476.

Palace, V.P. and J.F. Klaverkamp. 1993. Variation of heaptic enzymes in three species of freshwater fish from Precambrain Shield lakes and the effect of cadmium exposure. *Comp. Biochem. Physiol.* 104C:147-154.

Palace, V.P., H.S. Majewski and J.F. Klaverkamp. 1993. Interactions among antioxidant defenses in liver of rainbow trout (*Oncorhynchus mykiss*) exposed to cadmium. *Can. J. Fish. Aquat. Sci.* 50:156-162.

Palace, V.P., H.S. Majewski and J.F. Klaverkamp. 1990. Effects of sampling and storage conditions on the stability of biochemical parametersmeasured in rainbow trout (*Oncorhynchus mykiss*) liver. *Comp. Biochem. Physiol.* 95B:51-55.

Palozza, P. and N.I. Krinsky. 1991. The inhibition of radical initiated peroxidation of microsomal lipids by both α-tocopherol and β-carotene. *Free Radical Biol. Med.* 11:407-414.

Payne, J.F., L.L. Fancey, A.D. Rahimtula and E.L. Porter. 1987. Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring. *Comp. Biochem. Physiol.* 86C:233-245.

Payne, J.F., C. Bauld, A.C. Dey, J.W. Kiceniuk and V. Williams. 1984. Selectivity of mixed-function oxygenase enzyme induction in flounder (*Pseudopleuronectes americanus*) collected at the site of the Baje Verte, Newfoundland, oil spill. *Comp. Biochem. Physiol.* 79C:15-19.

Perdu-Durand, E.F. and J.P. Cravedi. 1989. Characterization of xenobiotic metabolizing enzymes in sturgeon (*Acipenser baeri*). *Comp. Biochem. Physiol.* 93B:921-928.

Peters, L.D., C. Porte, J. Albaigés and D.R. Livingstone. 1994. 7-ethoxyresorufin O-deethylase (EROD) and antioxidant enzyme activities in larvae of sardine (*Sardina pilchardus*) from the North coast of Spain. *Mar. Poll. Bull.* 28:299-304.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Analyt. Biochem.* 83:346-356.

Pettersson, A. 1995. Decreased astaxanthin levels in the Baltic salmon and the M74 syndrome. *Proceedings of the Second Workshop on Reproduction Disturbances in Fish.* Swedish Environmental Protection Agency, Stockholm, Sweden. Nov. 20-23.

Pieri, C., M. Mazza, F. Moroni, R. Recchioni and F. Marscheselli. 1994. Melatonin: a peroxyl radical scavenger more effective than vitamin E. *Life Sci.* 55:PL271-276.

Pohjanvirta, R., S. Sankari, T. Kulju, A. Naukkarinen, M. Ylinen and J. Tuomisto. 1990. Studies on the role of lipid peroxidation in the acute toxicity of TCDD in rats. *Pharmacol. Toxicol.* 66:399-408.

Poston, H.A., R.C. Riis, G.L. Rumsey and H.G. Ketola. 1977. The effect of supplemental dietary amino acids, minerals and vitamins on salmonids fed cataractogenic diets. *Cornell Vet.* 67:472-509.

Rabie, F., M. Magid, K. Gima'a and K. Karrar. 1972. Evolution of catalase in fish. *Comp. Biochem. Physiol.* 43A:1053-1055.

Radi, A.A., B. Matkovics and I. Csengeri. 1987. Comparative studies of the phospholipid fatty acids and the antioxidant enzyme activities in fish with different feeding habits. *Comp. Biochem. Physiol.* 87B:49-54.

Rappe, C. and H.R. Buser. 1989. Chemical and physical properties, analytical methods, sources and environmental levels of halogenated dibenzodioxins and dibenzofurans. In *Halogenated Biphenyls, Terphenyls, Naphthalenes, Dibenzodioxins and Related Products.* R.D. Kimbrough and A.A. Jensen [Eds.] Elsevier, New York.

Reiter, R.J., D. Tan, B. Poeggeler, A. Menedez-Paleaz, L. Chen and S. Saarela. 1994. Melatonin as a free radical scavenger: implications for aging and age-related diseases. *Ann. N.Y. Acad. Sci.* 719:1-12.

Ribera, D., J.F. Narbonne, X. Michel, D.R. Livingstone and S. O'Hara. 1991. Responses of antioxidants and lipid peroxidation in mussels to oxidative damage exposure. *Comp. Biochem. Physiol.* 100C:177-181.

Roberfroid, M.B. 1995. Pharmacology of antioxidant molecules: analysis of their mechanisms of action. In *Free Radicals and Oxidation Phenomena in Biological Systems.* Marcel Dekker Inc., New York, NY. pp.237-252.

Roberts, E.S., A.D. Vaz and M.J. Coon. 1992. Role of isozymes of rabbit microsomal cytochrome P-450 in the metabolism of retinoic acid, retinol and retinal. *Mol Pharmacol.* 41:427-433.

Roberts, M.H., D.W. Sved and S.P. Felton. 1987. Temporal changes in AHH and SOD activities in feral spot from the Elizabeth River, a polluted sub-estuary. *Mar. Environ. Res.* 23:89-101.

Rodriguez-Ariza, A., J. Peinado, C. Pueyo and J. Lopez-Barea. 1993. Biochemical indicators of oxidative stress in fish from polluted littoral areas. *Can. J. Fish. Aquat. Sci.* 50:2568-2573.

Roesijadi, G. 1992. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat. Toxicol.* 22:81-114.

Rousseau, E.J., A.J. Davidson and B. Dunn. 1992. Protection by β-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: implications for carcinogenesis and anticarcinogenesis. *Free Rad. Biol. Med.* 13:407-433.

Rousseaux, C.G., A. Branchaud and P.A. Spear. 1995. Evaluation of liver histopathology and EROD activity in St. Lawrence lake sturgeon (*Acipenser fulvescens*) in comparison with a reference population. *Environ. Toxicol. Chem.* 14:843-849.

Rowan, D.J. and J.B. Rasmussen. 1992. Why don't Great Lakes fish reflect environmental concentrations of organic contaminants?-an analyis of between-lake variability in the ecological partitioning of PCBs and DDT. *J. Great Lakes Res.* 18:724-742.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs) dibenzofurans (PCDFs) and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors. *CRC Crit. Rev. Toxicol.* 21:51-88.

Safe, S. 1986. Comparative toxicology and mechanism of action of poylchlorinated dibenzo-p-dioxins and dibenzofurans. *Ann. Rev. Pharmacol. Toxicol.* 26:371-399.

Safe, S., L. Safe and M. Mullin. 1987. Polychlorinated biphenyls: environmental occurrence and analysis. In *Polychlorinated Biphenyls. Vol. I.* Springer-Verlag, Heidelberg.

Sass, J.O., A. Forster, K.W. Bock and H. Nau. 1994. Glucuronidation and isomerization of all-trans and 13-cis-retinoic acid by liver microsomes of phenobarbital or 3-methylcholanthrene-treated rats. *Biochem. Pharmacol.* 47:485-492.

Sato, M. and I. Bremner. 1993. Oxygen free radicals and metallothionein. *Free Rad. Biol. Med.* 14:325-337.

Schiedt, K., M. Vecchi and E. Glinz. 1986. Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Sci.*83B:9-12.

Schiedt, K., F.J. Leuenberger, M. Vecchi and E. Glinz. 1985. Absorbtion, retention and metabolic transformations of carotenoids in rainbow trout, salmon and chicken. *Pure. Appl. Chem.* 57:685-692.

Scott, W.B. and E.J. Crossman. 1973. Freshwater Fishes of Canada. *Fish Res. Bd. Can. Bulletin* #184.

Serbinova, E., V. Kagan, D. Han and L. Packer. 1991. Free radical recycling and intramembrane mobility in the antioxidant properties of alpha-tocopherol and alpha-tocotrienol. *Free Rad. Biol. Med.* 10:263-276.

Shara, M.A. and S.J. Stohs. 1987. Biochemical and toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) congeners in female rats. *Arch. Environ. Contam. Toxicol.* 16:599-605.

Sijm, D.T.H.M., A. Yarechewski, D.C.G. Muir, G.R.B. Webster, W. Seinen and A. Opperhuizen. 1990. Biotransformation and tissue distribution of 1,2,3,7-tetrachlorodibenzo-p-dioxin, 1,2,3,4,7-pentachlorodibenzofuran in rainbow trout. *Chemosphere*. 21:845-866.

Singh, Y., G.L. Hall and M. Miller. 1992. Species differences in membrane susceptibility to lipid peroxidation. *J. Biochem. Toxicol*. 7:97-105.

Skea, J.C., J. Symula and J. Miccoli. 1985. Separating starvation losses from other early feeding fry mortality in steelhead trout *Salmo gairdneri*, chinook salmon *Oncorhynchus tsawytscha* and lake trout *Salvelinus namycush*. *Bull. Environ. Contam. Toxicol*. 35:82-91.

Smith, A.G. and F. De Matteis. 1990. Oxidative injury mediated by the hepatic cytochrome P-450 system in conjunction with cellular iron. Effects on the pathway of haem biosynthesis. *Xenobiotica*. 20:865-877.

Smith, I.R., C.B. Portt and D.A. Rokosh. 1990. Hepatic mixed function oxidases induced in populations of white sucker, *Catostomus commersoni*, from areas of Lake Superior and the St. Marys River. *J. Great Lakes Res.* 17:382-393.

Smith, L.M., T.R. Schwartz and K. Feltz. 1990. Determination and occurrence of AHH-active polychlorinated biphenyls, 2,3,7,8-tetrachloro-p-dioxin and 2,3,7,8-tetrachlorodibenzofuran in Lake Michigan sediment and biota. The question of their relative toxicological significance. *Chemosphere.* 21:1063-1085.

Smolowitz, R.M., M.E. Hahn and J.J. Stegeman. 1991. Immunohistochemical localization of cytochrome P4501A1 induced by 3,3',4,4'-tetrachlorobiphenyl and by 2,3,7,8-tetrachlorodibenzofuran in liver and extrahepatic tissues of the teleost *Stenotomus chrysops* (scup). *Drug. Metab. Dispos.* 19:113-123.

Spear, P.A., P. Higueret and H. Garcin. 1994. Effects of fasting and 3,3',4,4',5,5'-hexabromobiphenyl on plasma transport of thyroxine and retinol. *J. Toxicol. Environ. Saft.* 42:173-183.

Spear, P.A., A.Y. Bilodeau and A. Branchaud. 1992. Retinoids: from metabolism to environmental monitoring. *Chemosphere.* 25:1733-1738.

Spear, P.A., D.H. Bourbonnais, R.J. Norstrom and T.W. Moon. 1990. Yolk retinoids (vitamin A) in eggs of the herring gull and correlations with polychlorinated dibenzo-p-dioxins and dibenzofurans. *Environ. Toxicol. Chem.* 9:1053-1061.

Spear, P.A., D.H. Bourbonnais, D.B. Peakall and T.W. Moon. 1989. Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3',4,4'-tetrachlorobiphenyl. *Can. J. Zool.* 67:908-913.

Spear, P.A. and T.W. Moon. 1986. Liver retinoid concentrations in natural populations of herring gulls (*Larus argentatus*) contaminated by 2,3,7,8-tetrachlorodibenzo-p-dioxin and in ring doves (*Stretopelia risoria*) injected with a dioxin analogue. *Can. J. Zool.* 64:204-208.

Spies, R.B. and D.W. Rice. 1988. Effects of organic contaminants on reproduction of the starry flounder (*Platichthyes stellatus*) in San Francisco Bay. II. Reproductive success of fish captured in San Francisco Bay and spawned in the laboratory. *Mar. Biol.* 98:191-200.

Spies, R.B., D.W. Rice, P.A. Motagna and R.R. Ireland. 1985. Reproductive success, xenobiotic contaminants and hepatic mixed function oxidase (MFO) activity in *Platichthyes stellatus* population from San Francisco Bay. *Mar. Environ. Res.* 17:117-121.

Spitsbergen, J.M., M.K. Walker , J.R. Olson and R.E. Peterson. 1991. Pathologic alterations in early life stages of lake trout, *Salvelinus namaycush*, exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin as fertilized eggs. *Aquat. Toxicol.* 19:41-72.

Staats, D.A., and H.D. Colby. 1989. Modulation of the effects of ascorbic acid on lipid peroxidation by tocopherol in adrenocortical mitochondria. *J. Steroid Biochem.* 32:609-611.

Stancher, B., and F. Zonata. 1984. Quantitative high performance liquid chromatographic method for determining the isomer distribution of retinol (vitamin A_1) and 3-dehydroretinol (vitamin A_2) in fish oils. *J. Chromatog.* 312:423-434.

Stegeman, J.J. 1979. Temperature influence on basal activity and induction of mixed function oxygenase activity in *Fundulus heteroclitus*. *J. Fish. Res. Bd. Can.* 36:1400-1404.

Stocker, R. and E. Peterhans. 1989. Synergistic interaction between vitamin E and the bile pigments bilirubin and biliverdin. *Bioch. Biophys. Acta.* 1002:238-241.

Stohs, S.J., M.A. Shara, N.Z. Alsharif, Z.Z. Wahba and Z.A. Al-Bayati. 1990. 2,3,7,8-tetrachlorodibenzo-p-dioxin induced oxidative stress in female rats. *Toxicol. Appl. Pharmacol.* 106:126-135.

Suguhara, N., Y. Tsuruta, Y. Date, K. Furuno and K. Kohashi. 1994. High peroxidative susceptibility of fish oil polyunsaturated fatty acids in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* 126:124-128.

Sun, M. and S. Zigman. 1978. An improved spectrophotometric assay for superoxide dismutase based on epinephrine autooxidation. *Analyt. Biochem.* 90:81-86.

Symula, J., J. Meade, J.C. Skea, L. Cummings, J.R. Colquhoun, H.J. Dean and J. Miccoli. 1990. Blue-sac disease in Lake Ontario lake trout. *J. Great Lakes Res.* 16:41-52.

Tanabe, S. 1988. PCB Problems in the future: foresight from current knowledge. *Environ. Pollut.* 50:5-28.

Tappel, A.L. 1978. Glutathione peroxidase and hydroperoxides. *Meth. Enzymol.* 52:506-513.

Terao, J., P.L. Boey, F. Ojima, A. Nagao, T. Suzuki and K. Takama. 1992. Astaxanthin as a chain-breaking antioxidant in phospholipid peroxidation. In *Oxygen Radicals.* K. Yagi, M. Kondo and T. Yoshikawa. [Eds]. Elsevier Science Publishers.

Thomas, P. and H.W. Wofford. 1993. Effects of cadmium and Arochlor 1254 on lipid peroxidation, glutathione peroxidase activity and selected antioxidants in Atlantic croaker tissues. *Aquat. Toxicol.* 27:159-178.

Thomas, P. 1987. Influence of some environmental variables on the ascorbic acid status of striped mullet (*Mugil cephalus*) tissues. III. Effects of exposure to oil. *J. Fish Biol.* 30:485-494.

Thornburn, G. 1994. International Joint Commission recommends comprehensive approach to persistant toxic substances. *Focus on International Joint Commission Activities.* 19:1-4.

Tzimas, G., J.O. Sass, W. Wittfoht, M.M.A. Elmazar, K. Ehlers and H. Nau. 1994. Identification of 9,13 dicis-retinoic acid as a major metabolite of 9-cis retinoic acid and limited transfer of 9-cis retinoic acid and 9,13-dicis retinoic acid to the mouse and rat embryos. *Drug Metab. Dispos.* 22:928-936.

Uchiyama, M. and M. Mihara. 1978. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Analyt. Biochem.* 86:271-278.

U.S.E.P.A. 1991. *Bioaccumulation of Selected Pollutants in Fish, A National Study. Vol. 1.* Office of Water Regulation and Standards, Wash., D.C.

Van Birgelen, A.P.J.M., J. van der Kolk, K.M. Fase, I. Bol, H. Poiger, A. Brouwer and M. Van den Berg. 1994. Toxic potency of 3,3',4,4',5-pentachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-p-dioxin in a subchronic feeding study in rat. *Toxicol. Appl. Pharmacol.* 127:209-221.

Vanden Bossche, H. and G. Willemsens. 1991. Vitamin A. In J.H. Saurat, ed., *Retinoids: 10 years On.* S. Karger, Basel, Switzerland, pp.79-88.

Van der Kraak, G.J., K.R. Munkittrick, M.E. McMaster, C.B. Portt and J.P. Chang. 1992. Exposure to bleached kraft mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxicol. Appl. Pharmacol.* 115:224-233.

Vivien-Roels, B. and P. Pévet. 1993. Melatonin: presence and formation in vertebrates. *Experientia.* 49:642-647.

Walker, M.K., P.M. Cook, A.R. Batterman, B.C. Butterworth, C. Berini, J.J. Libal, L.C. Hufnagle and R.E. Peterson. 1994. Translocation of 2,3,7,8-tetrachlorodibenzo-p-dioxin from adult female lake trout (*Salvelinus namaycush*) to oocytes: effects on early life stage development and sac fry survival. *Can. J. Fish. Aquat. Sci.* 51:1410-1419.

Walker, M.K., L.C. Hufnagle, M.K. Clayton and R.E. Peterson. 1992. An egg injection method for assessing early life stage mortality of polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 22:15-38.

Walker, M.K. and R.E. Peterson. 1991. Potencies of polychlorinated dibenzo-p-dioxin, dibenzofuran and biphenyl congeners, relative to 2,3,7,8-tetrachlorodibenzo-p-dioxin, for producing early life stage mortality in rainbow trout (*Onchorhynchus mykiss*) *Aquat. Toxicol.* 21:219-238.

Walker, M.K. and R.E. Peterson. 1990. 2,3,7,8-tetrachlorodibenzo-p-dioxin produces a blue-sac syndrome during early life stage developmentof lake trout (*Salvelinus namaycush*). In *Rountable on Caontaminant Caused Reproductive Problems in Salmonids*. International Joint Commission. Windsor, Ontario.

Walker, M.K., J.M. Spitsbergen, J.R. Olson and R.E. Peterson. 1991. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) toxicity during early life stage development of lake trout (*Salvelinus namaycush*). *Can. J. Fish. Aquat. Sci.* 48:875-883.

Wendel, A. 1988. Enzymes acting against reactive oxygen. *Adv. Clin. Enzymol.* 6:161-167.

Whittle, D.M., C. Mageau, R.K. Duncan, D.B. Sargeant, M.D. Nassichuk, J. Morrison and J. Piuze. 1993. Canadian national dioxin sampling program: dioxins and furans in biota near 46 pulp and paper mills using the chlorine bleaching process. *Chemosphere*. 27:279-286.

Whittle, D.M., D.B. Sargeant, S.Y. Huestis and W.H. Hyatt. 1992. Foodchain accumulation of PCDD and PCDF isomers in the Great Lakes aquatic community. *Chemosphere*. 25:1559-1563.

Williams, D.E., H.M. Carpenter, D.R. Buhler, J.D. Kelly and M. Dutchuk. 1992. Alterations in lipid peroxidation, antioxidant enzymes, and carcinogen metabolism in liver microsomes of vitamin E-deficient trout and rat. *Toxicol. Appl. Pharmacol.* 116:78-84.

Willis, D.E., A.J. Edwards and R.F. Addison. 1991. Effects of environemtnal pH on the hepatic mixed function oxidases in Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.* 48:445-447.

Winston, G.W. 1991. Oxidants and antioxidants in aquatic animals. *Comp. Biochem. Physiol.* 100C:173-176.

Winston, G.W. and R.T. Di Giulio. 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.* 19:137-161.

Wolf, G. 1984. Multiple functions of vitamin A. *Physiol. Rev.* 64:873-937.

Zile, M.H. 1992. Vitamin A homeostasis endangered by environmental pollutants. *Proc. Soc. Exper. Biol. Med.* 201:141-153.