THE UNIVERSITY OF MANITOBA

SUPEROXIDE DISMUTASE, CATALASE AND GLUTATHIONE PEROXIDASE ANTIOXIDANT ENZYME ACTIVITIES AS INDICATORS OF LIPID PEROXIDATIVE STRESS IN FRESHWATER FISH

by

VINCE P. PALACE

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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ABSTRACT

The distribution of several biochemical parameters, including the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), were investigated in rainbow trout (Onchorhynchus mykiss) liver quadrants. Activities of SOD, CAT and GPx did not vary significantly between quadrants. Variation of the enzyme activities and effects of Cd exposure in a whole lake experiment on these enzymes were studied in liver from three freshwater species; pearl dace (Semotilus margarita), white sucker (Catostomus commersoni) and lake charr (Salvelinus namaycush). Enzymatic activity varied significantly between different lakes in geographic proximity. Other than GPx activity, which was lower in lake charr, activities of the enzymes were not different between the three fish species. Seasonal variation was observed in both CAT and SOD activity. Cadmium exposure resulted in increased liver concentrations of the metal, but metallothionein protein did not significantly increase. SOD activity in liver of fish exposed to Cd was consistently higher than in liver of fish from reference lakes.

Rainbow trout fed diets deficient in ascorbic acid (AsA), tocopherol, both or neither were exposed to one of three cadmium concentrations for 181 days. Cadmium exposure was found to significantly increase the Cd content of liver, especially in fish fed an AsA deficient diet. Hepatic stores of ascorbic acid decreased in response to both Cd exposure and tocopherol and AsA deficient diets. Depletion of tocopherol in liver occurred only in those fish fed a tocopherol deficient diet and exposed to Cd. Acid-soluble thiols were elevated in both liver and kidney of fish exposed to Cd. SOD activity in liver increased with Cd exposure and with dietary deficiencies of tocopherol and AsA, while CAT was inhibited by exposure to Cd alone. GPx activity was unaffected by Cd, but

was reduced in fish fed a diet deficient in both tocopherol and AsA. Erythrocyte fragility, a measure of lipid peroxidative membrane damage, was increased by Cd exposure and the absence of dietary tocopherol and AsA.

ACKNOWLEDGEMENTS

The work represented in this thesis includes not only my efforts, but also vital contributions from many people at the Freshwater Institute and the University of Manitoba. I would especially like to thank the staff at the Freshwater Institute annex, H. Majewski, R. Mcnichol, S. Harrison, B. Evans, D. Majewski (for her guided tours of the ELA district), L. Wesson, S. Brown and C. Catt for their endless patience and assistance both in the lab and in preparing manuscripts.

My supervisor, Dr. J.F. Klaverkamp (Jack), through his guidance and diplomatic criticism ensured that this project was brought to a successful conclusion. I value my experiences over the last several years and look forward to continuing my work and friendship with him. I would also like to express my gratitude to the other members of my commitee, Drs. C. Pinsky and L. Lockhart, for their constructive comments and suggestions.

The field collections at the Experimental Lakes Area were completed under the guise of "a fishing trip" with the help of my friends D. Champagne, P. Lemoine and J. Gross.

Finally this section would not be entirely complete without acknowledging the contribution of those closest to me. Thank you mother for instilling the confidence in me to pursue this goal and to my wife, Barbara, for continuing to love the "perpetual student."

INTRODUCTION

Because of its toxicity to biological systems, cadmium (Cd) has been increasingly monitored in air, soil and water for the past 2 decades. Cd is a group IIB element (Atomic number 48, Atomic weight = 112.40) that is widely distributed in both terrestrial and aquatic environments. Concentrations in rock formations range from 0.001 µg Cd/gm in igneous rocks to 90 µg Cd/gm in shale. Soils generally contain less than 1 µg/gm (GESAMP 1985), however more contaminated soils do exist in central Europe and Japan (Nriagu 1988). Cadmium in freshwater systems is for the most part, associated with particulate matter and is soluble predominantly as Cd^{++} with small amounts associated with anions (eg. $CdCl_2$, $CdSO_4$, $CdCO_3$ and $Cd(OH)_2$) (GESAMP 1985). Canadian freshwaters have a median concentration of 0.01 µg Cd/l with a maximum of 0.27 µg Cd/l reported in contaminated waters. Lake Ontario and Lake Erie contain between 0.015 and 0.108 µg Cd/l (Coale and Flegal 1989). Harrison et al (1989) reported Cd concentrations of <1 to 62 µg Cd/gm wet weight for the top 2 cm of sediment from lakes near Flin Flon, Manitoba. They correlated this with concentrations of Cd in pike (Esox lucius) and sucker (Catostomus commersoni) of <0.01 µg Cd/gm in muscle and 0.01 to 0.71 µg Cd/gm in liver. GESAMP (1985) calculates that Cd is 300 times higher in North American winds and 1900 times higher in urban air than in the earth's crust. Air samples from over the Atlantic Ocean range from 0.003 to 0.62 ng Cd/m^3.

Cadmium is mobilized by a variety of natural and anthropogenic sources including volcanic activity, forest fires, windblown dusts, burning of fossil fuels (eg. wood, coal, and waste) ore smelting (eg. lead, copper, zinc and nickel), sewage sludges, urban and motorway dusts, fertilizers, electroplating,

batteries, alloys, plastics and as pigment in yellow and brown paints. The influence of anthropogenic activities on the release of cadmium to the environment has been estimated to be 19 times greater than natural weathering processes which emit 2.9×10^8 gm yr^{-1} (Freedman 1989). In 1985 primary production accounted for 19,000 tonnes of Cd compared to 1300 tonnes in 1930 (Nriagu 1988). Cadmium released from these sources may enter aquatic environments from the air in particulate form as well as dissolved in precipitation (GESAMP 1985).

Recognizing the threat of Cd to aquatic biota, acceptable limits of Cd in freshwater ecosystems have been established in many countries. The United States Environmental Protection Agency (EPA) has set a maximum acceptable limit for protecting freshwater fish at 1.2 and 0.4 µg Cd/l in hard and softwater respectively (Hellawell 1988). Canadian Water Quality Guidelines (CWQG), meanwhile, set maximal limits of 0.2 µg Cd/l for softwater ($CaCO_3$ = 0-60mg/l) and <1.8 µg Cd/l for hardwater ($CaCO_3$ = >180mg/l) in order to protect freshwater aquatic life, and 5µg Cd/l for drinking water (Canadian Council of Resource and Environment Ministers (CCREM) 1987). Emphasis is placed on the "hardness" of water because calcium and other divalent cations are known to antagonize cadmium toxicity in aquatic organisms (Wicklund and Runn 1988).

Despite existing regulatory mechanisms and the fact that only one large scale incidence of human Cd poisoning has been documented (Japan 1947) (Mance 1987), toxicity of the metal to biological systems is a major concern. Lazerte et al (1989) report Cd concentrations approaching the 0.2 µg Cd/l Canadian Water Quality Guideline in Plastic Lake, which receives atmospheric deposition from the smelter in nearby Sudbury, Ontario. In a softwater tributary of Slate River in Colorado, concentrations are as much as 20 times higher (Mance 1987). Within this concentration range, relatively short exposures (4 to 180 days) to

Cd elicited responses in fish including increased infection (Knittel 1980), delayed sexual maturation, increased frequency of deformities, reduced growth (Petersen et al 1983), and hyperactivity (Benoit et al 1976).

While it is generally accepted that the toxicity of Cd is dependent on modifying factors including water hardness, pH, temperature and the presence of organic compounds and other metals (CCREM 1987), the critical toxic mechanism of Cd in freshwater fish is unknown. Suggestions include inhibition of acetyl cholinesterase enzyme activity followed by paralysis and death (Cearley and Coleman 1974), and renal failure (Hawkins et al 1980), while Giles (1984) notes electrolyte imbalance as a consequence of Cd exposure in fish. Stromberg et al (1983) reported histopathological evidence linking Cd exposure to gill damage, but in a review of the effects of Cd on freshwater fish, Sprague (1987) indicated that this link has not yet been clearly established.

Because Cd accumulates most in liver and kidney (Stromberg et al 1983), it seems reasonable to consider effects in these organs. Their importance as uptake, absorbtion and excretion centers for metals has been previously discussed (Suzuki et al 1990). Specifically, Sprague (1987) reports bioconcentration of Cd in freshwater fish liver ranging from 3 to 100X the water concentration, depending on the test species and water hardness. The kidney is also a major absorbtion site for Cd (Nriagu 1988), and exposure has been correlated with histological abnormalities of renal tubular cells in rainbow trout (Kumada et al 1980).

Lipid peroxidation, a process by which cellular and subcellular membranes are oxidatively degraded (Maiorino et al 1989), is increasingly being recognized as a mechanism of heavy metal toxicity in liver and kidney. Andersen and Andersen (1988), for example, found a dose dependent increase in lipid peroxidative metabolites in Cd exposed mice, but data specific to fish

are scarce. This thesis considers the potential of Cd as an inducer of lipid peroxidation in fish , and its effects on both enzymatic and non-enzymatic cellular antioxidants opposing this process.

This thesis is written in the style of manuscripts submitted to primary scientific journals. The thesis is comprised of three separate chapters, each consisting of a separate introduction, materials and methods and results and discussion sections. A general conclusion and reference section follows. The first chapter, "Effects of Sampling and Storage Conditions on the Stability of Biochemical Parameters Measured in Rainbow Trout (Oncorhynchus mykiss) Liver", was undertaken to examine the possible interference of anesthetization and of sampling and storage conditions on both enzymatic and non-enzymatic antioxidant concentrations to be considered in further studies. Having resolved the tactics for sampling, Chapter 2, "Variation of Hepatic Enzymes in Three Species of Freshwater Fish from Precambrian Shield Lakes and the Effect of Cadmium Exposure", focuses on the enzymatic antioxidant stores in natural populations of fish. In addition, the effects of Cd on enzymatic antioxidants are considered in fish from a whole lake experiment on Cd additions conducted at the Experimental Lakes Area in Northwestern Ontario.field setting. Chapter 3, "Interactions between Antioxidant Defenses in Liver and Kidney , and Cd Accumulation and Toxicity in the Rainbow Trout (Oncorhynchus mykiss)", examines the complex cellular interactions involved in lipid peroxidative defense through dietary manipulation of two non-enzymatic antioxidants.

EFFECTS OF SAMPLING AND STORAGE CONDITIONS ON THE STABILITY OF BIOCHEMICAL PARAMETERS MEASURED IN RAINBOW TROUT (*Oncorhynchus mykiss*) LIVER

ABSTRACT

Effects of prolonged immersion in the anesthetic, tricaine methanesulfonate (MS222) on liver glycogen concentrations, and the effects of storage temperature and duration on liver concentrations of glycogen, ascorbic acid, protein and acid-soluble thiols were investigated using rainbow trout (Oncorhynchus mykiss). Uniformity of concentrations of glycogen, ascorbic acid, protein and acid-soluble thiols; and of activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase were investigated in liver quadrants. Immersion in MS222 for more than 15 minutes significantly decreased liver glycogen concentrations. Acid-soluble thiols, but not protein declined under conventional conditions of -15 and -38°C and 6 months of storage. Ascorbic acid and glycogen declined when stored on ice, dry ice or liquid nitrogen, while protein and acid-soluble thiols remained stable in all three media. Only ascorbic acid and acid-soluble thiols were not uniformly distributed throughout liver quadrants.

INTRODUCTION

Environmental pollutants exert their direct effects at the cellular membrane or subcellular level (Larsson et al 1985). Consequently, early indicators of stress may often be shifts in metabolites or molecular functions (Nriagu 1988). Coupled with the fact that sublethal pollutant stress is first manifested in individuals rather than populations, monitoring contaminant-induced stress through the use of biochemical indicators seems promising (Neff 1985)

Laboratory and field applied studies have established that protein, ascorbic acid, acid-soluble thiols (AST), glycogen, superoxide dismutase, catalase and glutathione peroxidase are sensitive biochemical indicators of stress. For example, ascorbic acid concentrations decrease in liver, kidney and gill of mullet (Mugil cephalus) exposed to Cd (Neff 1985) and similar results are reported when the same species is exposed to oil or capture stress (Thomas 1987, Wedemeyer 1972). Losses of liver and muscle glycogen in Cd-exposed rainbow trout (Oncorhynchus mykiss) also occur with acute or chronic stress (Larsson et al 1985). Protein in liver of rainbow trout decreases in the presence of water-borne Cd, Cr, Ni as well as with Pb in carp (Cyprinus carpio) (Neff 1985). AST, a measure of sulfhydryl groups including glutathione and metallothionein, increase with exposure to Cd in mullet (Neff 1985, Wofford and Thomas 1984). Superoxide dismutase, catalase and glutathione peroxidase are three enzymes involved in protecting cellular membranes from toxic oxidation (Aksnes and Njaa 1981).

In order to establish the effects of sampling, rainbow trout were exposed to a pH-neutralized anesthetic, tricaine methanesulfonate (MS222), for up to 120 minutes after which livers were removed and analyzed for glycogen. In

addition, rainbow trout livers were sectioned into quadrants, each being analyzed and compared for uniformity of concentrations of all the above parameters. Short term storage effects were investigated by holding whole livers on ice, dry ice and liquid nitrogen for up to 4 days, while long-term effects were evaluated by storing whole livers at -15, -38 and -72°C for up to 6 months. In both long and short-term procedures, the samples were analyzed for glycogen, ascorbic acid, AST and protein.

MATERIALS AND METHODS

Juvenile rainbow trout (37±9gm) were starved and held in 140 litre fiberglass tanks at 12 (±1)°C in dechlorinated Winnipeg city tap water ($CaCO_3$ = 82.5 mg/l; conductivity = 165 μm hr/cm; pH = 7.8), for a period of at least 10 days prior to sampling. A 12 hr light:12 hr dark photoperiod was maintained during this acclimation period. All fish (except where noted) were anesthetized for 5 minutes (±20s) with ammonium hydroxide neutralized MS222 (pH = 7.0) (Wedemeyer 1970) at a concentration of 0.25 gm/l. Whole livers were removed, immediately frozen on dry ice and dispatched to their appropriate media for storage.

Larger rainbow trout (125 ± 43gm) treated in the same manner were used to obtain samples for subsampling and short term storage. Livers were sectioned into quadrants before freezing on dry ice, with sectioning criteria illustrated in Figure 1. All samples were stored in sterile plastic bags, except for those in liquid nitrogen, where aluminum foil was used.

Peterson's (1977) method of protein determination was modified so that initial incubation was prolonged from 10 to 20 minutes. Ascorbic acid, glycogen and AST contents were determined by the colorimetric methods of Jagota and Dani

(1982), Montgomery (1957) and Thomas et al (1982), respectively. Superoxide dismutase, catalase and glutathione peroxidase were assayed using the methods of Sun and Zigman (1978), Rabie et al (1972) and Tappel (1978), respectively. All data were evaluated using ANOVA.

RESULTS AND DISCUSSION

All liver quadrants contained approximately equal amounts of protein and glycogen (Figure 2A, B). This contrasts with independent findings by Richards (1982) and Sasse (1975) of a glycogen gradient, set up by differential glycogenolysis, from highest in the periportal region to lowest in the centrilobular region of starved rats and hamsters. High ascorbic acid levels in quadrant 2 (Figure 2C) are likely to be the result of differential transport, and not increased synthesis in that section of the liver, as teleosts, with the exception of some cyprinids, lack the rate limiting enzyme, L-gulonolactone oxidase, for ascorbic acid synthesis (Chatterjee 1973). Specifically, Hilton et al (1979a,b) showed inconclusive evidence of the ability of rainbow trout to synthesize the vitamin from glucose. While quadrant 2 contained about 151% of the mean ascorbic acid of the other three quadrants, it also represented only 63% of the mean AST (Figure 2D). Smith et al (1979) reported that glutathione was not uniformly distributed in rat liver, and that lowest concentrations were furthest from the periportal region, as in quadrant 2 of our results.

There are at least two interactions between ascorbic acid and glutathione which can account for their inversely related concentrations in quadrant 2. Firstly, ascorbic acid may oxidize glutathione so that it would not react with DTNB colorimetrically in the AST assay, and an artificially low reading would result. However, Baysal et al (1988) have shown, glucose present

in the homogenate would have produced enough reducing power in NADPH, through the hexose monophosphate shunt, to re-reduce glutathione by 70 to 90%. Trichloroacetic acid (TCA) incorporated in the homogenate (Thomas et al 1982) would also drive glutathione from oxidized to reduced form, and thus a total GSH/GSSG count would be obtained. A second possible interaction involves the theory that ascorbic acid is a cofactor in the synthesis of glutathione. Deana et al (1975) reported decreasing glutathione synthesis in ascorbic acid deficient guinea pigs, leading Thomas et al (1982) to suggest that ascorbic acid is necessary for glutathione synthesis. Superoxide dismutase, catalase and glutathione peroxidase were approximately equal in all liver quadrants (Figure 3A, B and C).

Altering the immersion time of rainbow trout in neutralized MS222 was found to have a pronounced effect on liver glycogen (Figure 4). Assuming a reference level of glycogen at 15 minutes of immersion, there is a general decrease, so that after an additional 45 minutes of immersion, a 60% loss of glycogen is incurred ($p<0.001$). While Wedemeyer and McLeay (1981) reported decreased glycogen concentrations in response to acute stress corresponding to a three to four fold increase in blood glucose, Black and Connor (1964) found no significant changes in muscle glycogen with rapid anesthesia. Stress such as anesthesia may act on the hypothalmus causing an increase of catecholamine release from chromaffin cells of the posterior cardinal veins and the head kidney, or the release of adrenocorticotropic hormone from the pituitary. The net result of each is an increase in blood glucose and a decrease in liver glycogen. Mazeaud and Mazeaud (1981) reported that these processes can decrease liver glycogen in a matter of hours. Catecholamine discharge latency is 2-3 minutes from the time of stress, while we observed continuation of the heartbeat for 15 to 30 minutes during anesthetization, indicating that the

decrease in liver glycogen may be much more rapid. This is likely to be due to increased levels of circulating catecholamines, continued phosphorylase-a breakdown of glycogen (Arias et al 1988) while none is being accumulated, and the time interval involved in removing the tissue from the carcass.

Protein levels remained comparable in all three short term storage media (Figure 5A). Although there were slightly higher mean AST levels in samples stored in liquid nitrogen, there were no significant differences between the three media (Figure 5B). Ascorbic acid levels declined significantly in dry ice and liquid nitrogen as did the ice-stored samples with the exception of the 4-day samples (Figure 5C). Similar to our declines of ascorbic acid in liver tissue, ascorbic acid in fish feed has been quantitatively identified as decreasing with exposure to light, higher temperatures and longer storage duration (Soliman et al 1987, Hilton et al 1977). Glycogen levels remained highest in liquid nitrogen (Figure 5D). After 2 days of storage, the dry ice samples retained only 42% of the mean glycogen content of the liquid nitrogen samples, while those on ice retained an average of 16%. Both liquid nitrogen and dry ice stored samples showed significant declines in glycogen with increasing storage time ($p<0.05$) but the lack of significant losses in ice-stored samples is probably due to the rapid initial decline. Enzymatic breakdown of glycogen by phosphorylase-a is likely to be responsible for the depletion (Arias et al 1988), although at present there is no additional literature dealing with specific liver glycogen loss from stored samples.

The benefits of lower temperature storage are illustrated in Figure 6A. While AST levels in samples stored at -72°C remained stable for six months, those stored at -15°C and -38°C assayed progressively less AST with increasing storage time ($p<0.05$), so that after 6 months they retained 20% and 53%

respectively of their original mean concentration. Enzymatic breakdown of glutathione by gamma-glutamyltranspeptidase and gamma-glutamylcyclotransferase (Meister 1983, Anderson 1985) is likely to be responsible for the depletions at higher temperatures, while at -72°C these enzymes are largely inhibited (Anderson 1985, Akerboom and Sies 1981). Protein concentrations remained stable at all three temperatures over the 6 month trial (Figure 6B). Peterson (1977) reported similar results for protein standards stored at 0°C and lower. Quantitative errors that may result from prolonged anesthesia are evident in Figure 6C, where the glycogen concentrations of the -72°C samples are lower than the -15°C samples. However, the -72°C sampled fish were left exposed to MS222 containing water for approximately 40min longer than the -15°C samples. Ascorbic acid data were anomalous and therefore not presented here.

Ascorbic acid, glycogen and AST all exhibit declines with increased storage duration, while protein levels remained stable for at least 6 months under the conditions tested. Only ascorbic acid and AST showed uneven distribution in the rainbow trout liver. Although exposure to neutralized MS222 was found not to affect liver glycogen levels within 12 minutes (Wedemeyer 1970), continued exposure beyond 15 minutes resulted in rapid and significant losses.

Figure 1 : Liver sectioning criteria (ventral view)

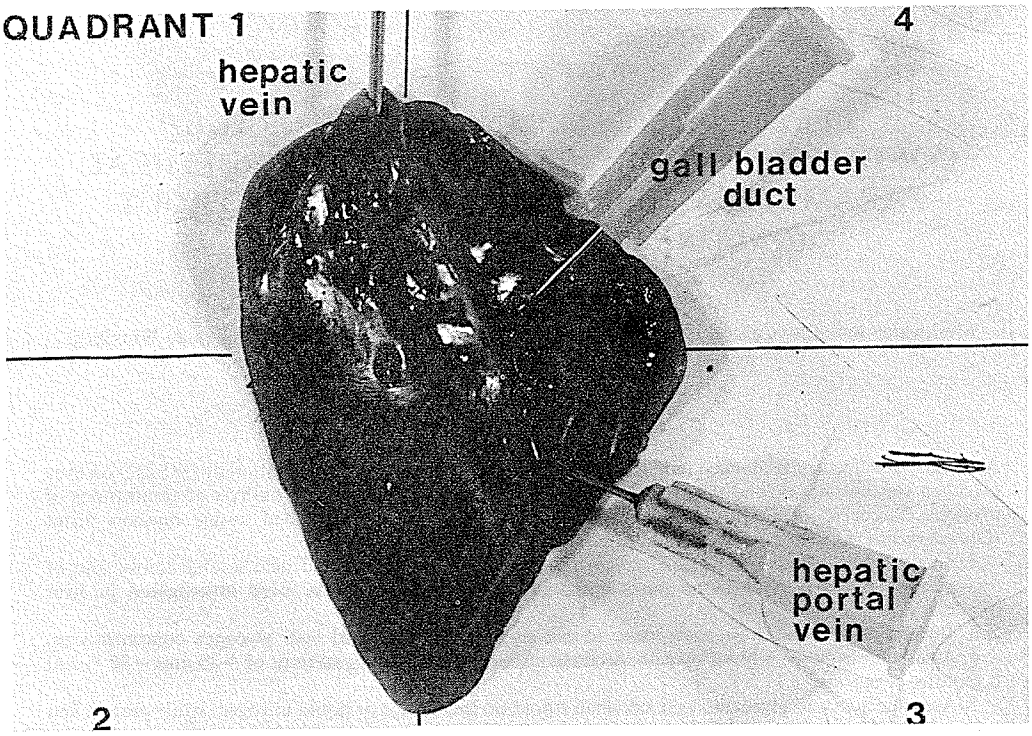

QUADRANT 1
hepatic vein
gall bladder duct
hepatic portal vein
2
3
4

Figure 2: Distribution of glycogen (panel A), protein (panel B), ascorbic acid (panel C) and acid-soluble thiols (panel D) in quadrants of rainbow trout liver. Data are expressed as X±SE for n=8 fish. * = significantly ($p<0.05$) different from other quadrants.

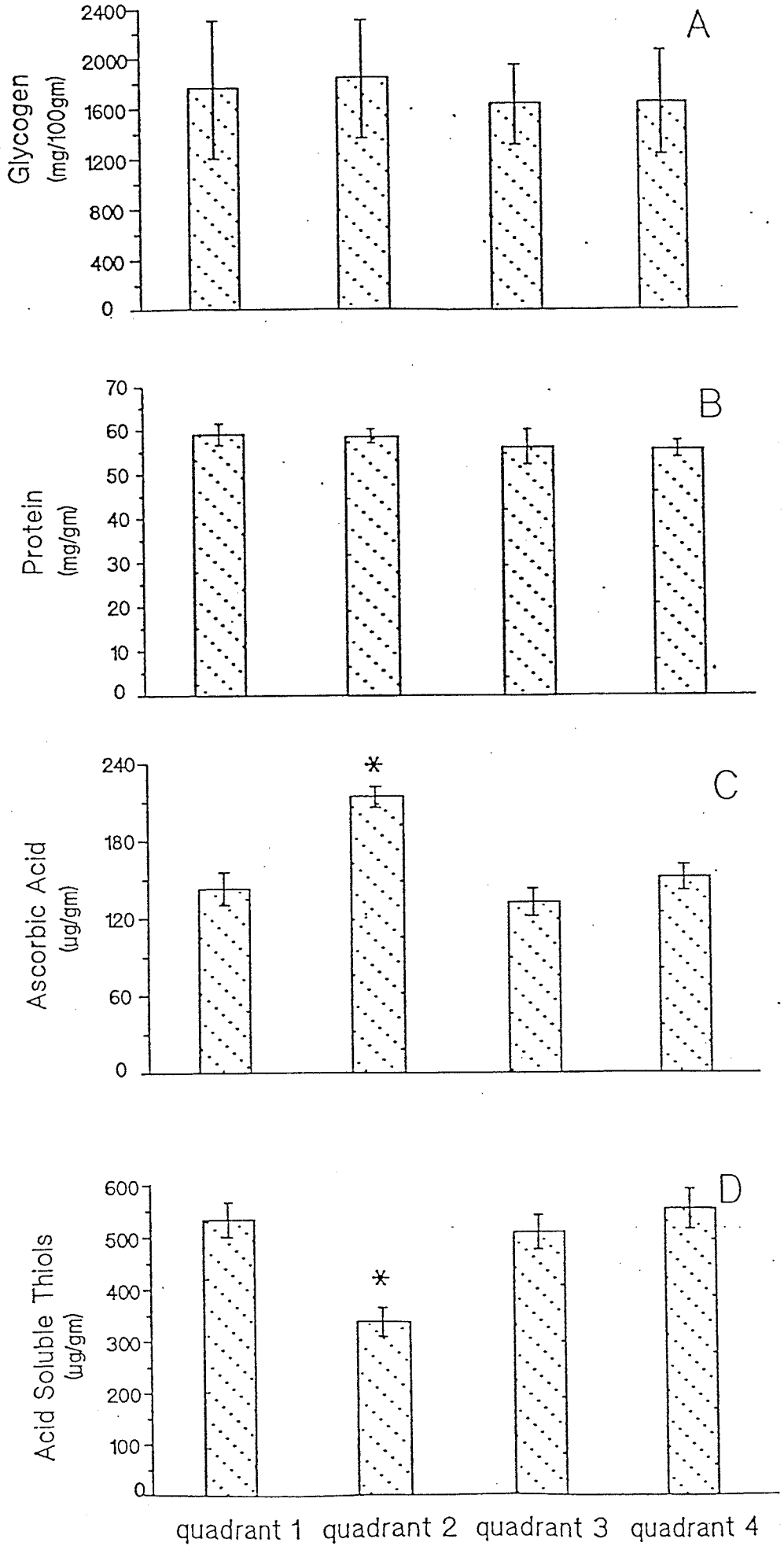

A
Glycogen (mg/100gm)
2400
2000
1600
1200
800
400
0
B
Protein (mg/gm)
70
60
50
40
30
20
10
0
C
Ascorbic Acid (ug/gm)
240
180
120
60
0
*
D
Acid Soluble Thiols (ug/gm)
600
500
400
300
200
100
0
*
quadrant 1
quadrant 2
quadrant 3
quadrant 4

Figure 3: Distribution of superoxide dismutase (panel A), catalase (panel B) and glutathione peroxidase (panel C) in quadrants of rainbow trout liver. Data are expressed as X±SE for n=8 fish. One unit of enzyme is defined for each as: SOD - 1 unit inhibits autoxidation of epinephrine by 50% at pH = 10.2, 25°C. CAT - 1 unit decomposes 1.0 µmol H_2O_2 at pH = 7.0, 25°C. GPx - 1 unit catalyses the oxidation of 1.0 µmol of reduced glutathione by H_2O_2 to oxidized glutathione per minute at pH = 7.0, 25°C.

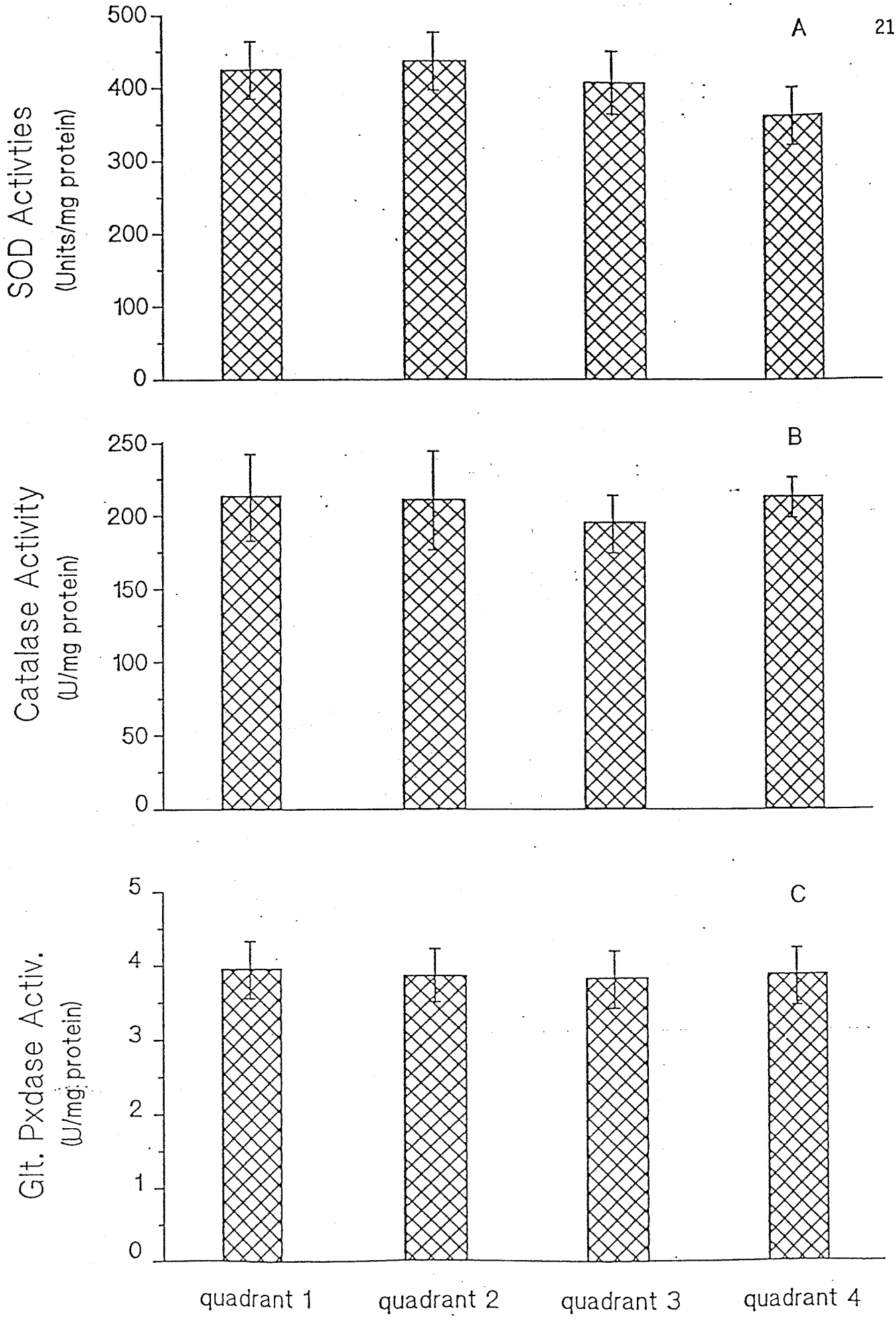

A
SOD Activties
(Units/mg protein)
500
400
300
200
100
0
B
Catalase Activity
(U/mg protein)
250
200
150
100
50
0
C
Glt. Pxdase Activ.
(U/mg protein)
5
4
3
2
1
0
quadrant 1
quadrant 2
quadrant 3
quadrant 4

Figure 4: Effect of immersion in MS222 on liver glycogen. Data are expressed as X±SE for n=8 fish.
* = significantly ($p<0.05$) different from 15 minute samples.

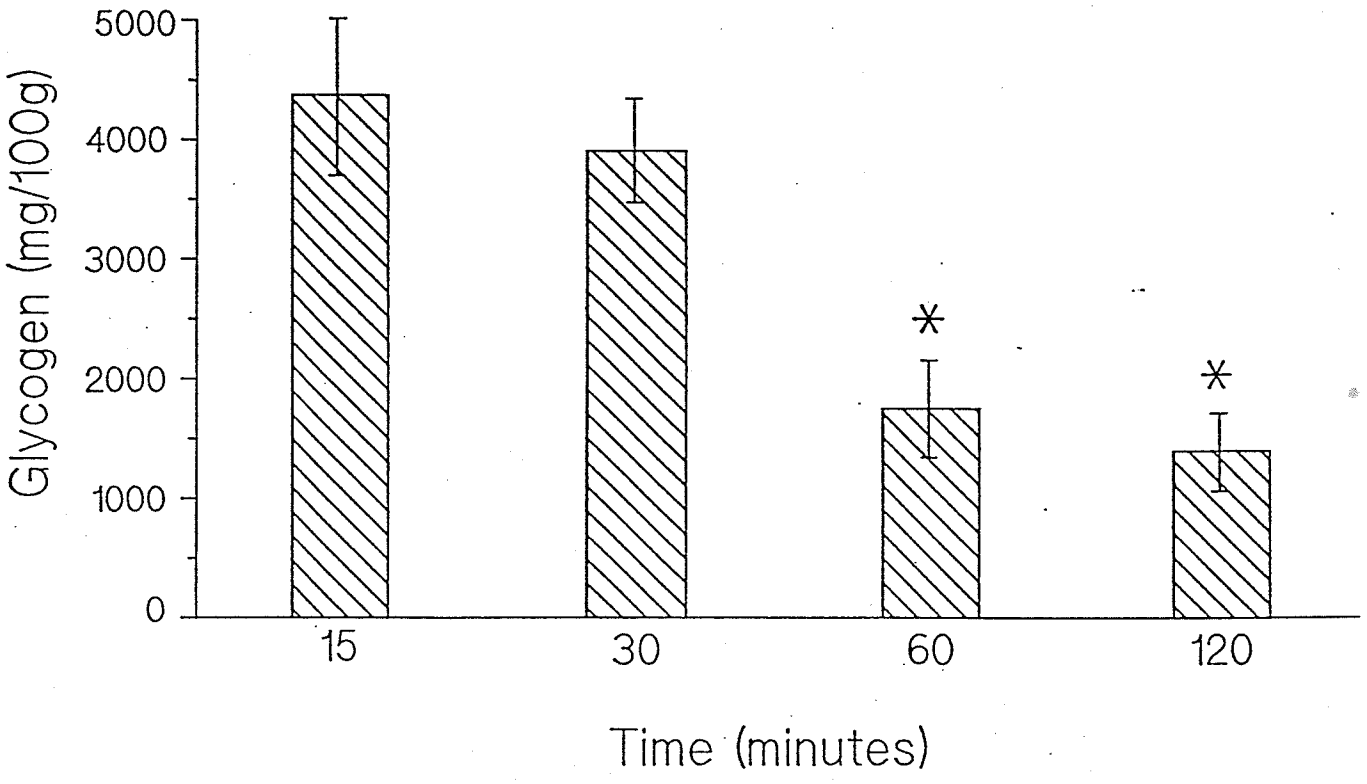

5000
4000
3000
2000
1000
0
Glycogen (mg/100g)
15
30
60
120
Time (minutes)
*
*

Figure 5: Short-term storage effects of ice, dry ice and liquid nitrogen on protein (panel A), acid-soluble thiols (panel B), ascorbic acid (panel C) and glycogen (panel D). Data are expressed as X±SE for n=8 fish.
* = significantly ($p<0.05$) different from 1 day samples of the same treatment.

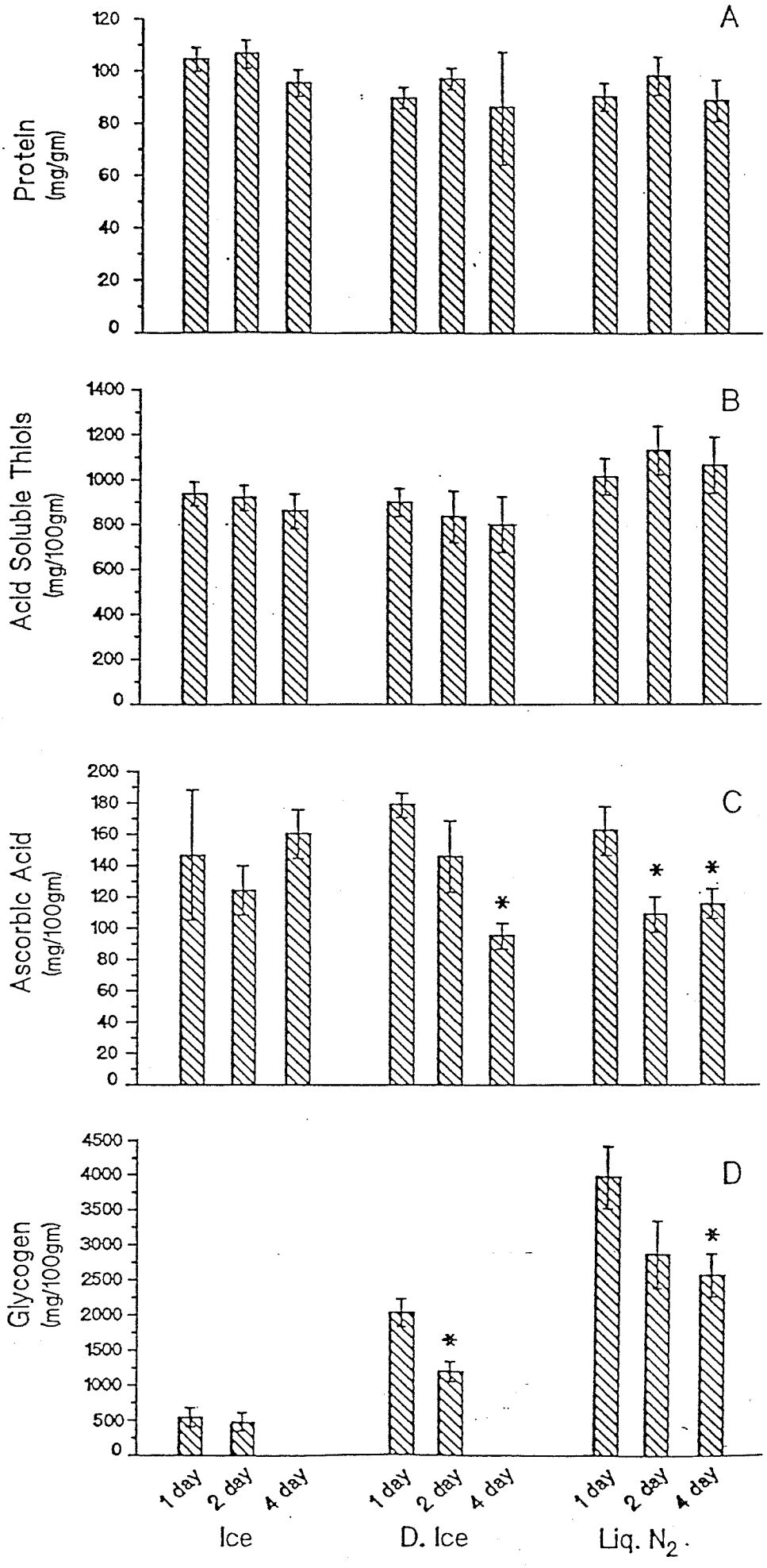
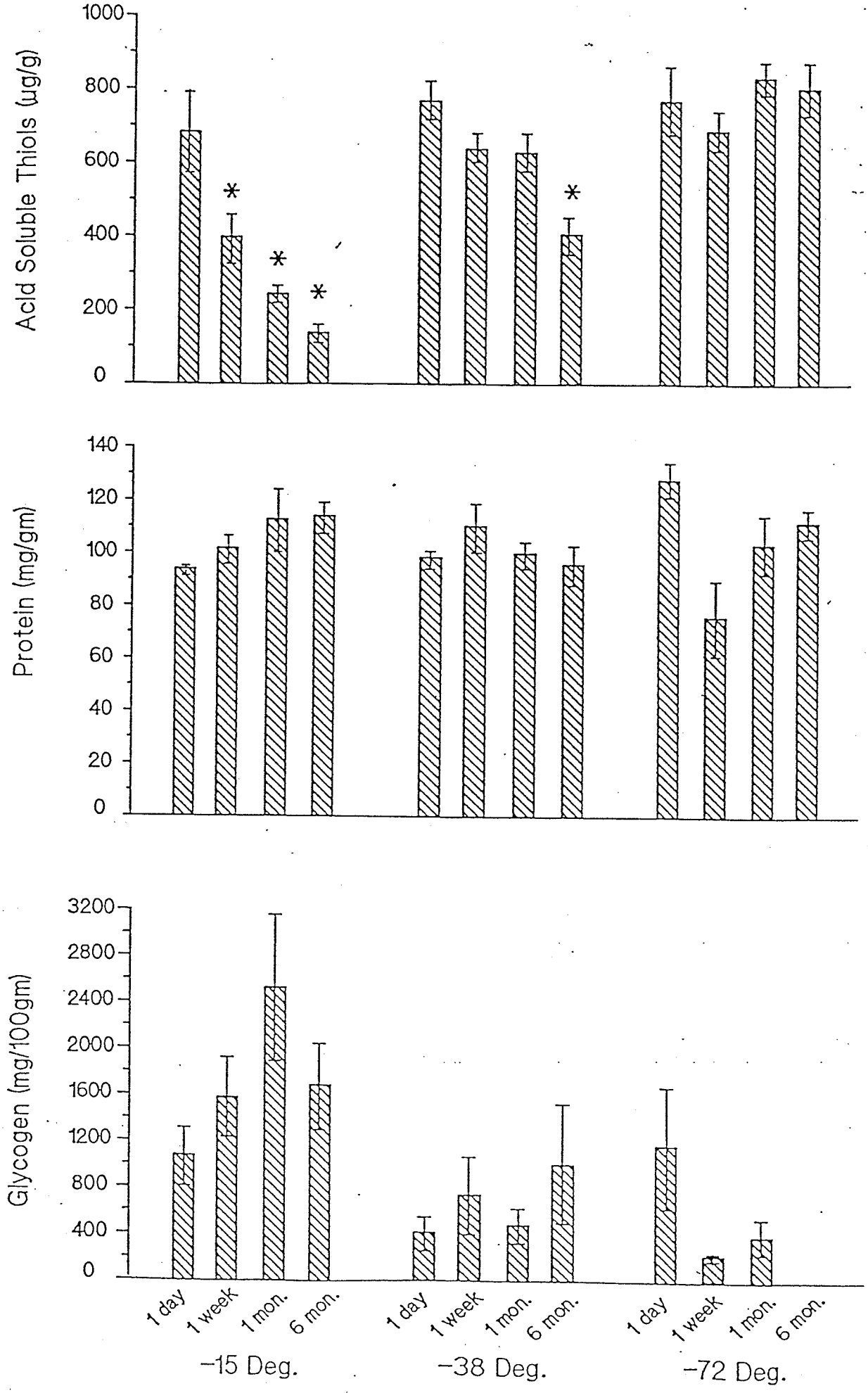

A
Protein (mg/gm)
120
100
80
60
40
20
0
B
Acid Soluble Thiols (mg/100gm)
1400
1200
1000
800
600
400
200
0
C
Ascorbic Acid (mg/100gm)
200
180
160
140
120
100
80
60
40
20
0
*
D
Glycogen (mg/100gm)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1 day
2 day
4 day
Ice
D. Ice
Liq. N2

Figure 6: Long-term storage effects of -15, -38 and -72°C on acid soluble thiols (panel A), protein (panel B) and glycogen (panel C). Data are expressed as X±SE for n=8 fish.
* = significantly ($p<0.05$) different from 1 day samples of the same treatment.


Acid Soluble Thiols (µg/g)
1000
800
600
400
200
0
*
*
*
*
Protein (mg/gm)
140
120
100
80
60
40
20
0
Glycogen (mg/100gm)
3200
2800
2400
2000
1600
1200
800
400
0
1 day
1 week
1 mon.
6 mon.
1 day
1 week
1 mon.
6 mon.
1 day
1 week
1 mon.
6 mon.
−15 Deg.
−38 Deg.
−72 Deg.

VARIATION OF HEPATIC ENZYMES IN THREE SPECIES OF FRESHWATER FISH FROM PRECAMBRIAN SHIELD LAKES AND THE EFFECT OF CADMIUM EXPOSURE

ABSTRACT

Variation of the three antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), and the effects of Cd exposure in a whole lake experiment, on these enzymes were investigated in liver from three freshwater species; pearl dace (Semotilus margarita), white sucker (Catostomus commersoni) and lake charr (Salvelinus namaycush). Enzymatic activity varied significantly between different lakes in geographical proximity. Other than GPx activity, which was lower in lake charr, activities of the three enzymes were not different between the three fish species. Seasonal variation was observed in both CAT and SOD activities. Cd exposure resulted in increased liver concentrations of the metal, but metallothionein protein did not significantly increase. SOD activity in liver of fish exposed to Cd was consistently higher than in liver of fish from reference lakes.

INTRODUCTION

In addition to involvement in ageing and radiation-mediated cellular damage, lipid peroxidation has been receiving increasing attention as a mechanism of toxicity for a variety of organic and inorganic environmental pollutants (Gutteridge and Halliwell 1990, Palace et al 1991). Lipid peroxidation is initiated in membrane polyunsaturated fatty acids (PUFA) when a reactive chemical species removes a hydrogen atom from a methylene carbon in the PUFA structure (Figure 1). The PUFA radical that is generated, then undergoes rearrangement, followed by incorporation of molecular oxygen to create a lipid peroxy radical. This peroxyl radical is highly reactive and can attack membrane proteins or reinitiate the cycle by abstracting another hydrogen atom from an adjacent PUFA (Horton and Fairhurst 1987). In this manner, lipid peroxidation may lead to disruption of cellular and subcellular membranes, and to altered activities of enzymatic membrane proteins (Krinsky 1988).

Although a wide range of environmental toxicants are reported to induce lipid peroxidation, the actual initiating species for the process in membranes is thought to be the hydroxyl radical ($OH^{\bullet}$). As indicated in Figure 1, the production of $OH^{\bullet}$ arises most often through the interaction of other oxygen radicals (H_2O_2, O_2^{-}) with transition metal ions (Fe^{++}, Cu^{+}) (Gutteridge and Halliwell 1990). The availability of these oxygen radicals to initiate indirectly lipid peroxidation is largely dependent on the activity of cellular antioxidants, including the three enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Misra et al 1990).

SOD, CAT and GPx contribute to protection against lipid peroxidation by scavenging oxygen radicals that can induce the process (Mather-Mihaich and DiGiulio 1986). SOD is a metalloenzyme incorporating either Cu, Zn, Fe or Mn in its structure depending on its cellular location. For example, MnSOD is localized in mitochondrion while Cu and ZnSOD are found in cytosol. SOD scavenges superoxide anion (O_2^-), a common byproduct of oxygen metabolism (Figure 1) that can play a role in membrane degradation (Hassan 1988). Despite the in situ metal present, SOD transforms the radical in the following manner.

$$O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The cellular activity of SOD is dependent on a variety of environmental factors including changes in pO_2 (Gregory and Fridovich 1973) and exposure to pollutants such as heavy metals (Mylroie et al 1986) and organochlorines (Roberts et al 1987).

Although consumption of superoxide anion by SOD removes one potential threat, H_2O_2 can also indirectly initiate lipid peroxidation. The cytosolic enzymes CAT and GPx are capable of consuming this reactive species by the following reactions.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$H_2O_2 \xrightarrow{GPx} H_2O + H_2O$$

In addition to consuming H_2O_2, GPx reduces lipid hydroperoxides to hydroxy fatty acids. Both GPx functions are dependent on the presence of glutathione,

another cellular antioxidant, as a source of reducing power (Flohe 1982). Similar to SOD, the activities of both CAT and GPx may be altered by exposure to environmental pollutants (Singh and Savilingam 1982, George and Young 1986).

Because environmental toxicants are present in sublethal concentrations (Nriagu 1988), there is increasing use of subcellular biochemical indicators to predict population stresses arising from chronic exposure to a given toxicant (Larsson et al 1985). The earliest biological indicators of toxicant-induced stress are usually shifts in metabolites or changes in other biochemical systems (Nriagu 1988). Changes in SOD, CAT and GPx protective mechanisms have been identified as early indicators of cellular susceptibility to oxidant injury from toxicant stress (Ardelt et al 1989).

To establish the variability of SOD, CAT and GPx activity, livers from three species of freshwater fish were sampled from several precambrian shield lakes in the Experimental Lakes Area (ELA) of northwestern Ontario (Cleugh and Hauser 1971). Additionally, the sensitivity of the enzymes to an environmental toxicant was investigated by sampling a lake which was experimentally treated with cadmium. As metallothionein is involved in sequestering metals away from sites of potential toxic action (Bremner and Beattie 1990) and is also reported to scavenge free radicals (Shi 1990), analysis of this low molecular weight protein was performed on livers from all three species.

MATERIALS AND METHODS

Lake charr (Salvelinus namaycush) ($\bar{X} \pm SE = 589.9 \pm 21.6$gm) and white sucker (Catostomus commersoni) ($\bar{X} \pm SE = 778.4 \pm 34.0$gm) livers of both sexes were sampled in fish from lakes 305, 468 and 382 in May, June, September and October of 1988. In 1989 both species were again sampled from lakes 468 and 382

for three months, and in addition, fish were sampled once in lake 375 in May, 224 in June, 259 in September and 625 in October. Pearl dace (Semotilus margarita) were sampled in 1989 during May, July, September and October for each of lakes 114, 375, 260 and 382. As part of a larger study of metal toxicity in natural freshwater systems, Cd was added in concentrations less than the Canadian Water Quality Guideline of 200 ng Cd/l to lake 382, achieving water concentrations shown in Figure 2. Table 1 outlines locations as well as physical and chemical characteristics for each of the lakes. A detailed map of the ELA area can be found as a supplement in the Journal of Fisheries Research Board of Canada 1971, volume 2.

Lake charr and white suckers were collected with gill nets retrieved at 30 minute intervals, while pearl dace were sampled using conical minnow traps set for a 24 hour period. Lake charr and white sucker were anesthetized for 5 minutes immediately after capture with buffered (pH = 7.0) tricaine methanesulphonate. The livers were then removed and immediately frozen on dry ice for transportation. Pearl dace were removed from the traps, placed whole in sterile plastic bags, and plunged into acetone cooled with dry ice until frozen. They were also put on dry ice for transportation. Following transportation to the Freshwater Institute (not more than 96 hours from capture) all samples were kept at -120 ± 10°C until analysed to prevent changes in biochemical activity (Palace et al 1990).

SOD, CAT and GPx were assayed as described in Palace et al (1990) using the methods of Sun and Zigman (1978), Rabie et al (1972) and Tappel (1978) respectively. Metallothionein was measured using a 203Hg displacement method (Dutton et al 1990) and Cd in liver tissue was analyzed using a Varian GTA-95 graphite tube atomizer. All data were evaluated with the SAS package using ANOVA with statistical significance set at $p = 0.05$.

RESULTS AND DISCUSSION

Pearl dace, white sucker and lake charr taken from lake 382 accumulated significantly more Cd in their livers than fish caught in the reference lakes (Figure 2). Cadmium was added to lake 382 between May and October; and during the 1988 and 1989 addition seasons, both white sucker and lake charr livers increased in Cd concentration. Although both species eliminated some Cd from the liver between the 1988 and 1989 addition seasons, the highest concentrations occured at the end of 1989. This increase is likely due to increased Cd addition to lake 382 during 1989 (Figure 3) as well as relatively slow elimination of Cd compared with accumulation (Hellawell 1988). Pearl Dace had the greatest accumulation of the three species, with a six fold increase from May to October of 1989.

It is well established that Cd in fish liver is often bound to the low molecular weight protein metallothionein (Klaverkamp et al 1984). Metallothionein synthesis is induced in the liver of Cd-exposed fish, and thought to function primarily in the metabolism of trace metal ions or in sequestering metals away from sites of potential toxic action (Hamilton and Mehrle 1986). Our results indicate no consistent increase in metallothionein concentration for any of the three species in lake 382 over those fish in reference lakes (Figure 4). Without increasing concentrations of metallothionein, however, Cd may still be associated with the protein by displacing Cu and Zn from its structure (Klaverkamp et al 1984). Metallothionein has frequently been measured in tissues of relatively large salmonids and cyprinids, however data for smaller species such as Pearl Dace are scarce. Benson and Birge (1985) suggested increased tolerance to Cd and Cu

in fathead minnows (Pimephales promelas) was due to metallothionein, but no actual analysis of the protein was performed. Surprisingly, after accumulating similar or higher concentrations of Cd in liver, concentrations of metallothionein in Pearl Dace liver were much lower than in lake charr and white sucker (Figure 4).

Whereas studies exist showing variation in activities of antioxidant enzymes for different fish species (Aksnes and Njaa 1981), tissues (Wdzieczak et al 1981, Mazeaud et al 1979), seasons (Gabryelak et al 1983) and for tissues from fish with different feeding habits (Radi et al 1987), little information is available analyzing variation between lakes within a given geographical area. Interspecies variation of activities for SOD, CAT and GPx can be 4 to 5 fold with values approaching this for interspecies variation. For example, Gabryelak et al (1983) reported a greater than 5 fold increase of SOD activity for goldfish (Carrassius auratus) erythrocytes sampled in two different years. Morris and Albright (1984) found CAT to vary as much as 4 times and GPx 2 to $2\frac{1}{2}$ times in gas gland and rete mirabile of six different marine fish.

In some instances large variations of enzymatic activity were observed between fish of the same species captured at the same sampling time from different reference lakes (Figures 5,6 and 7). SOD was least variable in lake charr where the largest difference of activity in liver of fish from reference lakes was 21% between lakes 259 (4313 ± 434 units) and 468 (5444 ± 1104) in September of 1989. White sucker showed a maximum difference of 29% (lakes 224 and 468, June 1989), while pearl dace varied as much as 32% (lakes 114 and 375, July 1989) (Figure 5). CAT and GPx had similar variation of 20-42% and 31-52% respectively, within the three species (<Figures 6 and 7). Whether these observations represent different physical or chemical parameters of the lakes

or biological differences between fish of different lakes requires more consideration.

Differences between fish species may, in fact, play a large role in determining the activities of SOD, CAT and GPx. Effects of ambient temperature and foraging habits may be factors in establishing and maintaining membrane content of an array of polyunsaturated fatty acids (PUFA) (Radi et al 1987). The PUFA content of even closely related species may be vastly different, requiring varying concentrations of SOD, CAT and GPx to protect them against oxidative damage (Witas et al 1984). With the exception of GPx activity, which was consistently $1\frac{1}{2}$ to 3 fold higher in the cyprinids pearl dace and white sucker than lake charr (Figure 7), our data indicate comparatively little variation in enzymatic activities of fish sampled from the same lakes. Specifically, SOD varied to a maximum of 17% between lake charr and pearl dace (lake 375 May 1989) (Figure 5) while CAT differed by 25% between lake charr and white sucker in lake 468 (October 1989) (Figure 6). Aside from isolated cases of significant differences, no consistent patterns for different activities of the enzymes were observed between lake charr, white sucker and pearl dace.

Whereas published data specific to liver are unavailable, previous reports indicate seasonal variability of antioxidant enzyme activity in other tissues. Gabryelak et al (1983) reported that red blood cells obtained from 3 different cyprinid species had slightly higher activities of SOD, CAT and peroxidase in spring than autumn. An examination of seasonal variability in our study excludes lake 382, because of the seasonal addition of Cd and its potential interference with antioxidant enzymatic activities. Significant temporal variation of SOD activity ocurred between sample months in three species in random fashion (Figure 5). Lake charr, however, consistently had

slightly higher activity of SOD in September as did pearl dace in May and September samples. It is interesting to note that pearl dace sampled here would have experienced the largest ambient temperature fluctuations in May and September as they are largely found in shallow water habits (Scott and Crossman 1973). While CAT also had random significant variation in activity between sample periods, the pattern found for SOD was conserved with lake charr assaying slightly higher CAT activity in May and September (Figure 6). Consistent with a link observed between changing ambient temperature and altered enzyme activity, Kolupaev and Putintseva (1984) report complex changes of CAT activity in erythrocytes from dace (Phoxinus phoxinus) exposed to different water temperatures. However, they also report the same effects for peroxidase whereas our results indicate only random changes in activity for GPx, with no pattern between sample periods.

Variation of the three antioxidant enzymes is not exclusively associated with natural factors, but increasingly with the presence of inorganic and organic contaminats (Mather-Mihaich and DiGuilio 1986). Heavy metals including Cd may mediate their damage by producing radicals capable of inducing oxidative breakdown in membranes. Myung-Ho et al (1989) report that superoxide production is enhanced as much as 10 fold in rat liver homogenates exposed to Cd. Similar results of increased peroxidative metabolites with metal exposure have been observed for rats in vivo (Misra et al 1990). Concurrent with the increase of oxidative radicals, antioxidant enzyme activities from organisms exposed to metals may vary. Canada and Calabrese (1989) indicate that SOD activity of mammalian hepatic systems can be inhibited or induced by exposure to various xenobiotics. Feral spot (Leiostomus xanthurus) collected from an estuary polluted with heavy metals and polyaromatic hydrocarbons had elevated hepatic SOD activity (Roberts et al 1987). Alternately, rats ingesting lead had their

SOD activity in erythrocytes depleted (Mylroie et al 1986). Killifish (Fundulus heteroclitus) exposed to 1 mg Cd/l had reduced activity of CAT in liver (Pruell and Engelhardt 1980). A similar study using rainbow trout, however, reported no significant change in CAT activity after 4 months of exposure to 1 µg Cd/l (Arillo et al 1984). Plaice (Pleuronectes platessa) showed decreased GPx activity in liver after intraperitoneal injection of Cd (George and Young 1986). Noting that SOD activity did not significantly change throughout the study period for lake charr or white sucker (Figure 5), the activities of SOD in livers of fish from lake 382 were generally higher than those from reference lakes, with approximately half of these samples being significantly different. Although no other significant differences were found, further support for the possibility of being at response thresholds was also observed in pearl dace where slightly higher activities ocurred for the last two sample periods in 1989. None of the three species showed consistent Cd efffects on hepatic CAT or GPx activities in lake 382 (Figures 6 and 7). We found no evidence to support the suggestion of Wdzieczak et al (1987) that lower activities of one enzyme may be replaced by higher activities of another.

Assuming Cd exposure produced increased formation of peroxidative radicals in livers of fish from lake 382, the relatively small response of the inducible antioxidant enzymes may have been accounted for by an otherwise adequate supply of non-enzymatic cellular antioxidants. Such cellular antioxidants include tocopherol (Burton and Traber 1990), glutathione (Kawabata et al 1989) and ascorbic acid (Miyazawa et al 1986). Gardner and Fridovich (1987) documented that glutathione actually may control transcription of SOD. Whether this indicates cellular ability to substitute one antioxidant for another in its inventory is unclear.

Results from our study of antioxidant enzyme activity in livers from three freshwater fish species indicate that significant spatial variation may occur between samples collected from relatively small differences in geographical location. While CAT and SOD did not vary in a consistent manner between pearl dace, white sucker and lake charr, GPx was $1\frac{1}{2}$ to 3 times higher in livers of the cyprinids than lake charr. Cadmium exposure resulted in significantly higher concentrations of the metal in liver of all three fish species, but concurrent rises in metallothionein concentrations were not evident. SOD activity was generally increased in liver of all three species with Cd exposure while CAT and GPx showed no such response. Both SOD and CAT activities demonstrated temporal variations.

These changes in enzymatic activity indicate that tolerance threshold to Cd was reached at concentrations lower than the Canadian Water Quality Guideline. Having established that the antioxidant enzymes SOD, CAT and GPx may vary seasonally, geographically, between species and with exposure to Cd, the actual mechanisms controlling these changes remain unclear. An examination of the balance between both non-enzymatic and enzymatic cellular antioxidants would further our understanding of cellular defense mechanisms against xenobiotic exposure. Further study, especialy on this balance and on indicators of lipid peroxidation, is needed to consider the interaction of physical and chemical properties of the specific aquatic environment with biological mechanisms of antioxidant control.

Table 1: Physical and Chemical properties of some ELA lakes.

Lake	Lat.	Long.	Lake Area (ha)	Avg. pH	Max. Dpth. (m)	DOC (µg/l)	DIC (µg/l)	Ca^{++} (µg/l)	Alkal. (µg/l)
114	49°41'	93°46'	12.1	6.80	4.0	735	80	1.59	74.6
375	49°44'	93°47'	23.9	7.54	26.0	460	103	6.27	372.0
260	49°41'	93°46'	32.4	7.18	14.0	440	83	2.60	155.0
468	49°41'	93°43'	290.0	7.33	34.1	490	110	2.12	104.5
305	49°42'	93°42'	52.0	7.15	32.7	450	135	2.57	128.0
224	49°42'	93°43'	25.4	6.96	27.4	290	59	1.92	76.0
259	49°42'	93°47'	96.9	6.50	20.3	535	84	1.69	66.9
625	49°45'	93°48'	78.3						
382	49°42'	93°40'	37.3	6.96	13.0	700	88	2.27	83.0

Figure 1: Cadmium involvement in the initiation of lipid peroxidation in polyunsaturated fatty acid lipids (PUFA) and consumption of oxygen radicals by the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

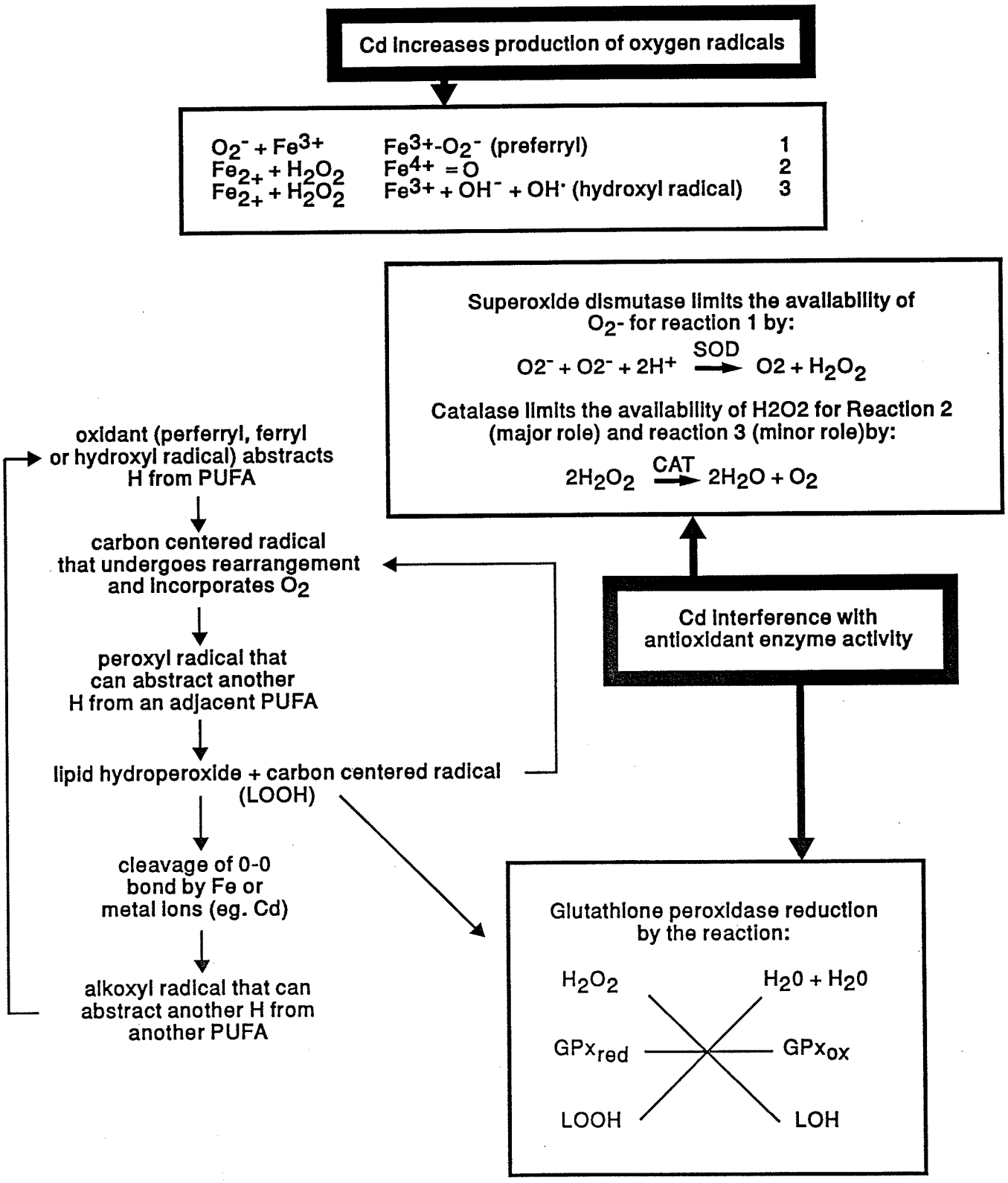

Cd increases production of oxygen radicals
$O_2^- + Fe^{3+}$ Fe^{3+}-O_2^- (preferryl) 1
$Fe_{2+} + H_2O_2$ $Fe^{4+} = O$ 2
$Fe_{2+} + H_2O_2$ $Fe^{3+} + OH^- + OH^{\cdot}$ (hydroxyl radical) 3
Superoxide dismutase limits the availability of O_2- for reaction 1 by:
$O2^- + O2^- + 2H^+ \xrightarrow{SOD} O2 + H_2O_2$
Catalase limits the availability of H2O2 for Reaction 2 (major role) and reaction 3 (minor role)by:
$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$
oxidant (perferryl, ferryl or hydroxyl radical) abstracts H from PUFA
carbon centered radical that undergoes rearrangement and incorporates O_2
peroxyl radical that can abstract another H from an adjacent PUFA
lipid hydroperoxide + carbon centered radical (LOOH)
cleavage of 0-0 bond by Fe or metal ions (eg. Cd)
alkoxyl radical that can abstract another H from another PUFA
Cd interference with antioxidant enzyme activity
Glutathione peroxidase reduction by the reaction:
H_2O_2
$H_20 + H_20$
GPx_{red}
GPx_{ox}
LOOH
LOH

Figure 2: Water cadmium concentrations of lake 382 during 1988 and 1989.

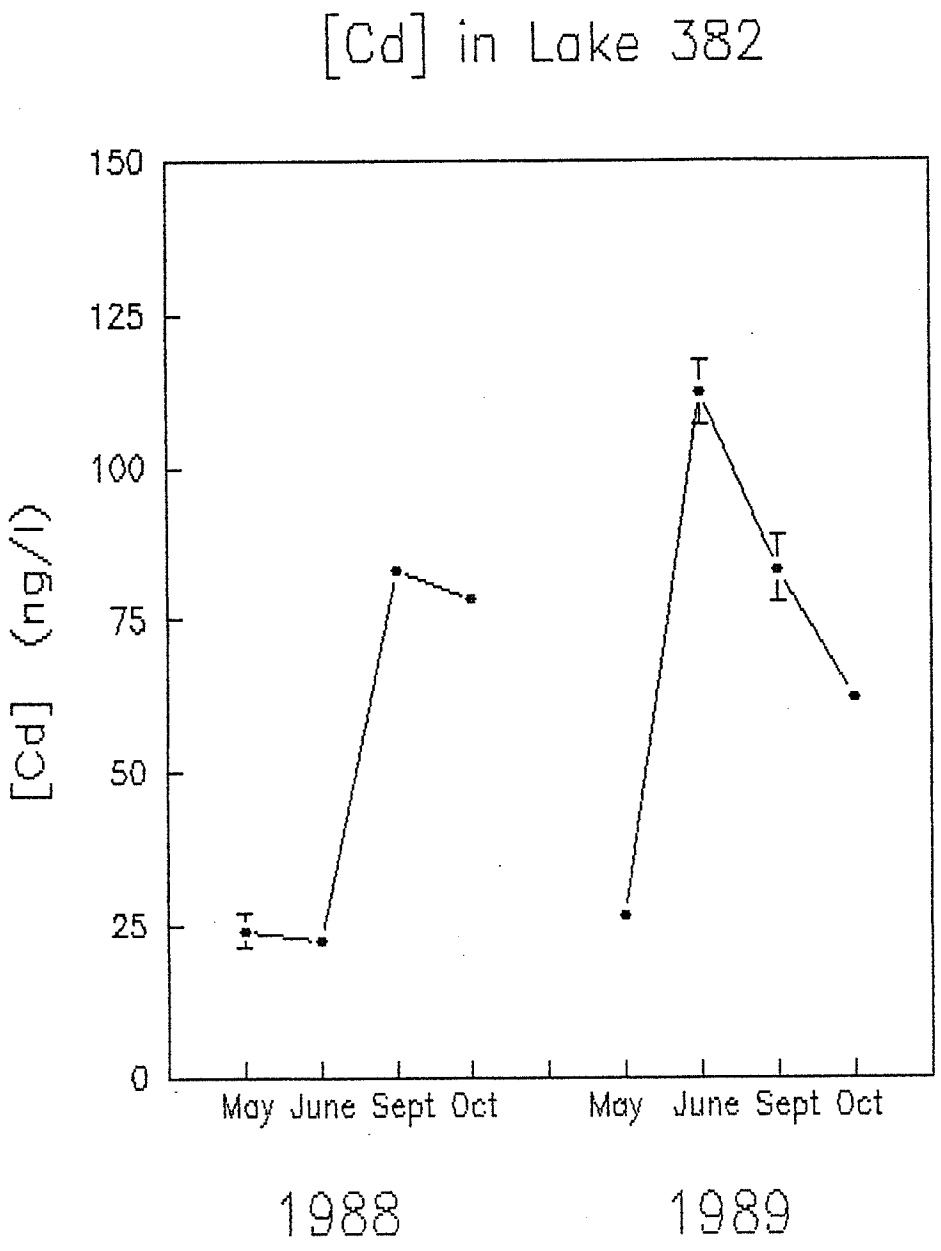
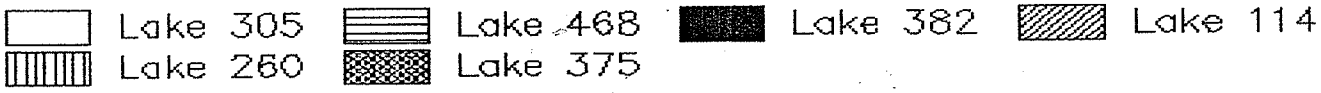

[Cd] in Lake 382
150
125
100
75
50
25
0
[Cd] (ng/l)
May June Sept Oct
May June Sept Oct
1988
1989

Figure 3: Cadmium in livers of lake charr (Salvelinus namaycush) (panel A), white sucker (Catostomus commersoni) (panel B) and pearl dace (Semotilus margarita) (panel C) from some ELA lakes. Data are expressed as X±SE. (n=5, p=0.05).

(M = May, J = June, S = September and O = October)

c = significantly higher Cd accumulation in livers of Lake 382 fish than fish from other lakes.

d = Lakes 375 (May), 224 (June), 259 (September) and 625 (October) were sampled in place of Lake 305 for 1989.



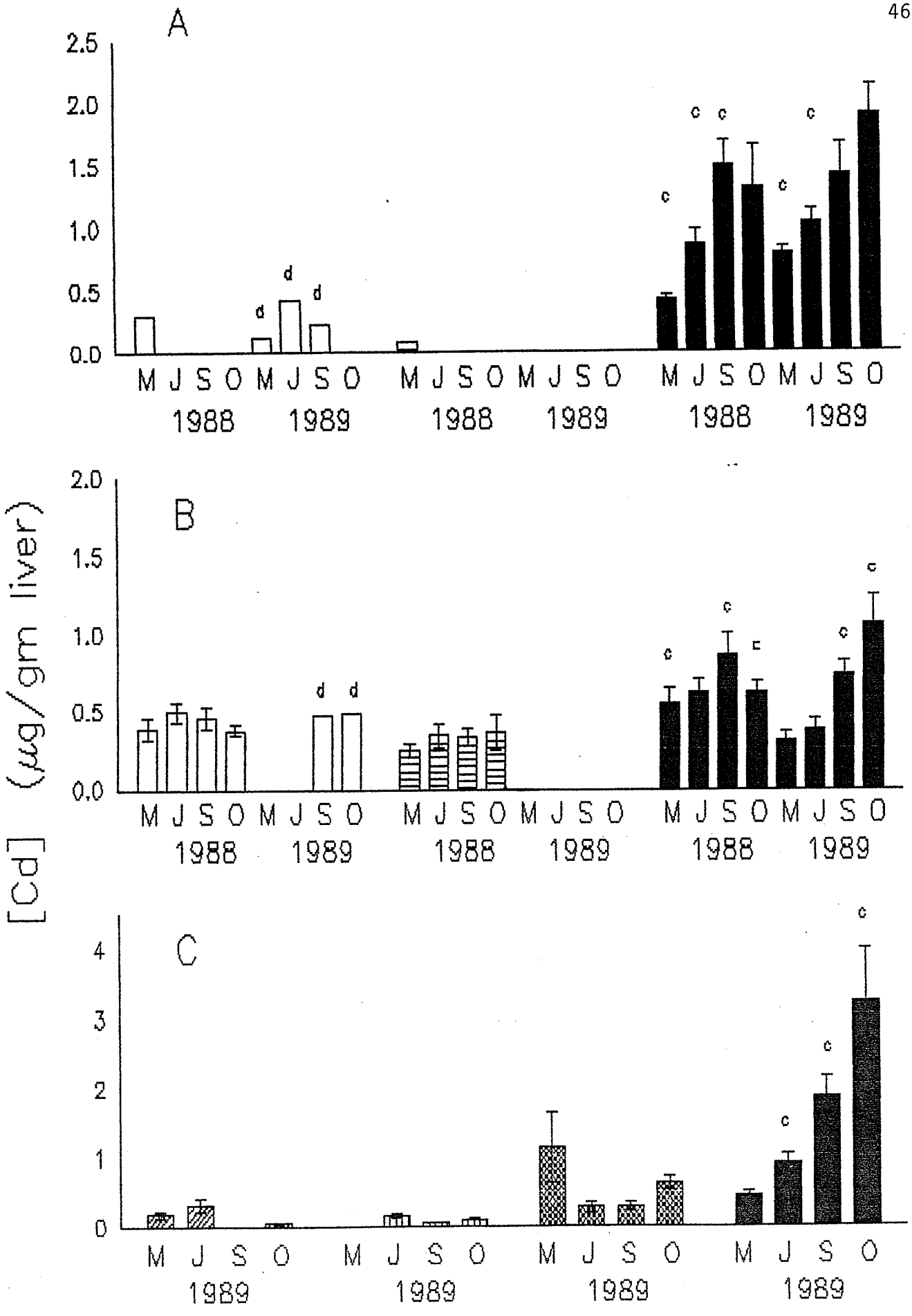
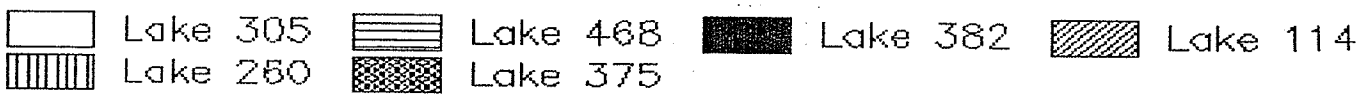

A
B
C
[Cd] (µg/gm liver)
2.5
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
M J S O M J S O M J S O M J S O M J S O M J S O
1988 1989 1988 1989 1988 1989
M J S O M J S O M J S O M J S O
1989 1989 1989 1989

Figure 4: Metallothionein in livers of lake charr (Salvelinus namaycush) (panel A), white sucker (Catostomus commersoni) (panel B) and pearl dace (Semotilus margarita) (panel C) from some ELA lakes. Data are expressed as X±SE. (n=8, p=0.05).

(M = May, J = June, S = September and O = October)

m = significantly higher metallothionein production in livers of Lake 382 fish than fish from other lakes.

d = Lakes 375 (May), 224 (June), 259 (September) and 625 (October) were sampled in place of Lake 305 for 1989.



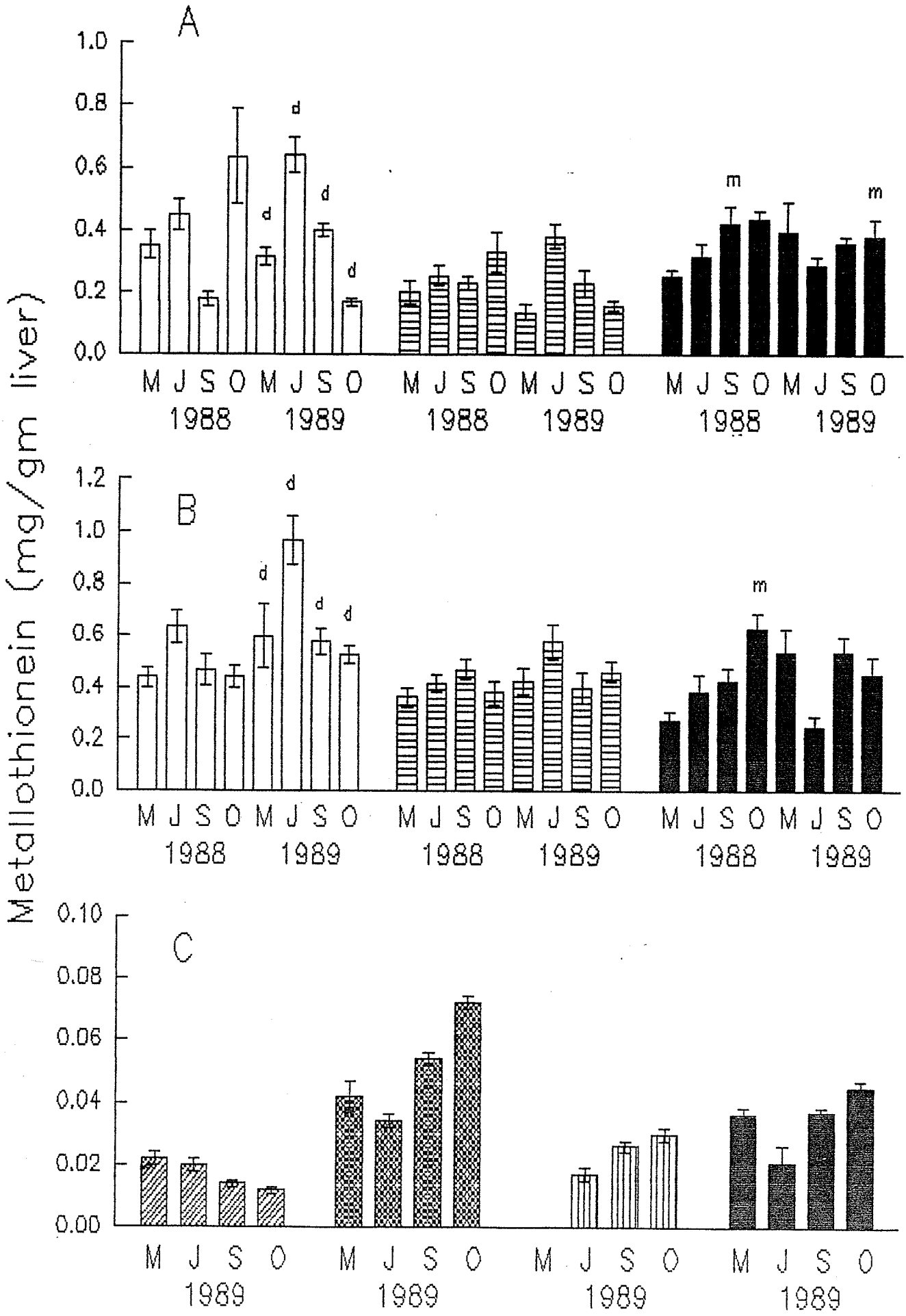

A
B
C
Metallothionein (mg/gm liver)
1.0
0.8
0.6
0.4
0.2
0.0
1.2
0.10
0.08
0.06
0.04
0.02
0.00
d
m
M J S O M J S O
1988 1989

Figure 5: Superoxide dismutase activity in livers of lake charr (Salvelinus namaycush) (panel A), white sucker (Catostomus commersoni) (panel B) and pearl dace (Semotilus margarita) (panel C) from some ELA lakes. Data are expressed as X±SE. (n=8, p=0.05).

(M = May, J = June, S = September and O = October)

l = significantly different enzyme activity between fish sampled from different lakes at the same time.

s = significantly different enzyme activity between fish sampled from the same lake at different times.

d = Lakes 375 (May), 224 (June), 259 (September) and 625 (October) were sampled in place of Lake 305 for 1989.

One unit of SOD inhibits autoxidation of epinephrine by 50% at pH=10.2, 25°C.

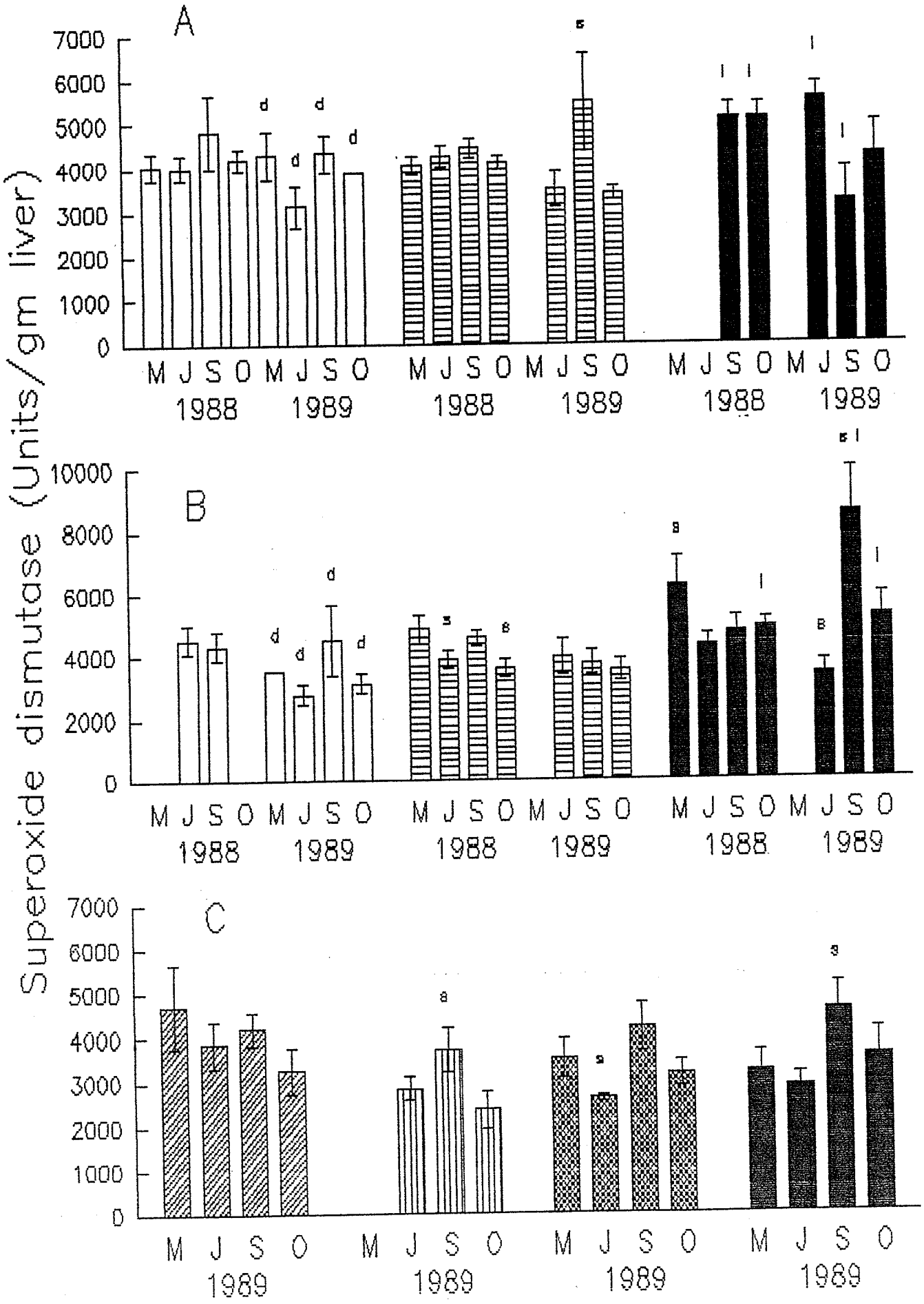

A
B
C
Superoxide dismutase (Units/gm liver)
7000
6000
5000
4000
3000
2000
1000
0
10000
8000
M J S O M J S O M J S O M J S O M J S O M J S O M J S O M J S O
1988 1989 1988 1989 1988 1989
1989 1989 1989 1989

Figure 6: Catalase activity in livers of lake charr (Salvelinus namycush) (panel A), white sucker (Catostomus commersoni) (panel B) and pearl dace (Semotilus margarita) (panel C) from some ELA lakes. Data are expressed as X±SE. (n=8, p=0.05).

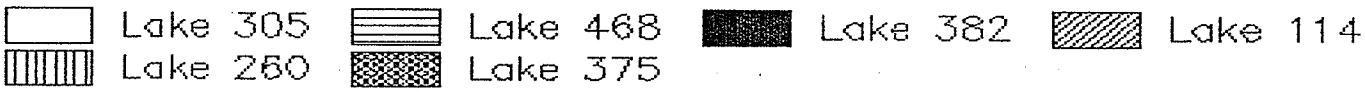
(M = May, J = June, S = September and O = October)

l = significantly different enzyme activity between fish sampled from different lakes at the same time.

s = significantly different enzyme activity between fish sampled from the same lake at different times.

d = Lakes 375 (May), 224 (June), 259 (September) and 625 (October) were sampled in place of Lake 305 for 1989.

One unit of CAT decomposes 1.0 µmol H_2O_2 at pH=7.0, 25°C.



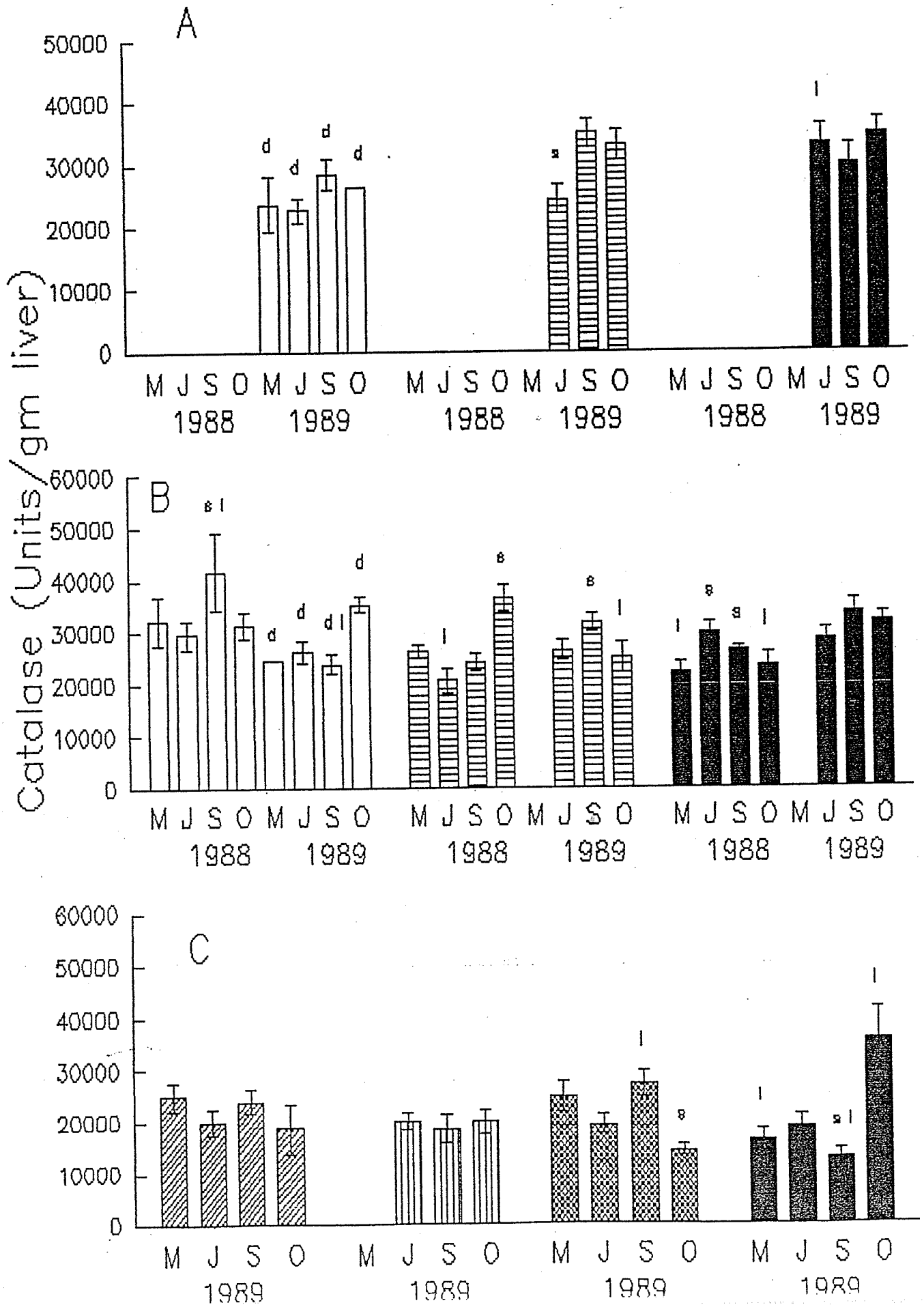

A
B
C
Catalase (Units/gm liver)
50000
40000
30000
20000
10000
0
60000
M J S O M J S O M J S O M J S O M J S O M J S O M J S O M J S O
1988 1989 1988 1989 1988 1989
M J S O M J S O M J S O M J S O
1989 1989 1989 1989

Figure 7: Glutathione peroxidase activity in livers of lake charr (Salvelinus namaycush) (panel A), white sucker (Catostomus commersoni) (panel B) and pearl dace (Semotilus margarita) (panel C) from some ELA lakes. Data are expressed as X±SE. (n=8, p=0.05).

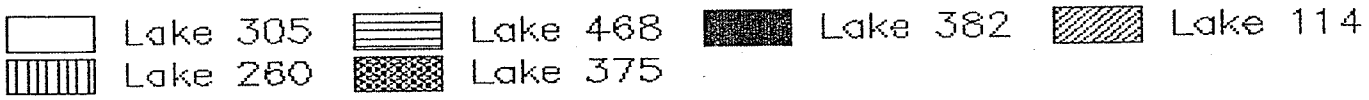
(M = May, J = June, S = September and O = October)

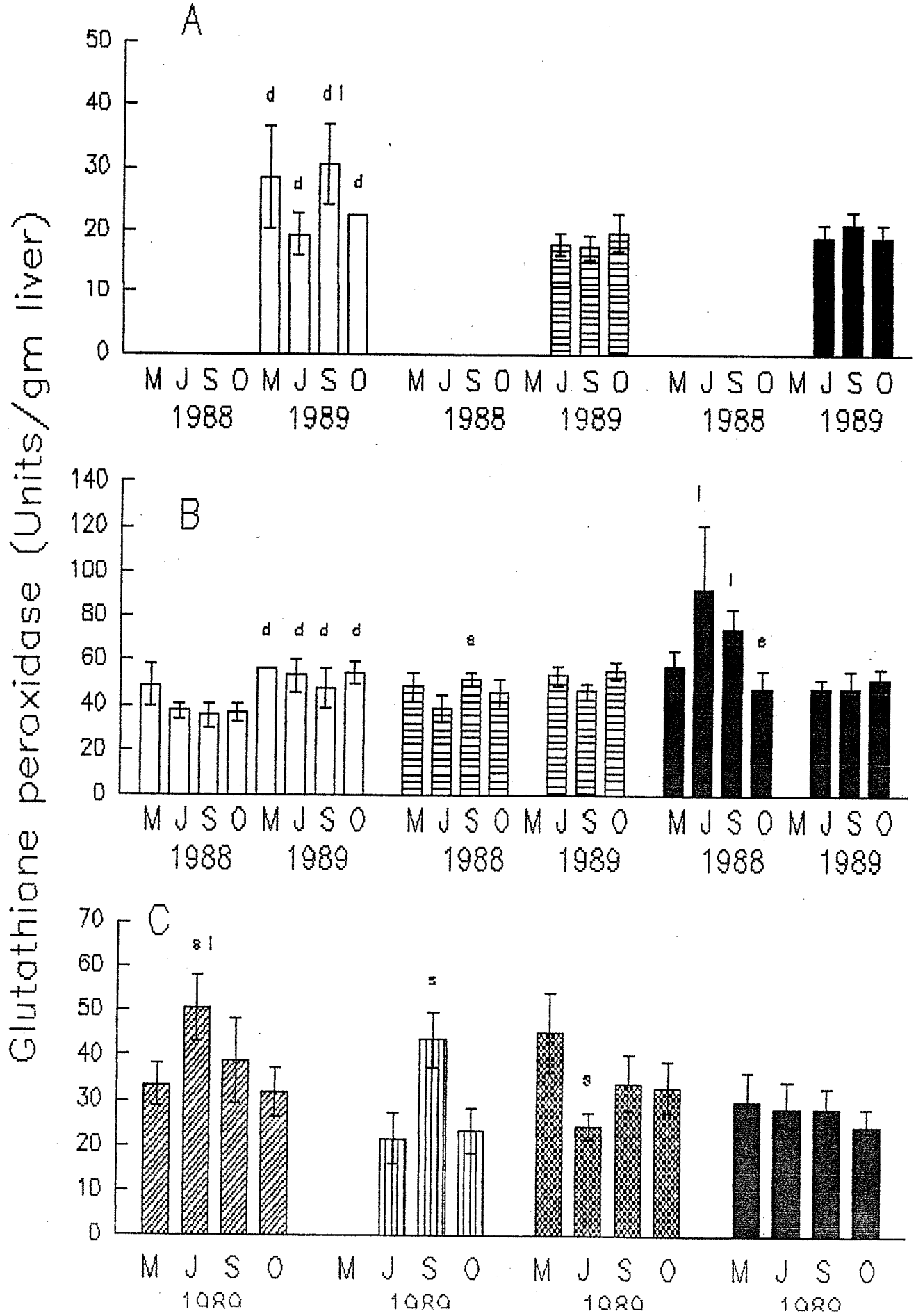
l = significantly different enzyme activity between fish sampled from different lakes at the same time.

s = significantly different enzyme activity between fish sampled from the same lake at different times.

d = Lakes 375 (May), 224 (June), 259 (September) and 625 (October) were sampled in place of Lake 305 for 1989.

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.




A
B
C
Glutathione peroxidase (Units/gm liver)
M J S O M J S O M J S O M J S O
1988 1989 1988 1989 1988 1989

INTERACTIONS BETWEEN ANTI-OXIDANT DEFENSES IN LIVER AND KIDNEY, AND CADMIUM ACCUMULATION AND TOXICITY IN THE RAINBOW TROUT (*Onchorhynchus mykiss*)

ABSTRACT

Rainbow trout (Onchorhynchus mykiss) (X ± SE = 180 ± 10 gm) fed diets deficient in ascorbic acid, tocopherol, both or neither, were exposed to one of three cadmium concentrations (0, 2 or 4 μg/L). After 181 days of exposure, liver, kidney and whole blood were sampled. Cadmium exposure was found to significantly increase the Cd content in liver, especially in fish fed an ascorbic acid deficient diet. Hepatic stores of ascorbic acid decreased in response to Cd exposure and tocopherol-ascorbic acid deficient diets. Depletion of tocopherol in liver occurred only in those fish fed a tocopherol deficient diet and exposed to Cd. Acid-soluble thiols were elevated in both liver and kidney of fish exposed to Cd. Superoxide dismutase activity in liver increased with Cd exposure and with dietary deficiencies of tocopherol and ascorbic acid, while catalase was inhibited by exposure to Cd alone. Glutathione peroxidase activity was unaffected by Cd, but was reduced in fish fed a diet deficient in both tocopherol and ascorbic acid. Hemoglobin, hematocrit, and plasma concentrations of sodium, chloride, calcium and glucose were unaffected by either Cd exposure or dietary deficiencies. Erythrocyte fragility measured by spontaneous hemolysis of red blood cells in physiological saline, was increased by Cd exposure and the absence of dietary tocopherol and ascorbic acid.

INTRODUCTION

Cadmium is increasingly recognized as threatening to aquatic systems because of its acute toxicity to aquatic organisms (Larsson et al 1985, Kumada et al 1980, Hall and Burton 1982) and its presence in toxic concentrations in the environment (Nriagu 1988, Hellawell 1988, McFarlane and Franzin 1978). Previous studies (Majewski and Giles 1981, Giles 1984) exposing rainbow trout (Onchorhychus mykiss) to environmentally relevant concentrations of Cd (3.6 and 6.4 µg/L) have demonstrated disturbances in ionoregulation and oxygen carrying capacity, possibly indicating alterations in membrane integrity and fluidity.

Membrane fluidity is essential for a variety of cellular functions and is maintained through incorporation of polunsaturated fatty acids (PUFA) in membrane lipids. Due to the presence of high numbers of methylene interrupted double bonds, PUFA are susceptible to breakdown through a radical generated process known as lipid peroxidation (Horton and Fairhurst 1987). Cadmium has been identified as a potential lipid peroxidation inducer for PUFA (Stacey et al 1980) and fish in particular are rich in PUFA (Gibson 1989, Hearn et al 1987).

Enzymatic defenses against lipid peroxidation include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These three enzymes inhibit lipid peroxidation by detoxifying reactive oxygen species generated by processes throughout the cell (Mather-Mihaich and DiGiulio 1986). Chronic exposure to environmental pollutants such as cyanide and cadmium has been shown to affect the activities of these enzymes corresponding to an increase of lipid peroxidation (Stacey et al 1980, Ardelt et al 1989).

Non-enzymatic antioxidants, including tocopherol, ascorbic acid and glutathione, are also important for defense against lipid peroxidative

breakdown of membrane lipids. Lipid radicals that arise from exposure to environmental pollutants and which propogate lipid peroxidation are detoxified by tocopherols (Mezes and Vadasz 1984). As one of its functions, ascorbic acid may regenerate tocopherol from the tocopheroxy radical which is produced during the initial detoxification of peroxidizing lipids (Geetha et al 1989). Glutathione is involved in a number of reactions that break down hydrogen peroxide, organic peroxides and free radicals, all potential inducers of lipid peroxidation (Meister 1988). In addition, glutathione is known to sequester heavy metals which are subsequently excreted in bile (Cherian and Vostall 1977). Neff (1985) indicated that pollutant stresses may result in depletion of one or more of these non-enzymatic defense parameters.

Metallothionein, a low molecular weight protein characterized by high cysteine and metal content (Noel-Lambot et al 1978), may be indirectly effective in reducing lipid peroxidation. Metallothionein may sequester heavy metals away from sites of potential toxic action such as membrane and cytosolic enzymes (Jenkins et al 1982) including SOD, CAT and GPx. In addition, metallothionein may prevent metals from contacting membrane lipids where they can induce lipid peroxidation (Andersen and Andersen 1988).

Increased lipid peroxidation may be manifested by the appearance of a variety of cellular alterations. One that is easily measured is a disturbance of membrane fluidity (Leighton et al 1985) which can be quantified by measuring the relative ease of lysis of erythrocytes (Draper and Csallany 1969). Damage to erythrocyte membranes due to lipid peroxidation may also influence osmotic homeostasis through changes in the activity of intrinsic proteins (Sorensen 1986) and membrane permeability (Leighton et al 1985). Hematocrit and hemoglobin may ultimately be reduced (Sorensen and Bauer 1983).

The objectives of this study included an examination of effects of Cd exposure (2 and 4 µg/L) and dietary deficiencies (tocopherol and ascorbic acid) on the status of hepatic enzymatic and non-enzymatic antioxidants. Hemolysis was used to measure the extent of oxidative erythrocyte membrane damage produced by Cd and dietary deficiencies. Sublethal effects of Cd on osmotic balance (plasma Na, K, Cl, Ca, glucose and osmolality) and oxygen carrying capacity (hemoglobin and hematocrit) were also examined.

MATERIALS AND METHODS

Rainbow trout (X ± SE= 182 ± 4gm; n=240) were acclimated in 140 litre fiberglass tanks at 12°C (± 1°C) in dechlorinated Winnipeg city tap water (Ca^{2+} = 20-25 mg/L), conductivity = 165 µmho/cm, pH = 7.8) for thirty days prior to Cd exposure. A 12 hr light: 12 hr dark photoperiod was maintained throughout acclimation and exposure periods. Each tank contained a group of 20 fish. Following acclimation, four groups were exposed to 2 µg Cd/L (actual concentration = 1.82 ± 0.03 µg/L) four groups to 4 µg/L (3.96 ± 0.35 µg/L) and four unexposed groups served as controls. Water exchange in each tank was at a rate of 1 L/min. The groups of 20 fish were fed one of four diets: tocopherol deficient, ascorbic acid deficient, tocopherol and ascorbic acid deficient and a reference diet deficient in neither ascorbic acid or tocopherol. A modification of the diet of Dabrowski et al (1990) was used as described in Table 1. For diet formulation, ingredients were mixed dry except for those noted (*) in Table 1 which were suspended in 200 ml of double distilled water. Ingredients were mixed in a Hobart mixer with an additional 750 ml of water to

form a paste that was extruded through a pelletizer of 2.0 mm diameter. The strands were cut into approxiametly 1 cm long pellets, frozen and stored at -120°C (± 10°C) (Palace et al 1990). The appropriate ration of food was removed from storage daily, thawed and fed immediately. Pellets were presented by slowly sprinkling on top of the water surface so that they were completely consumed in less than 5 seconds of contact with the water.

Ascorbic acid and α-tocopherol acetate were purchased from Sigma Chemical Company, St. Louis Mo.. Each group was fed 36.0 gm per day of the appropriate diet, approximately 1% of bodyweight, for a period of 181 days. At the end of this period, fish were anesthetized for 5 minutes in pH buffered tricaine methanesulphonate (MS222) (Wedemeyer 1970, Palace et al 1990). Blood was obtained through caudal vein puncture using a 1 inch 22G needle attached to a 1cc syringe. All livers and kidneys were dissected , placed in sterile plastic bags, immediately frozen on dry ice and the stored at -120°C (± 10°C) to avoid loss of biochemical parameters (Palace et al 1990).

Superoxide dismutase, catalase and glutathione peroxidase were assayed at 12°C (± 1°C) using the methods of Sun and Zigman (1978), Rabie et al (1972) and Tappel (1978), respectively. Total ascorbic acid was determined using the method of Jagota and Dani (1982). Glutathione was measured using the DTNB method of Moron et al (1979), which assays for acid-soluble thiols (AST). Glutathione comprises approxiametly 55% of these thiols in fish tissue (Wofford and Thomas 1984). Total tocopherol was analyzed using the extraction method of King (1985) followed by the colorimetric proceedure of Tsen (1961). Plasma ionic concentrations were measured using a Corning Chloride Analyser Model 925 for chloride, an Instrumentation Laboratory IL 943 Flame Photometer for sodium and pottassium and a Precision μ-Osmette for the determination of osmolality. Metallothionein content was analyzed using a ^{203}Hg displacement

method [M.D. Dutton, personal communication), while a Varian GTA-95 graphite tube atomizer was employed for determination of Cd, Cu and Zn. Draper and Csallany's (1969) method for measuring hemolysis was used as an indicator of erythrocyte fragility.

Data were analyzed using ANOVA and Tukey's test (SAS 1982) with statistical significance set at p= 0.05.

RESULTS AND DISCUSSION

CADMIUM EFFECTS

In this section, emphasis is placed on effects of Cd in fish receiving diets that were not deficient in ascorbic acid or tocopherol. In our laboratory, Cd has been shown to have an incipient lethal level (ILL) in rainbow trout of 16 µg/L (Majewski and Giles 1981). Our experimental exposures of 2 and 4 µg Cd/L correspond to 13% and 25% respectively of this ILL. After 181 days Cd accumulated significantly in the liver and correlated positively with ambient Cd exposure for all diets (Figure 1). Kidney was not analyzed for metal content. Although Cd is known to displace copper and zinc from metallothionein proteins as well as to produce increased hepatic zinc concentrations (Noel-Lambot et al 1978), no alterations in these metals were observed (Table 2) in trout exposed to Cd.

Although liver tocopherol concentrations were increased for fish fed the reference diet and exposed to 2 µg Cd/L, Cd showed no consistent effects on liver tocopherol in fish fed the reference diet (Figure 2). A decrease of

tocopherol concentrations was expected because Cd has been reported to induce lipid peroxidation by producing lipid hydroperoxides (Stacey et al 1980) which consume tocopherol (Stocker and Peterhans 1989).

Ascorbic acid concentrations were significantly depleted in fish exposed to Cd (Figure 3). Chronic exposure to Cd induces hyperglycemia in mullet (Mugil cephalus) and because high glucose concentrations can reduce uptake of ascorbic acid, tissue stores of the vitamin may be depleted (Stocker and Peterhans 1989). Thomas (1987) postulates that decreases of ascorbic acid are part of a non-specific stress response. The response involves hyperglycemia and the release of adrenocorticotropic hormone (ACTH) from the pituitary, thereby stimulating cortisol production from the interrenal gland (Wedemeyer 1969). Whether elevated cortisol is responsible for or simply accompanies the resultant hyperglycemia is unclear, however, the condition can significantly deplete ascorbic acid stores (Neff 1985). We found no significant ($p=0.05$) increase in plasma glucose for any of the exposure protocols (Table 2). Perhaps depletion of ascorbic acid is due to its increased utilization during glutathione synthesis (Deana et al 1975). Consideration of this possibility will follow.

Acid-soluble thiols in liver increased with exposure to Cd at 2 µg/L but not at 4 µg/L for fish fed the reference diet (Figure 4). However, in the kidney increases occurred at both 2 and 4 µg Cd/L exposures (Figure 5). Wofford and Thomas (1984) reported similar increases of AST in the liver and kidney of Cd exposed mullet (Mugil cephalus) and discuss possible mechanisms for this phenomenon. They conclude that glutathione does not act as a vehicle for Cd binding and excretion in mullet, but suggest that metallothionein induction may be more efficient in sequestering metals. Without metallothionein induction, therefore, metals may be available to glutathione for binding and

excretion as in mammalian systems. Glutathione has been identified as a source of reducing power for the enzyme glutathione peroxidase, which detoxifies lipid hydroperoxides (Ardelt et al 1989). A lack of hepatic induction of this enzyme, however, indicates that this interaction is not a significant factor (Figure 6).

It is well established that metallothioinein concentrations increase with exposure to Cd (Andersen and Andersen 1988, Bem et al 1989, Klaverkamp and Duncan 1987). However, no significant increases in metallothionein were found in liver of fish at either of the Cd concentrations (Table 2).

Unlike glutathione peroxidase activity, which was unaffected by Cd, superoxide dismutase and catalase activities were affected (Figures 7 and 8). Enhancement of SOD activity at 4 µg Cd/L may indicate that increased lipid peroxidation was occurring and that there was simultaneous demand for increased antioxidant enzymatic activities within the cell (Mather-Mihaich and DiGiulio 1986). Similar increases in SOD activity have been documented among feral spot (Leiostomus xanthurus) collected from a polluted estuary (Roberts and Sved 1987). Lower catalase activity with Cd exposure is also consistent with previous work (Singh and Savilingam 1982, Oh et al 1989). Possible mechanisms by which Cd produces lower catalase activity include direct metal mediated structural alteration of the enzyme (Arillo et al 1984) and depression of catalase synthesis (Pruell and Engelhardt 1980).

Hemoglobin and hematocrit have been identified as possible indicators of Cd toxicity and potential threats to lipid membrane integrity. Houston and Keen (1984), for example, found decreases in both for goldfish (Carrassius auratus) exposed to 18 µg Cd/L for 2 weeks. Majewski and Giles (1981) reported elevated hemoglobin and hematocrit in rainbow trout exposed for 178 days to 6.4 µg Cd/L, but not for those exposed to 3.6 µg Cd/L. Consistent with those results, our

data contain no evidence of pronounced hemoglobin or hematocrit disturbances at 2 or 4 µg Cd/L (Table 2).

Giles (1984) showed reductions of plasma sodium, pottassium and calcium for fish exposed to 6.4 µg Cd/L but not for fish exposed to 3.6 µg Cd/L. Additionally, he found plasma protein and osmolality were unaffected at both concentrations. With the exception of potassium, where significantly reduced concentrations were found in our study at 2 µg Cd/L and for 2 of the 4 diets at 4 µg Cd/L, our data support those of Giles (1984) where no significant alterations occurred in any of the plasma parameters at 3.6 µg Cd/L after 178 days.

Measuring the relative ease of hemolysis of erythrocytes is a useful indicator for evaluating the extent of lipid peroxidative damage at the cellular level and overall cellular antioxidant performance (Berry et al 1988). Red blood cell hemolysis was significantly increased with exposure to 4 µg Cd/L for 3 of the 4 diets (Figure 9). This hemolysis may indicate increased peroxidation of erythrocyte membranes because of Cd-induced depletion of enzymatic and non-enzymatic antioxidant capabilities.

DIET DEFICIENCY EFFECTS

Fish fed ascorbic acid deficient diets accumulated more Cd than fish fed the reference diet (Figure 1). This accumulation occurred even though the exposure took place in relatively hard water where calcium is known to inhibit Cd uptake (Wicklund and Runn 1988). Rambeck et al (1988) also demonstrated

elevated Cd uptake in ascorbic acid deficient chickens. They suggest that adequate stores of ascrobic acid inhibit Cd accumulation by increasing absorption of iron which competes with Cd for cellular binding and transport sites. There was no significant difference in Cd accumulation between tocopherol deficiently fed fish and fish receiving dietary tocopherol (Figure 1) with no Cd exposure.

In the presence of Cd, tocopherol concentrations decreased in liver of fish fed tocopherol deficient diets (Figure 2). As discussed in the Cd effects section, consumption of tocopherol could occur while detoxifying peroxy lipid radicals induced by Cd. Amongst the fish exposed to 4 μg Cd/L, the group fed diets deficient in tocopherol and ascorbic acid showed the greatest increase in concentrations of hepatic acid-soluble thiols (Figure 4). The same treatment, however, resulted in a significantly lower concentration of renal acid-soluble thiols (Figure 5). Previous reports (Ardelt et al 1989, Pentyuk et al 1989) have indicated that tocopherol deficiency decreases glutathione, probably by increasing its utilization as an antioxidant. Our results indicate that this process may have occurred in kidney but not in liver.

Consumption of diets deficient in ascorbic acid led to decreased hepatic concentrations of the vitamin, especially with Cd exposure (Figure 3). In the absence of Cd, ascorbic acid stores in liver were significantly ($p=0.05$) depleted only in fish fed diets deficient in both tocopherol and ascorbic acid. Because of a lack of L-gulunolactone oxidase enzyme activity, most teleost fish are unable to synthesize ascorbic acid (Chatterjee 1973), and therefore diets deficient in ascorbic acid would be expected to produce a decrease in tissue concentrations of the vitamin. Increased utilization of ascorbic acid has also been reported (Pentyuk et al 1989) in tocopherol deficient rats. These

investigators speculate that ascorbic acid decreases as it is utilized to make up for the loss of antioxidant activity from tocopherol. Our results show no consistent evidence for this interaction. Ascorbic acid probably acts in concert with tocopherol by regenerating it in a complex electron transport system (Stocker and Peterhans 1989). One peculiarity of this system is that ascorbic acid acts as an oxidant at physiological concentrations when tocopherol is depleted (Staats and Colby 1989). Therefore, a deficiency of tocopherol is damaging not only because of the loss of antioxidant activity, but also because of additional oxidant activity from ascorbic acid.

Ascorbic acid has been implicated as a cofactor in glutathione synthesis (Deana et al 1975). Deficiency of dietary ascorbic acid did not consistently affect glutathione concentrations in the liver and kidney (Figures 4 and 5). Surprisingly, metallothionein concentrations in liver were significantly ($p=0.05$) lower in ascorbic acid deficiently fed fish and fish fed diets deficient in ascorbic acid and tocopherol and exposed to Cd (Table 2). There were no significant differences in liver metallothionein of tocopherol deficiently fed trout. The role and dependency of ascorbic acid in the synthesis and metabolism of metallothionein in fish needs to be investigated further. Investigations in mammals (Onasaka et al 1987) have demonstrated that dietary supplementation of ascorbic acid induces metallothionein synthesis. This indicates a possible role of ascorbic acid in metallothionein synthesis and may explain why fish fed the diet deficient in ascorbic acid actually showed a decline of metallothionein concentration even when Cd was present.

Glutathione peroxidase activity was significantly lower for all fish fed tocopherol and ascorbic acid deficient diets (Figure 6). Previous studies in

rats and humans (Hill et al 1989, Jain et al 1988) have also demonstrated lower glutathione peroxidase activity during tocopherol deficiencies.

At both Cd concentrations, hepatic superoxide dismutase activity was higher in fish fed ascorbic acid and ascorbic acid and tocopherol deficient diets than in fish fed reference diets (Figure 7). Koul et al (1988), however, reported decreased SOD activity in the guinea pig with ascorbic acid deficiency. On the other hand, SOD induction has beeen documented in rats fed diets deficient in tocopherol (Hill et al 1989). Because ascorbic acid and tocopherol serve as antioxidants against lipid peroxidation, their absence along with Cd exposure probably increases demand for enzymatic antioxidants, such as SOD.

In addition to SOD inhibition produced by ascorbic acid deficiency, Koul et al (1988) also found decreased activity of catalase in guinea pigs. The elevation of catalase activity is significant for fish exposed to 4 μg Cd/L and fed ascorbic acid deficient diets (Figure 8). However, the effect is small and isolated given that the fish fed ascorbic acid and tocopherol deficient diets showed no such elevation.

Ascorbic acid and tocopherol deficiency coupled with Cd exposure led to higher spontaneous hemolysis of red blood cells, indicating lipid peroxidative weakening of the plasma membranes (Berry et al 1988). Whereas dietary deficiencies and exposure to Cd contributed to weakening of the membrane, this was not sufficient to produce consistent alterations in hemoglobin, hematocrit or plasma ion balance (Table 2).

Table 1: Diet composition

	gm/kg
Dextrin	157.31
Gelatin	60.08
Casein	581.29
Cellulose	27.94
Arginine	10.01
Methionine	5.01
Palmitic Acid	90.12
Linolenic Acid	0.14
Vitamin Premix	28.04
Mineral Premix	40.06

(*) indicates ingredients that were suspended in 200ml water before mixing

Vitamin Premix

(*)thiamine hydrochloride = 0.115gm, (*)riboflavin = 0.460gm, (*)pyridoxine hydrochloride = 0.115gm, niacin = 1.720gm, calcium pantothenoate = 1.150gm, myo-inositol = 4.590gm, (*)biotin = 0.011gm, (*)folic acid = 0.035gm, choline chloride = 19.714gm, (*)menadione = 0.090gm and (*)cyanocobalamin = 0.0002gm. Ascorbic acid where applicable was added at 1.0gm/kg and tocopherol acetate at 0.400gm/kg.

Mineral Premix

$Ca(H_2PO_4)_2$ = 27.5gm, $CaCo_3$ = 2.1gm, $MgSO_4 \cdot 7H_2O$ = 3.6gm, $FeSO_4 \cdot 7H_2O$ = 1.2gm, KCl = 2.0gm, NaCl = 3.2gm, (*)$Al_2(SO_4)_3$ = 0.008gm, (*)$ZnSO_4 \cdot 7H_2O$ = 0.16gm, (*)$CuSO_4 \cdot 5H_2O$ = 0.04gm, (*)$MnSO_4 \cdot 4H_2O$ = 0.14gm, (*)KI = 0.009gm, (*)Na3SeO3 = 0.003gm and (*)$CoSO_4 \cdot 7H_2O$ = 0.04gm

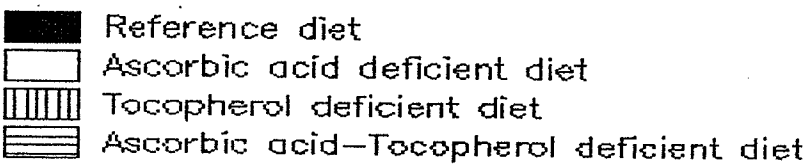
Table 2: Metals and metallothionein in liver, hemoglobin, hematocrit and plasma ionic concentrations. Data are expressed as X±SE (p=0.05).

c = indicates a significant difference between groups of fish receiving the same diet at different [Cd]

d = indicates a significant difference between fish receiving experimental diets and fish receiving the reference diet at the same [Cd]

DIET	Cd Exposure (µg/l)	Liver Zn (µg/gm) (n=7)	Liver Cu (µg/gm) (n=7)	Liver MTN (µg/gm) (n=7)	Hct (%) (n=12)	Hb (gm/dl) (n=12)	Plasma Na (mmol/l) (n=10)	Plasma K (mmol/l) (n=10)	Plasma Ca (mmol/l) (n=10)	Plasma Cl^- (mmol/l) (n=10)	Plasma Glc. (mmol/l) (n=10)	Osmolality (mosm/kg) (n=10)
Reference	0	27.33±3.28	173.7±35.2	0.76±0.24	36.1±2.7	7.46±0.48	154.13±0.66	1.56±0.04	2.25±0.04	131.70±0.42	2.90±0.06	289.9±2.9
	2	28.60±2.59	154.7±13.6	0.57±0.16	33.9±1.9	7.46±0.4	148.77±1.56	0.86±0.09 (c)	2.29±0.15	132.85±0.92	2.84±0.13	291.1±3.3
	4	31.69±1.63	211.9±33.9	0.54±0.05	30.5±2.0	6.63±0.48	153.80±0.90	1.28±0.09 (c)	2.20±0.05	132.10±0.64	3.07±0.09	297.3±4.4
Ascorbic acid deficient diet	0	29.30±3.33	187.0±30.3	0.60±0.24	31.7±1.8	6.32±0.37	152.36±1.12	1.56±0.07	2.10±0.03	129.00±1.34	3.31±0.41	286.4±1.3
	2	37.27±6.48	205.3±57.0	0.57±0.20	41.2±1.7 (c,d)	7.77±0.31	155.60±1.27	0.50±0.08 (c,d)	2.27±0.10	131.20±0.64	3.14±0.14	289.0±1.7
	4	30.16±1.41	152.0±41.3	0.35±0.04 (d)	29.2±1.3	6.11±0.25	153.53±1.00	1.36±0.09	1.99±0.09	131.65±0.42	3.14±0.06	288.6±1.3
Tocopherol deficient diet	0	32.67±3.88	227.1±42.5	0.60±0.20	31.4±2.6	6.93±0.45	157.42±1.07 (d)	1.56±0.10	2.04±0.06	130.65±0.56	3.01±0.11	286.3±1.0
	2	28.21±1.84	195.9±53.6	0.35±0.12	25.9±1.9 (c,d)	6.78±0.26	154.10±0.98	0.88±0.12 (c)	2.03±0.07	130.80±0.59	3.28±0.30	287.5±0.7
	4	30.45±2.20	191.0±41.3	0.49±0.09	31.9±1.7	6.56±0.35	154.82±1.06	0.77±0.08 (c)	2.10±0.08	129.25±0.82	3.42±0.22	287.1±1.2
Ascorbic acid and tocopherol deficient diet	0	25.64±1.65	199.1±29.0	0.27±0.05 (d)	32.0±2.2	6.50±0.51	149.85±0.91	1.55±0.09	1.96±0.05 (d)	129.65±0.64	3.06±0.04	285.8±1.4
	2	34.71±3.51	248.9±58.2 (c)	0.55±0.11 (c)	35.6±1.3	7.92±0.33	154.67±0.63	0.94±0.08 (c)	2.18±0.05	129.35±0.75	2.96±0.16	289.6±1.3
	4	27.17±1.32	160.1±37.5 (c)	0.32±0.05 (d)	29.8±2.6	6.14±0.47	158.01±1.07	1.83±0.21	2.00±0.07	132.25±0.43	3.22±0.07	293.0±1.8

Figure 1: Cadmium concentrations in liver. Data (X ± SE) are expressed as µg/gm wet weight. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).



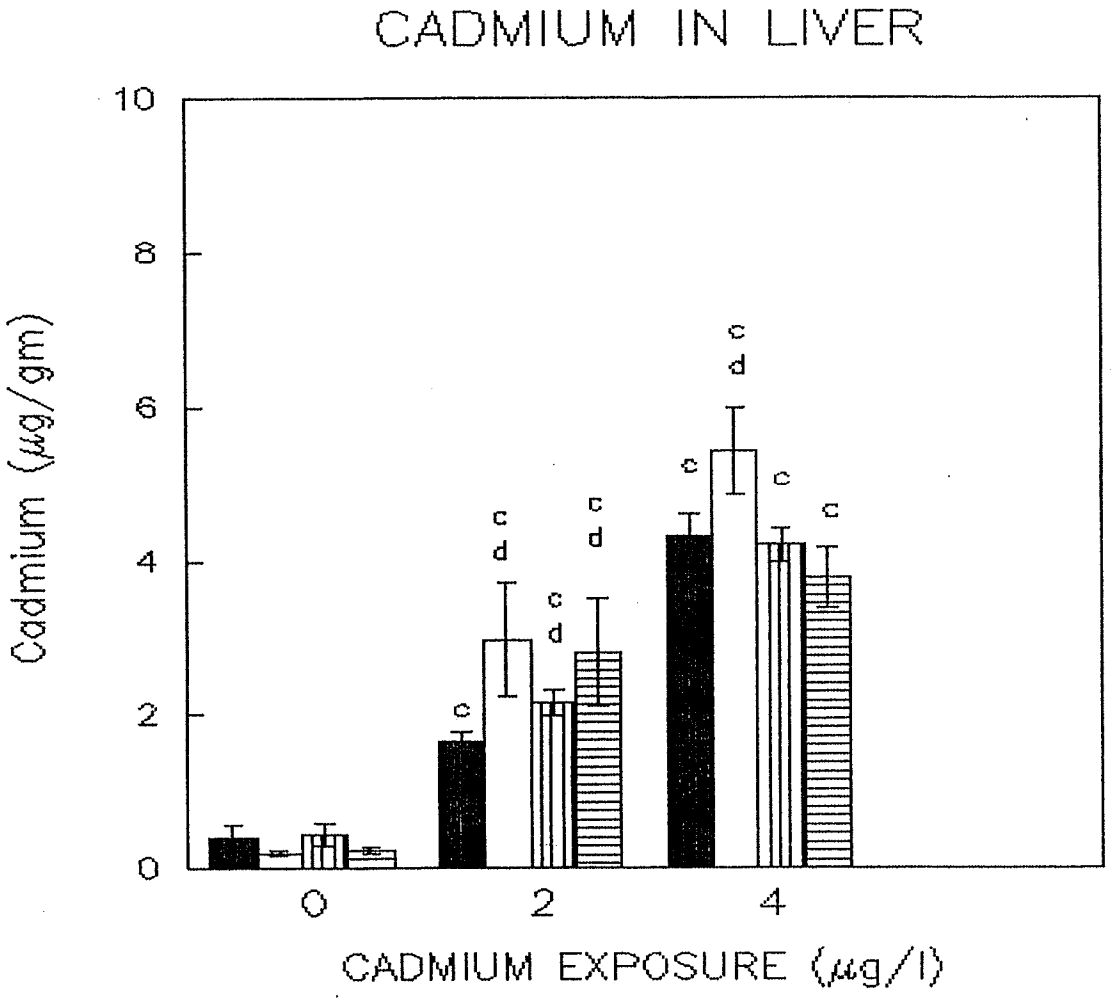
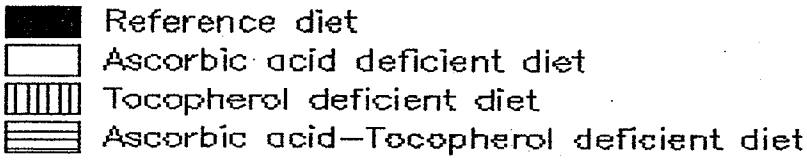

CADMIUM IN LIVER
Cadmium (µg/gm)
10
8
6
4
2
0
c
d
c
d
c
d
c
d
c
c
0
2
4
CADMIUM EXPOSURE (µg/l)

Figure 2: Ascorbic acid concentrations in liver. Data (X ± SE) are expressed as µg/gm wet weight. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7)



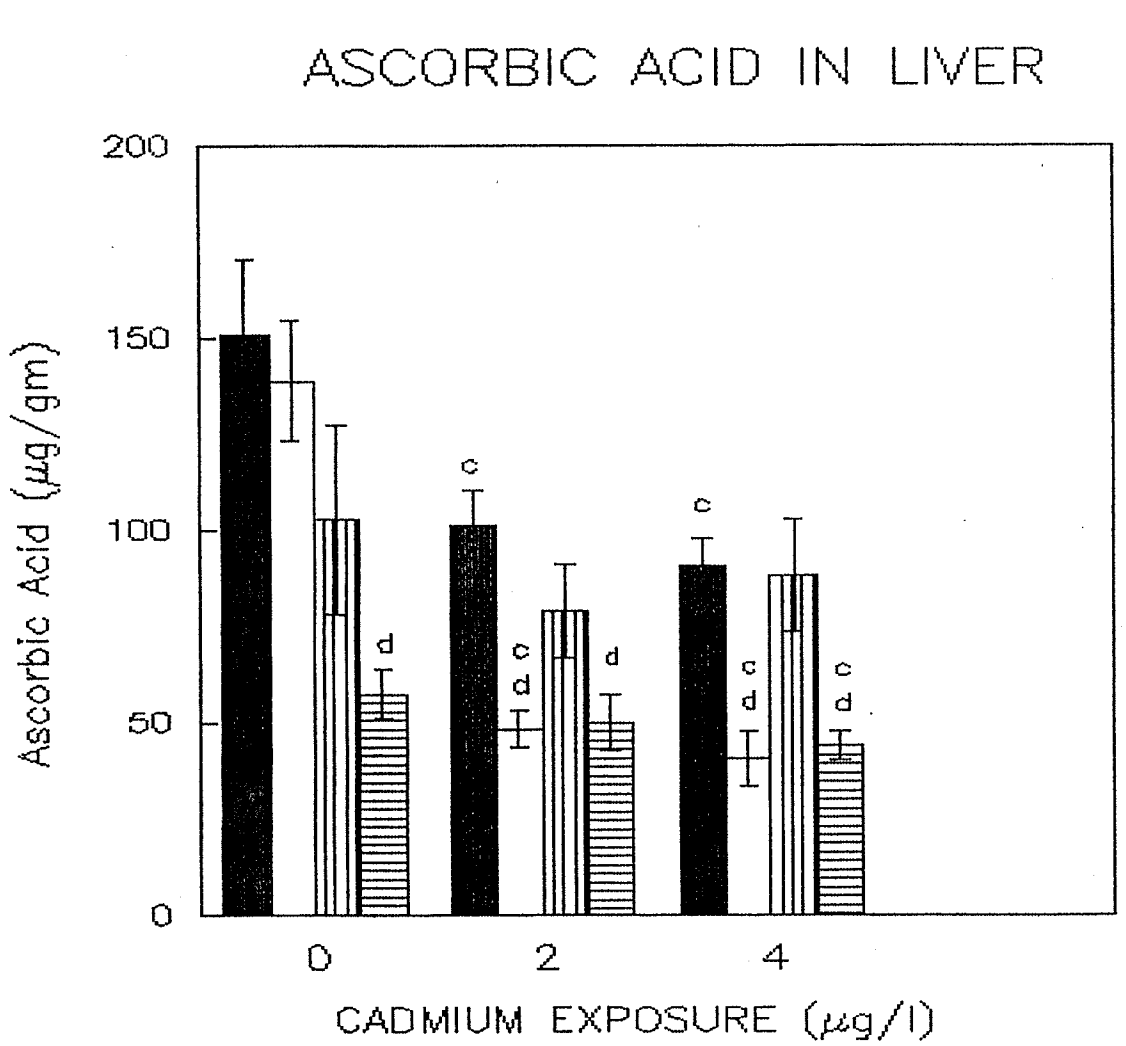
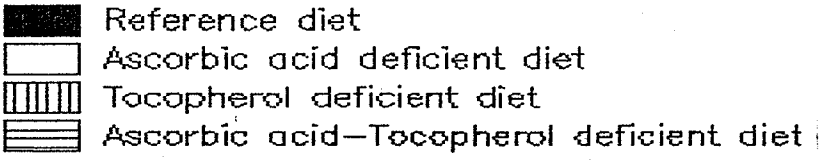

ASCORBIC ACID IN LIVER
200
150
100
50
0
Ascorbic Acid (µg/gm)
c
c
d
d
c
d
c
d
0
2
4
CADMIUM EXPOSURE (µg/l)

Figure 3: Tocopherol concentrations in liver. Data (X ± SE) are expressed as µg/gm wet weight. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).



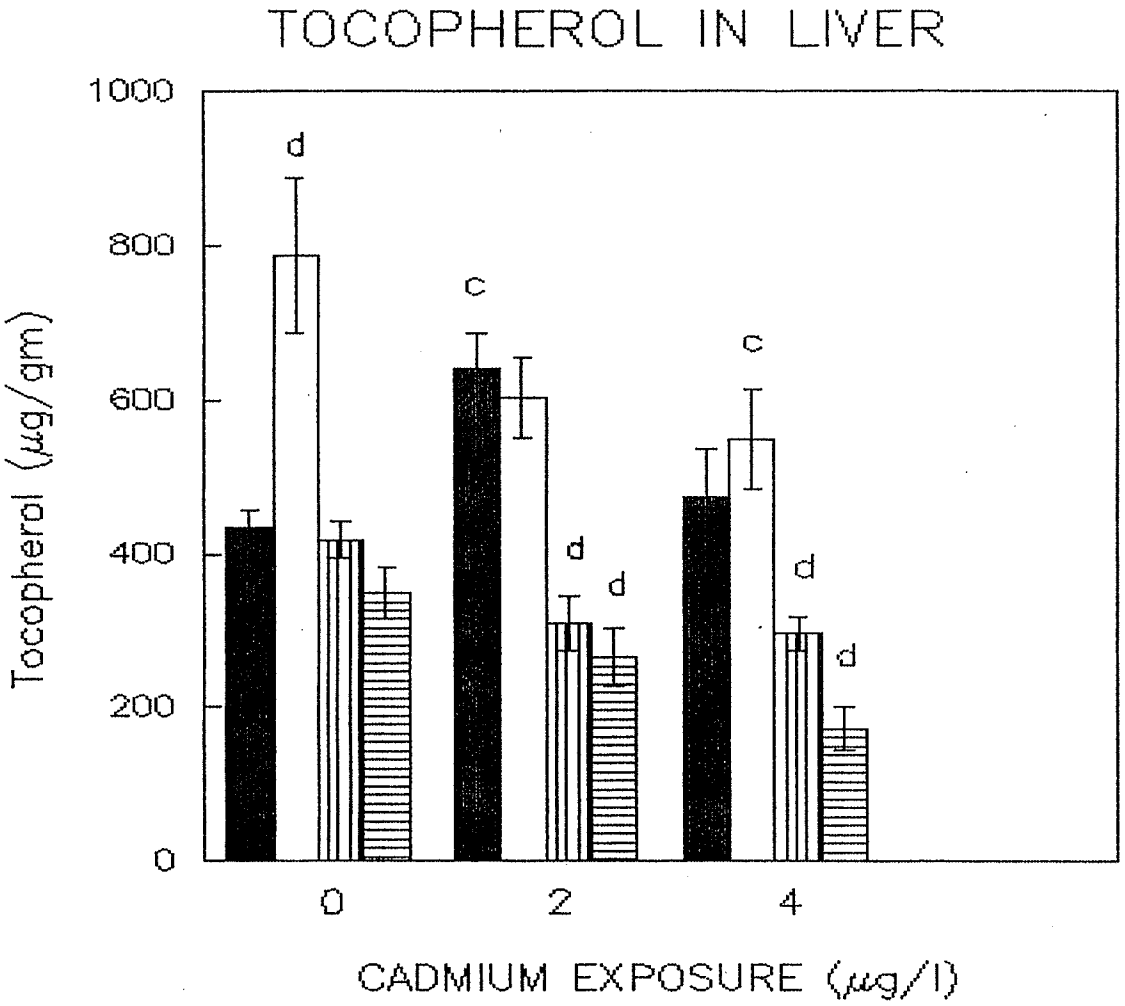
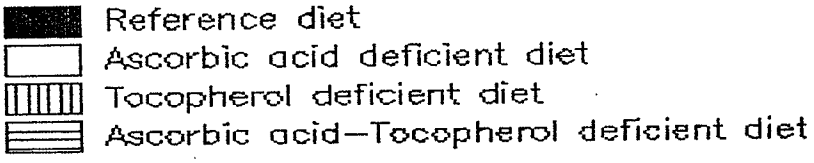

TOCOPHEROL IN LIVER
1000
800
600
400
200
0
Tocopherol (μg/gm)
d
c
d
d
c
d
d
0
2
4
CADMIUM EXPOSURE (μg/l)

Figure 4: Concentrations of acid-soluble thiols in liver. Data (X ±SE) are expressed as μg/gm wet weight. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=12)



ACID–SOLUBLE THIOLS IN LIVER

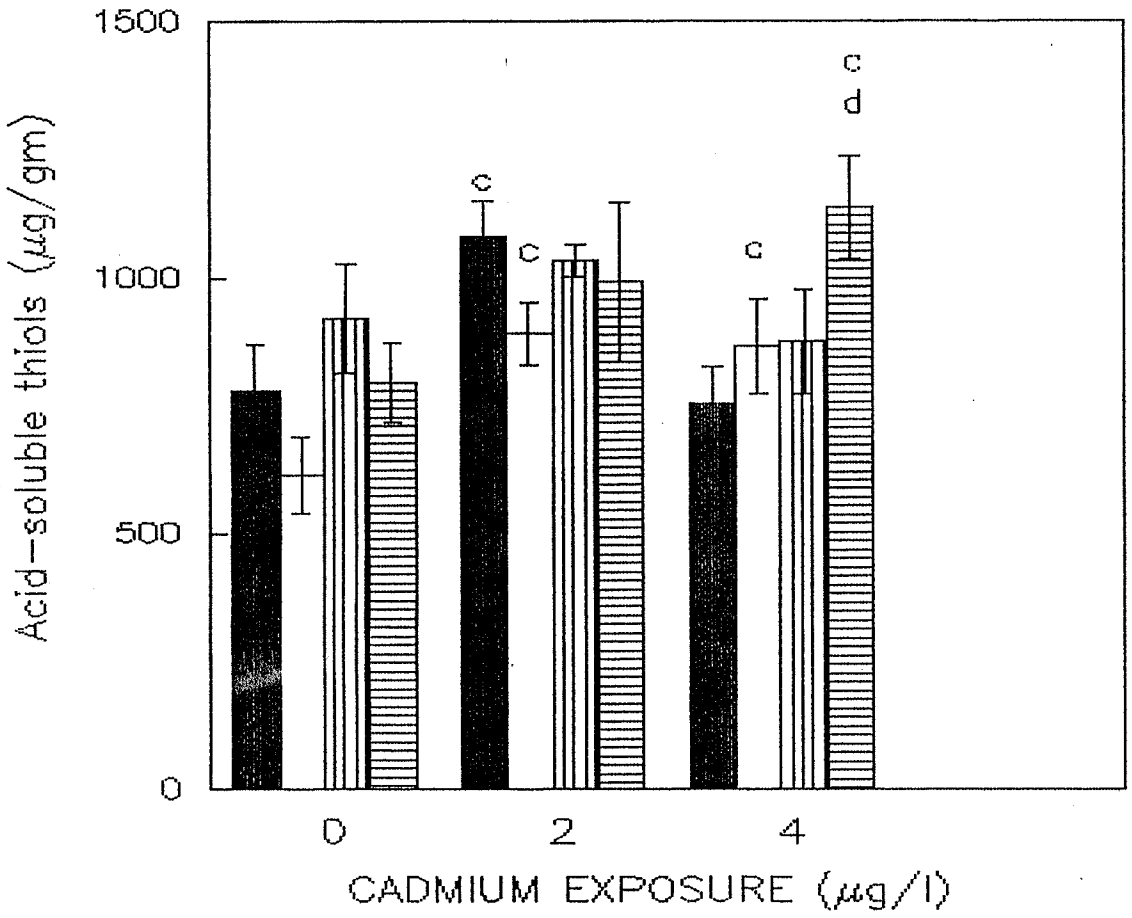
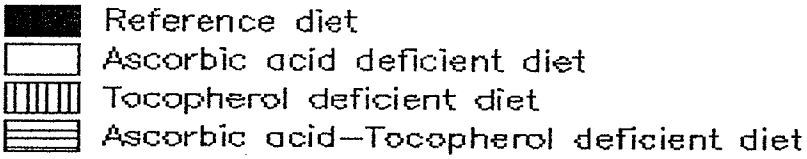


Figure 5: Concentrations of acid-soluble thiols in kidney. Data (X ± SE) are expressed as µg/gm wet weight. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significanmt differences from the reference diet at the same [Cd]. (p=0.05, n=12).



ACID–SOLUBLE THIOLS IN KIDNEY

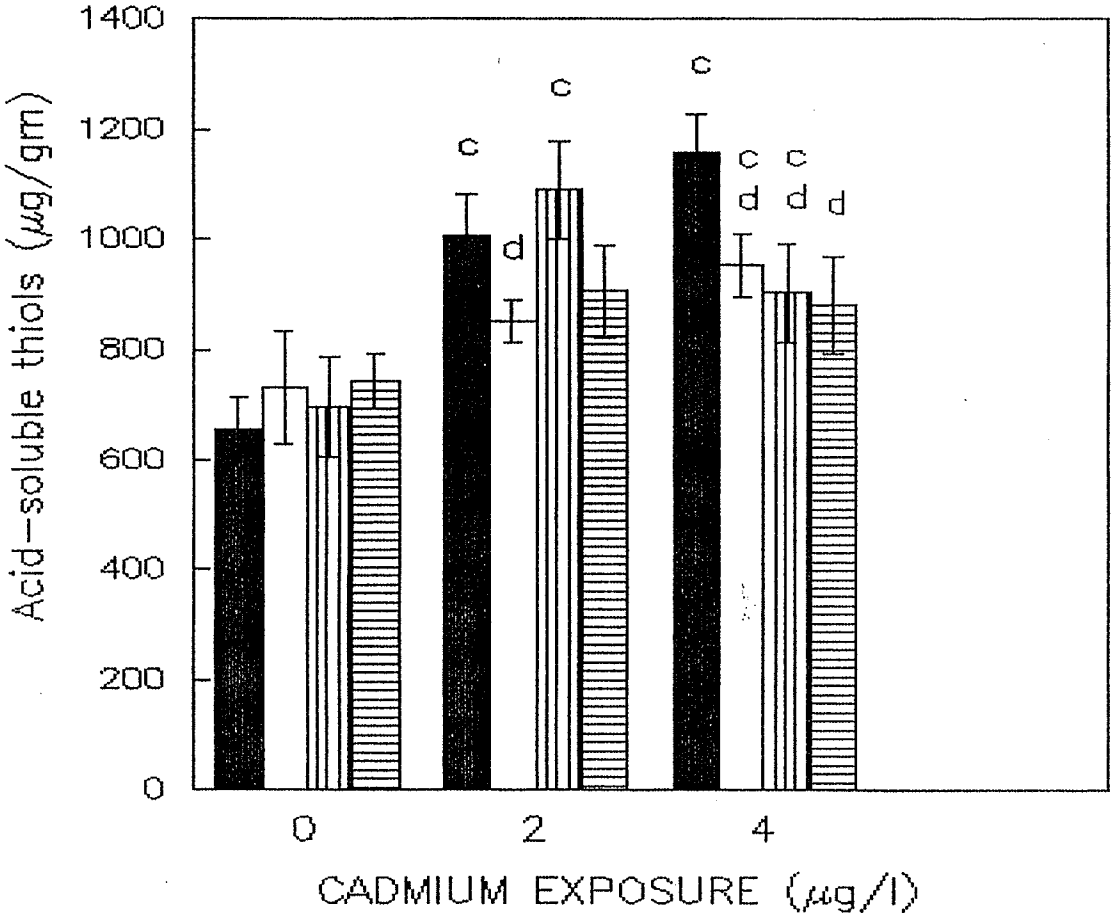
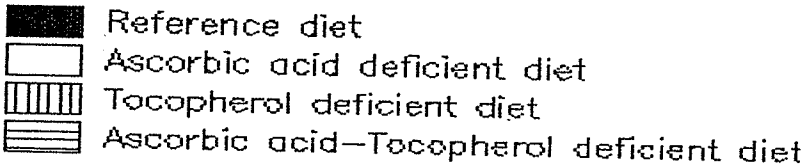


Figure 6: Glutathione peroxidase activity in liver. Data (X ± SE) are expressed as units/gm wet weight. One unit of glutathione peroxidase catalyzes the oxidation of 1.0 μmol of reduced glutathione by H_2O_2 to oxidized glutathione per minute at pH=7.0, 25°C. c= cadmiumn effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).



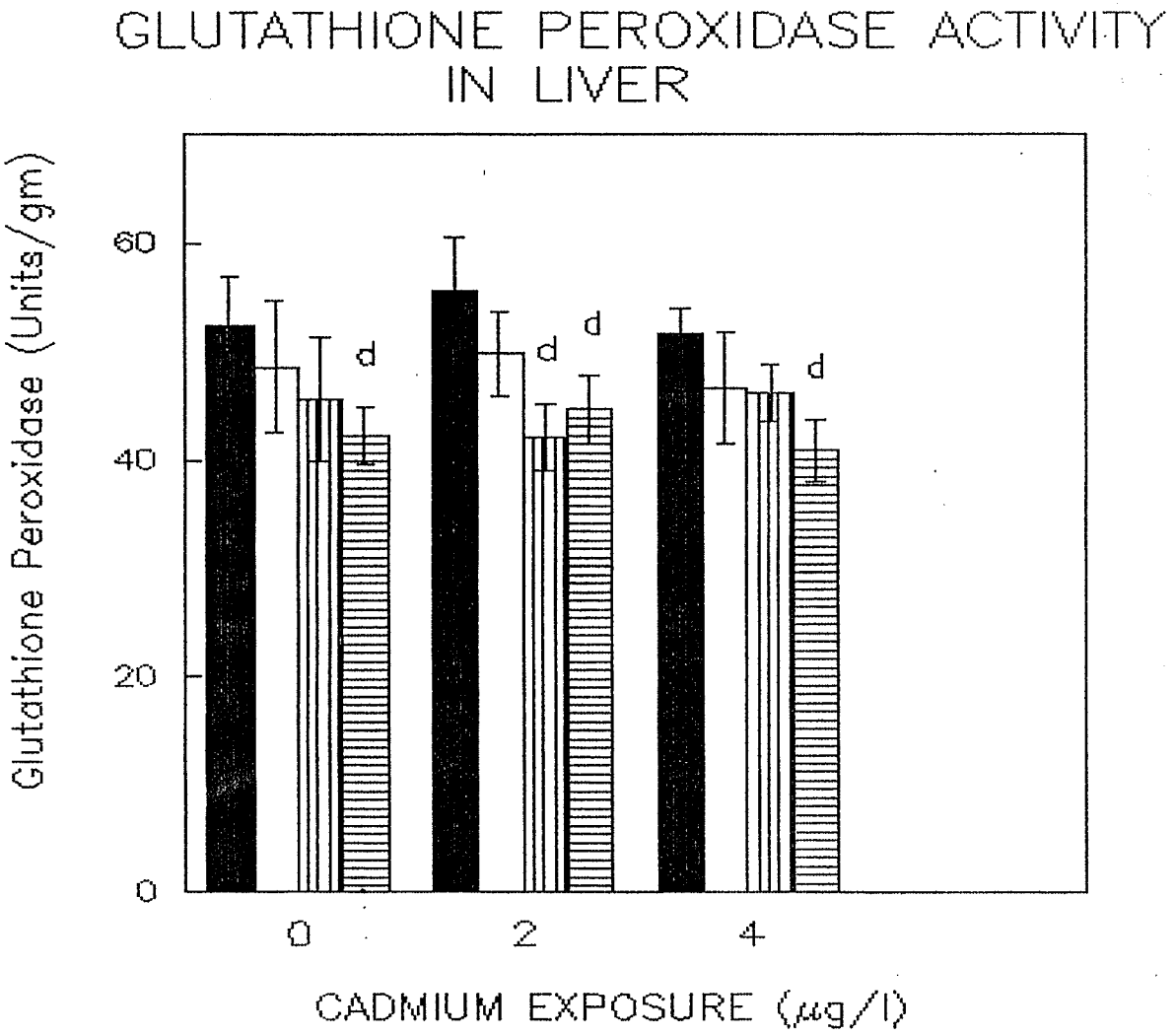
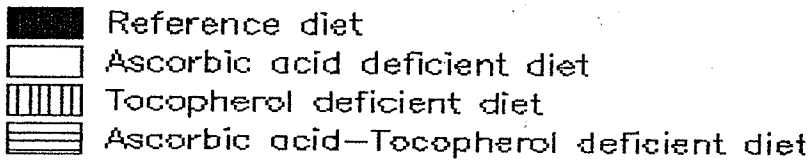

GLUTATHIONE PEROXIDASE ACTIVITY
IN LIVER
Glutathione Peroxidase (Units/gm)
60
40
20
0
d
d
d
d
0
2
4
CADMIUM EXPOSURE (μg/l)

Figure 7: Superoxide dismutase activity in liver. Data (X ± SE) are expressed as units/gm wet weight. One unit of superoxide dismutase inhibits autoxidation of epinephrine by 50%. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).



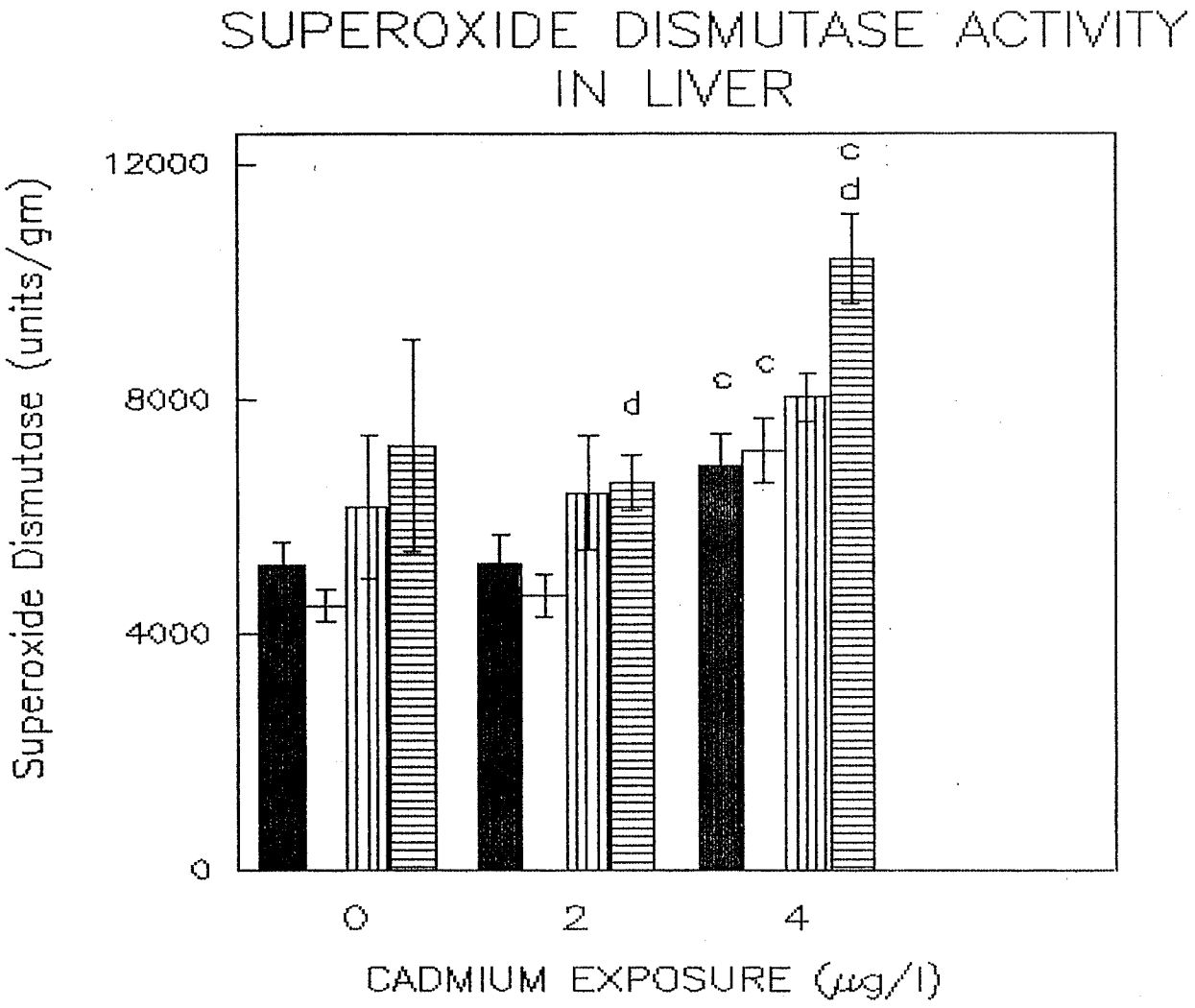
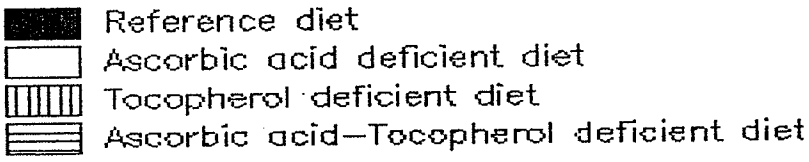

SUPEROXIDE DISMUTASE ACTIVITY
IN LIVER
Superoxide Dismutase (units/gm)
12000
8000
4000
0
c
d
d
c
c
0
2
4
CADMIUM EXPOSURE (µg/l)

Figure 8: Catalase activity in liver. Data (X ± SE) are expressed as units/gm wet weight. One unit of catalase decomposes 1.0 µmol H_2O_2 at pH=7.0, 25°C. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).



CATALASE ACTIVITY IN LIVER

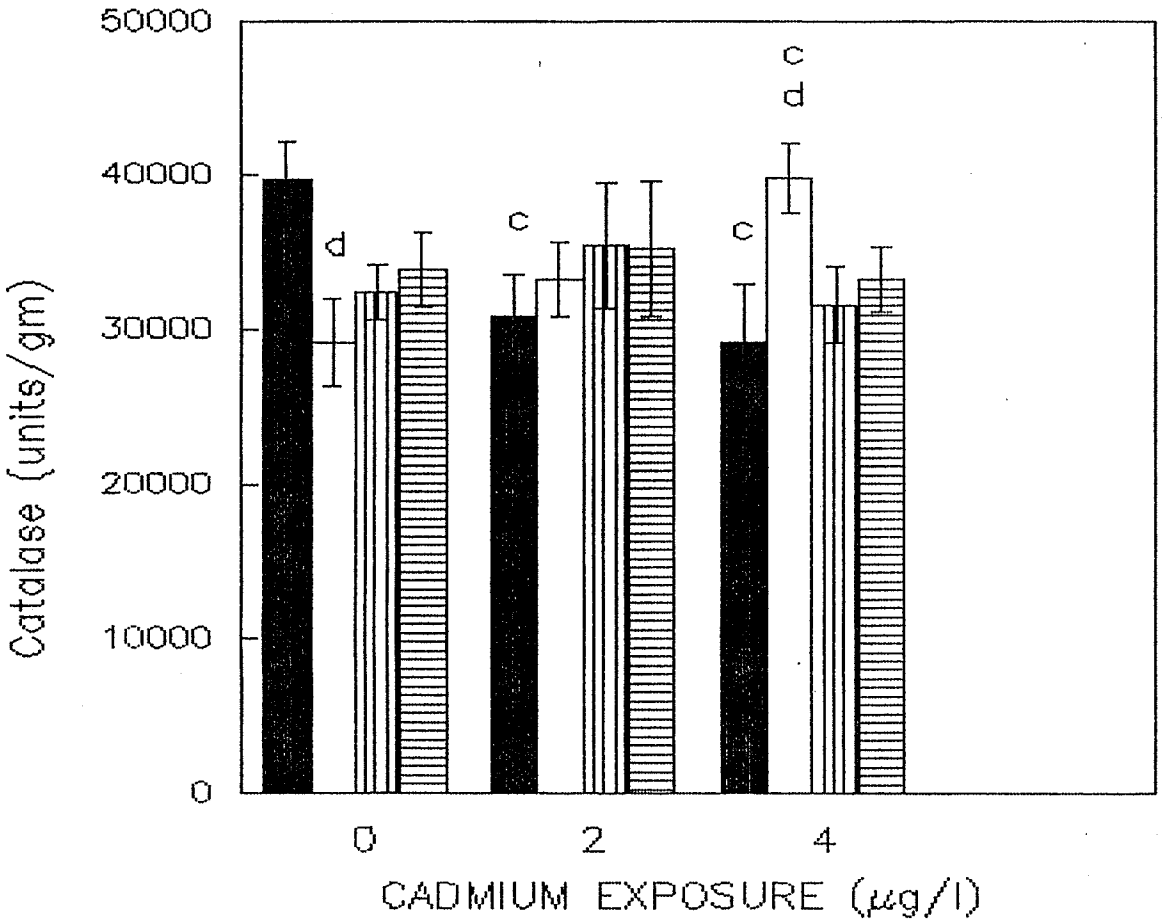
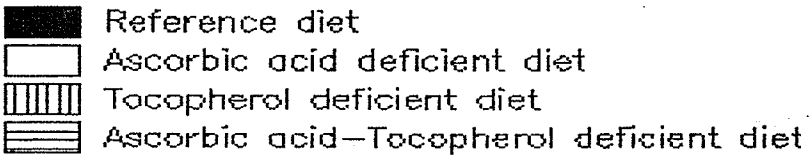
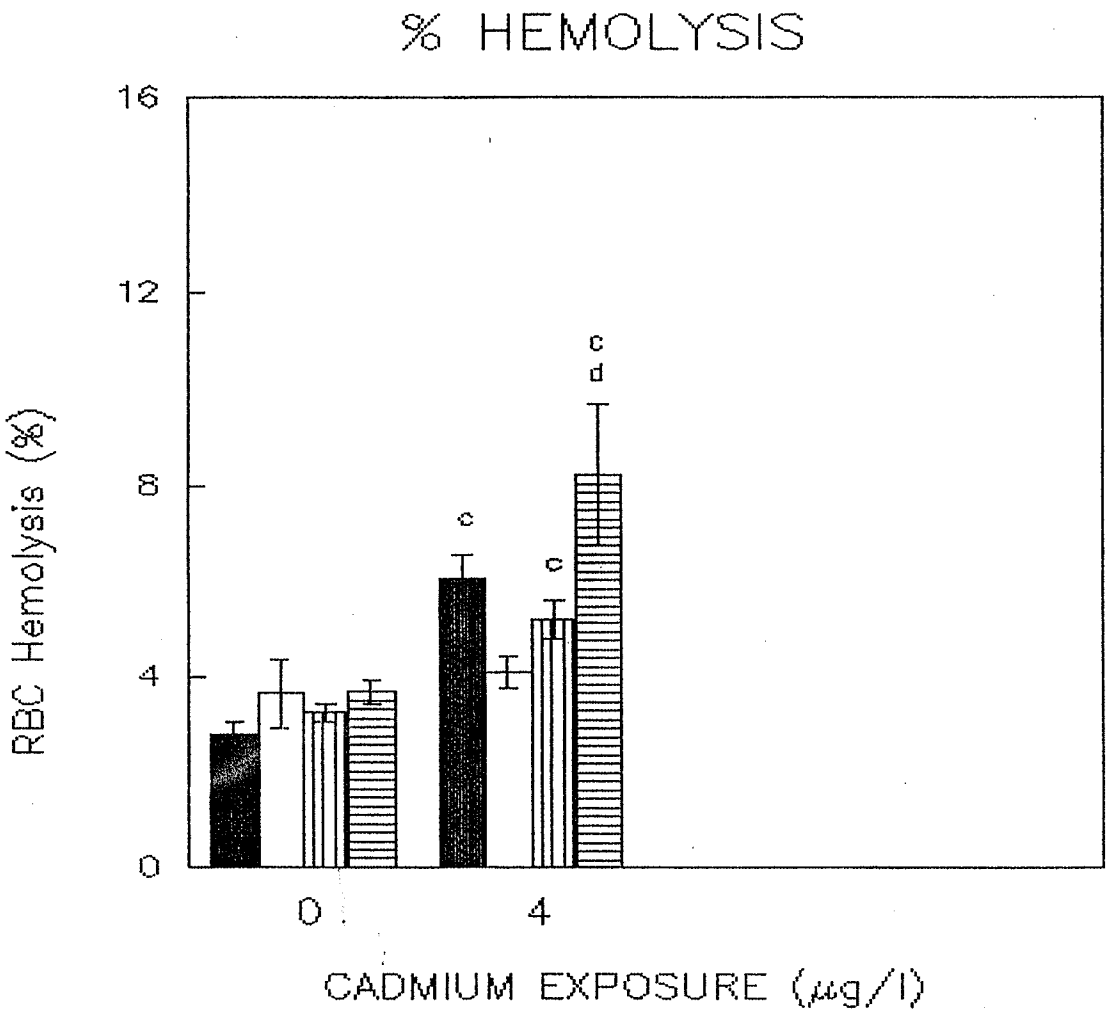


Figure 9: Spontaneous hemolysis of erythrocytes in physiological saline. Data (X ± SE) are expressed as a percentage of the total red blood cell population ,based on optical density, in 0.3 ml of whole blood. c= cadmium effects, ie. significant differences between groups receiving the same diet at different [Cd]. d= diet effects, ie. significant differences from the reference diet at the same [Cd]. (p=0.05, n=7).




% HEMOLYSIS
16
12
8
4
0
RBC Hemolysis (%)
c
d
c
c
0
4
CADMIUM EXPOSURE (µg/l)

CONCLUSIONS

The duration of storage and storage temperature of biological samples are especially important where lipid peroxidative indices are concerned. Andersen and Andersen (1988) report that mouse liver kept on ice will increase in lipid peroxidative metabolites by 25 to 50% in one hour. Whiteley et al (1988a) found lipid peroxidative breakdown of tissues to be significant at -20°C but not at -196°C, with further studies indicating that antioxidants present in stored tissues can alter the rate of this deterioration (Whiteley et al 1988b). Given this information, it is not surprising that recent studies monitoring enzymatic and non-enzymatic physiological parameters routinely use storage temperatures for tissues of -70°C (Salo et al 1990, Puntarulo and Cederbaum 1988) and lower (Dabrowski 1990). In addition to low temperature long-term storage, rapid excision and freezing of tissues is also a widely accepted practice (Forlin and Andersson 1985, Dabrowski et al 1990).

Data presented in Chapter 1 support the use of both rapid excision and low temperatures for long and short-term preservation of biochemical parameters. Concentration declines in higher storage temperatures were found for ascorbic acid, glycogen and acid-soluble thiols. Wedemeyer (1970, 1972, 1981) has compiled considerable data correlating changes of liver glycogen concentrations with general stress in fish. These findings along with the loss of glycogen in prolonged MS222 immersion found here, indicate that immediate dissection of tissues and storage at, or below, -72°C are critical.

Whereas low temperature storage of tissues used for enzymatic assays is standard practice, subsampling of organs is often not consistent. This is surprising, since several studies report non-homogeneous distributions of biochemical parameters in liver (Richards 1982, Sasse 1975, Smith et al 1979). Results from our studies indicate equal distribution of most enzymatic and non-

enzymatic factors, but not ascorbic acid and acid-soluble thiols. These two factors show an inverse relationship of high ascorbic acid and low acid-soluble thiols in quadrant 2 of the liver. This may indicate that ascorbic acid is required in glutathione (Deana et al 1975) or metallothionein synthesis (Onasaka et al 1987).

Having established that both low temperature storage and consistent subsampling of tissues are essential, variation of physiological parameters in natural fish populations is also an important consideration. Specifically, variation of antioxidant enzyme activities in fish reported by independent researchers can be large. This is compounded by non-standardized assay methods and different units of expression (See Appendices 1,2 and 3). Appendix 1 indicates that SOD, for example, is assayed by at least 7 popular methods, with various unit definitions being expressed either per gram of tissue or per mg of protein. Although Moss (1983) suggests enzyme activity be expressed as Units/mg of protein, seasonal variation (Claro 1983, Majewski unpubl. observ.) and effects of Cd on protein content in fish liver (Nriagu 1988), lead us to express our data as Units/gm of tissue.

By sampling fish from more than one reference lake, variation of antioxidant enzyme activity between seasons and species as well as between lakes could be investigated. Both SOD and CAT were found to vary seasonally while GPx did not. Glutathione peroxidase was, however, the only one of the 3 enzymes to show any significant interspecies variation, being $1\frac{1}{2}$ to 3 times higher in pearl dace than white sucker and lake charr. Comparing enzyme activities from fish sampled from different lakes at the same time, all three enzymes showed potential for differences of activity between 32 and 52%.

In addition to natural variations of antioxidant enzyme activity, low concentrations of Cd (<200ng Cd/l Canadian Water Quality Guideline) in Lake 382

may have been responsible for higher SOD activities in livers of fish from that lake. Catalase and GPx in fish from Lake 382 were unaffected. Surprisingly, metallothionein synthesis, which has been established as an early warning indicator of metal contamination (Nordberg 1989), was not induced in pearl dace from Lake 382, even though the concentration of Cd in liver was increased 6 fold during one Cd addition season. More study is needed to consider the interaction of physical and chemical properties of the environment with enzymatic and non-enzymatic antioxidants in biota. Specifically, the slight elevation of SOD activity in Lake 382 fish may indicate that toxicity threshold for Cd is being approached, and that biochemical and physiological alterations may occur with further exposure.

Considering the slight response of antioxidant enzymes to low concentrations of Cd, non-enzymatic antioxidants such as ascorbic acid, tocopherol and glutathione may be in sufficient cellular concentrations to alleviate the increased oxidative stress brought on by Cd exposure. Gardner and Fridovich (1987), in fact, suggest that the intracellular reducing equivalents exert transcriptional control on enzymatic antioxidants. Both ascorbic acid and tocopherol concentrations are reported to decrease when lipid peroxidation in liver increases (Thomas et al 1982, Maiorino et al 1989) and the elevation of SOD activity seen in Lake 382 fish may be prelude to a decline of activity as reported for fish exposed to other lipid peroxidative inducing xenobiotics (Matkovics et al 1984). The result would be a decline of cellular antioxidants and an increase of lipid peroxidation leading to disruption of cellular membranes and processes (Kappus 1985).

Confusion arising from reports of both induction (Roberts et al 1987) and inhibition (Koul et al 1988) of antioxidant enzyme activity following metal exposure may be accounted for by different cellular availability of alternative

antioxidants. Cells may maintain a certain level of antioxidant reducing power, and a decline in one factor may be compensated by an increase of another antioxidant (Canada and Calabrese 1989). In support of this interaction, Hill et al (1989) found induction of SOD in tocopherol deficient rats.

The experiment described in chapter 3 examined the interactions of enzymatic and non-enzymatic antioxidants, particularly biochemical responses to dietary deficiency of tocopherol and ascorbic acid. Ascorbic acid, soluble in the aqueous phase, forms an important link with tocopherol, soluble in the lipid phase, by a complex electron transport system that regenerates tocopherol's antioxidant capacity (Stocker and Peterhans 1989). As expected, the reduction of dietary ascorbic acid to simulate conditions of xenobiotic induced depletion, or dietary deficiency of this vitamin, was validated by a reduction of ascorbic acid in Cd exposed fish. This reduction was even greater when dietary tocopherol was absent, presumably because ascorbic acid was increasingly utilized as an antioxidant (Pentyuk et al 1989). In addition to the loss of antioxidant capacity as a result of ascorbic acid deficiency, the potential for lipid peroxidation is also increased by the enhancement of Cd uptake accompanying ascorbic acid deficiency (Rambeck et al 1988). This is supported by the high percentage of hemolysis of red blood cells observed in Cd exposed fish. Although tocopherol deficiency was achieved only with long dietary deficiency and Cd exposure, lipid peroxidation, measured by hemolysis, increased in its absence.

Having established that lipid peroxidation increases with Cd exposure and the reduction of certain cellular antioxidants, the ability of alternative antioxidants to ameliorate this adverse effect should be examined. While CAT activity declined only as a result of Cd exposure, GPx activity decreased only in tocopherol and ascorbic acid deficiently fed fish. Although these findings

are consistent with previous work (Koul et al 1988, Hill et al 1989), they would both tend to allow rather than antagonize lipid peroxidation in the liver. Alternatively, SOD activity was induced by both Cd exposure and dietary deficiency of ascorbic acid and tocopherol. Whether or not the increase of hepatic and renal acid-soluble thiols founds with Cd exposure contradicts the possibiity of glutathione exerting transcriptional control over Mn-SOD (Gardner and Fridovich 1987) is unclear, as only total SOD activity was measured (total SOD= Mn-SOD + Cu/Zn-SOD). Further study is required to examine interactions between Mn-SOD and Cu/Zn-SOD, and whether induction of one is able to compensate for loss of activity in the other (Canada and Calabrese 1989). For example, if glutathione were inhibiting Mn-SOD production, Cu/Zn-SOD may still be able to compensate and the net SOD activity could rise. Regardless of this relationship, the relevant point is that the increase of SOD activity and acid-soluble thiol concentration in liver would have acted against the proliferation of oxygen radicals capable of increasing lipid peroxidation (Hasssan 1988, Baysal et al 1988). Given the relatively short periods of Cd exposure studied as compared to chronic natural exposures, more study is required to consider whether these changes of cellular parameters are permanent or transient, and whether they are sufficient to prevent lipid peroxidation toxicity in the long term.

All three enzymatic antioxidants, SOD, CAT and GPx, have been identified as early indicators of pollutant mediated stress (Mather-Mihaich and DiGiulio 1986). Our studies indicate, however, that SOD is the most sensitive to increases of peroxidative breakdown due to Cd exposure and antioxidant depletion. Because of this, SOD may be a good indicator of stress produced by cadmium and possibly by other metals and xenobiotics (Neff 1985). Other parameters found to be sensitive to Cd stress were acid-soluble thiols,

hemolysis of red blood cells and to a lesser extent CAT activity. The relevance of enzymatic antioxidants as biochemical indicators is further strengthened by recent findings implicating lipid peroxidation as a mechanism of toxicity for organic pollutants (Mohammadpour et al 1988) and evidence that antioxidant enzyme activity may be affected (Shara and Stohs 1987). With sensitivity not limited to a single pollutant, antioxidant enzyme assays, particularly SOD, are applicable as biochemical indicators of stress in diverse environments where a number of pollutants are present.

APPENDIX 1: SUPEROXIDE DISMUTASE ACTIVITY IN FISH TISSUES

Species	Tissue	Method	Activity	Units and Reference
carp (*Cyprinus carpio*)	erythrocytes	nitroblue tetrazolium	823±19	Units/gm of Hb or wet tissue
	red muscle		100	1 Unit causes 50% inhibition
	white muscle		10	of luminescence due to the
	liver		569	accumulation of formazan
	spleen		83	(Mazeaud et al 1979)
	kidney		120	
	swim bladder		5	
	heart		50	
	gill		30	
	stomach		45	
	intestine		80	
	brain		30	
carp (*Cyprinus carpio*)	liver	inhibition of epinephrine autoxidation at 480nm	13.05 (±3.75)	Units/mg of protein
	kidney		15.16 (±3.79)	1 Unit causes 50% inhibition
	gill		17.10 (±1.80)	of autoxidation at 25°C, pH=10 (Radi et al 1987)
silver carp (*Carpiodes cyprinus*)	liver		14.47 (±0.18)	
	kidney		24.98 (±5.13)	
	gill		28.88 (±2.36)	
wels (*Siluris glanis*)	liver		60.70 (±3.61)	
	kidney		29.07 (±1.18)	
	gill		27.53 (±2.08)	

Appendix 1: cont.

Species	Tissue	Method	Activity	Units and Reference
saithe (Pollachius virens)	liver	inhibition of epinephrine autoxidation at 480nm	460	Units/gm wet tissue weight 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=10 (Aksnes and Njaa 1981)
	kidney		389	
	gill		152	
	white muscle		280	
mackerel (Scomber scombrus)	liver		716	
	kidney		459	
	gill		56	
	white muscle		300	
cod (Gadus morhua)	liver	inhibition of epinephrine autoxidation at 480nm	1928	Units/mg protein 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=10.2 (Bartkowiak et al 1981)
tench (Tinca tinca)	erythrocytes	inhibition of epinephrine autoxidation at 480nm	743	Units/gm of Hb 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=12 (Wdieczak et al 1981)
pike (Esox lucius)			557	
crucian carp (Carassius carassius)			817	
rainbow trout (Salmo gairdneri)	liver	inhibition of hydroxylamine oxidation to nitrites at 530nm	78.28 (±6.75)	Units/mg of protein 1 Unit inhibits maximum nitrite formation by 50% pH=7.8, room temp. (Desrochers and Hoffert 1983)
	brain		26.33 (±2.81)	
	retina		25.75 (±1.89)	
catfish (Ictalurus punctatus)	liver	inhibition of cytochrome c reduction at 480nm	488 (±53)	Units/gm of wet tissue 1 Unit causes 50% inhibition of reduction at 25°C, pH=7.8 (Gatlin and Wilson 1986)

Appendix 1: cont.

Species	Tissue	Method	Activity	Units and Reference
rainbow trout (*Salmo gairdneri*)	liver	inhibition of epinephrine autoxidation at 480nm	558.8 (±13.5)	Units/gm of wet tissue 1 Unit causes 50% inhibition of autoxidation at 20°C, pH=6.5 (Knox et al 1984)
feral spot (*Leistomus xanthurus*)	liver	chemilum-inescent technique	101.0 (±23.7)	Units/mg of protein (1 Unit inhibits chemiluminescence by 50% pH=7.8 (Roberts et al 1987)
carp (*Hypophthalmichths molitrix*)	liver gill muscle heart	inhibition of epinephrine autoxidation at 480nm	943.7 (±85.0) 320.3 (±31.0) 111.7 (±11.1) 127.4 (±11.3)	Units Mn-SOD/gm of wet tissue 1 Unit causes 50% inhibition of autoxidation at 30°C, pH=10.2 (Matkovics et al 1984)
catfish (*Ictalurus punctatus*)	liver gill	inhibition of cytochrome c reduction at 550nm	4.13 (±0.19) 1.33 (±0.05)	Units/mg of protein 1 Unit inhibits alkaline DMSO mediated cytochrome c reduction by 50% at 0°C, pH=9.5 (Mather-Mihaich and DiGiulio 1986)
rainbow trout (*Salmo gairdneri*)	liver	inhibition of 6-hydroxy-dopamine autoxidation at 490nm	661.5 (±18.1)	Units/gm of wet tissue 1 Unit causes 50% inhibition of autoxidation at 37°C, pH=6.5 (Knox et al 1982)

Appendix 1: cont.

Species	Tissue	Method	Activity	Units and Reference
scup (*Stenotomus chrysops*)	gas gland epithelium and rete mirabile	inhibition of cytochrome c reduction at 550nm	3.9 (±0.5)	Units/mg of protein 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=7.8 (Morris and Albright 1984)
seabass (*Centropristes striatus*)			2.6 (±0.2)	
tautog (*Tautoga onitis*)			5.2 (±1.0)	
sea robin (*Prionotus carolinus*)			6.7 (±0.5)	
toadfish (*Opsanus tau*)			9.9 (±0.7)	
eel (*Anguilla rostrata*)			8.6 (±0.7)	
rainbow trout (*Salmo gairdneri*)	brain	inhibition of superoxide generation by NADH in the presence of O_2 and subsequent reduction of NBT by superoxide at 550nm	14.4 to 16.0	Units/mg of protein 1 Unit causes 50% inhibition of NBT-NADH reaction at pH=8.3 (Van Balgooy and Roberts 1979)
carp (*Cyprinus carpio*)	erythrocytes	inhibition of epinephrine autoxidation at 480nm	801 (±132)	Units/gm of Hb 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=10.2 (Gabryelak et al 1983)
crucian carp (*Carassius carassius*)			1435 (±277)	
tench (*Tinca tinca*)			1072 (±172)	

Appendix 1: cont.

Species	Tissue	Method	Activity	Units and Reference
cod (Notothenia gibberifrons)	liver	inhibition of epinephrine autoxidation at 480nm	1600.4 (±69.3)	Units/gm of wet tissue 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=10.2 (Witas et al 1984)
cod (Notothenia coriicepsneglecta)			1707.5 (±118.7)	
cod (Notothenia nybelini)			1671.3 (±101.4)	
cod (Chaenocephalus aceratus)			263.1 (±28.8)	
carp (Cyrpinus carpio)			694.1 (±46.0)	
tench (Tinca tinca)			679.3 (±69.2)	
crucian carp (Carassius carassius)			669.1 (±88.8)	
toadfish (Opsanus tau)	gill	inhibition of cytochrome c reduction at 550nm	4.5 (±0.4)	Units/mg of protein 1 Unit causes 50% inhibition of cytochrome c reduction at 25°C, pH=7.8 (Morris and Albright 1981)
	kidney		4.8 (±0.3)	
	heart		6.3 (±0.2)	
	blood		0.6 (±0.02)	
	gas gland		11.2 (±1.0)	
	rete mirabile		8.4 (±0.7)	
rainbow trout (Salmo gairdneri)	liver	inhibition of epinephrine autoxidation at 480nm	798.9 (±25.9)	Units/gm of wet tissue 1 Unit causes 50% inhibition of autoxidation at 25°C, pH=7.8 (Knox et al 1981)
	heart		54.7 (±5.8)	

Appendix 1: cont.

Species	Tissue	Method	Activity	Units and Reference
plaice (*Pleuronectes platessa*)	liver	inhibition of 6-hydroxydopamine oxidation at 490nm	190.1 (±12.1)	Unit/gm of wet tissue
	kidney		95.7 (±6.5)	1 Unit causes 50% inhibition of autoxidation at 25°C, pH=6.5 (Syed and Coombs 1982)
	heart		61.6 (±2.4)	
	brain		64.8 (±2.6)	
	spleen		48.5 (±1.7)	
	muscle		22.6 (±4.6)	
	gills		10.1 (±0.7)	

APPENDIX 2: CATALASE ACTIVITY IN FISH TISSUES

Species	Tissue	Method	Activity	Units and Reference
catfish (*Ictalurus punctatus*)	liver	decrease of H_2O_2 at 240nm	14.46 (±0.37)	Bergmeyer Units/mg of protein 1 B.U. decomposes 1000mg H_2O_2 in one minute 25°C, pH=7.0 (Mather-Mihaich and DiGiuilio 1986)
	gill		0.56 (±0.04)	
tilapia (*Sarotherodon mossambicus*)	liver	decrease of H_2O_2 at 240nm	1.39 (±0.25)	Bergmeyer Units/mg of protein 1 B.U. decomposes 1000mg H_2O_2 in one minute 25°C, pH=7.0 (Singh and Savilingam 1982)
scup (*Stonotomus chrysops*)	gas gland and rete mirabile	decrease of H_2O_2 at 240nm	5.3 (±0.6)	Units/mg of protein 1 Unit degrades 1 µmole H_2O_2 per minute 26°C, pH=7.0 (Morris and Albright 1984)
seabass (*Centropristes striatus*)			5.7	
tautog (*Tautoga onitis*)			10.6	
searobin (*Prionotus carolinus*)			4.5 (±1.0)	
toadfish (*Opsanus tau*)			14.1 (±1.3)	
eel (*Anguilla rostrata*)			3.5	
killifish (*Fundulus heteroclitus*)	liver	decrease of H_2O_2 at 240nm	0.04 to 0.1	Specific activity Units/mg of protein (calculated from the change of optical density over a 25 second interval room temp.,pH=7.0 (Pruell and Engelhardt 1980)

Appendix 2: cont.

Species	Tissue	Method	Activity	Units and Reference
rainbow trout (*Salmo gairdneri*)	whole blood	decrease of H_2O_2 at 240nm	88.40 (±5.07)	Given as K (rate constant)/gm of Hb $K = 1/time\ (\ln S_1/S_2)$ where S_1=concentration of H_2O_2 at time 1 and S_2 is concentration at time 2 pH=7.0 (Arillo et al 1984)
killifish (*Fundulus heteroclitus*)	liver	decrease of H_2O_2 at 240nm	0.173	Given as the change of optical density /mg of protein over a 24 second interval 26°C, pH=7.0 (Jackim et al 1970)
milkfish (*Chanos chanos*)	erythrocytes	decrease of H_2O_2 at 240nm	0.0	Given as K, the rate constant 25°C,pH=7.0 (Smith 1976)
palani (*Acanthurus dussumieri*)			0.01	
naso (*Naso lituratus*)			0.02	
butterfly fish (*Chaetodon carassius*)			0.05	
carp (*Cyprinus carpio*)	erythrocytes	decrease of H_2O_2 at 240nm	4.6 (±1.5)	Bergmeyer Units/gm of Hb 1 B.U. decomposes 1000mg of H_2O_2 in one minute 25°C,pH=7.0 (Gabryelak 1983)
tench (*Tinca tinca*)			6.1 (±1.8)	
crucian carp (*Carassius carassius*)			7.8 (±0.5)	

Appendix 2: cont.

Species	Tissue	Method	Activity	Units and Reference
cod ice fish (*Notothenia gibberifrons*)	liver	decrease of H_2O_2 at 240nm	0.71 (±0.10)	Bergmeyer Units/gm of wet tissue 1 B.U. decomposes 1000mg of H_2O_2/min 25°C, pH=7.0 (Witas et al 1984)
cod ice fish (*Notothenia coriicepsneglecta*)			0.65 (±0.07)	
cod ice fish (*Notothenia nybelini*)			1.22 (±0.43)	
cod ice fish (*Chaenocephalus aceratus*)			0.72 (±0.10)	
carp (*Cyprinus carpio*)			1.73 (±0.19)	
tench (*Tinca tinca*)			1.36 (±0.28)	
crucian carp (*Carassius carassius*)			1.38 (±0.25)	
catfish (*Heterpneustes fossilis*)	liver	manometric	769 (±63)	Given as ml of O_2 evolved/mg tissue/minute (Thomas and Murthy 1978)
	kidney		207 (±22)	
dace (*Phoxinus phoxinus*)	erythrocytes	decrease of H_2O_2 at 240nm	0.77 (±0.08)	Given as the catalase number (the quantity of blood in mg required to disintegrate 1 µmol H_2O_2) 4°C, pH=7.4 (Kolupaev and Putintseva 1984)
Pacific mackerel (*Scomber japonicus*)	muscle	decrease of H_2O_2 at 240nm	2.5	Given as Relative Specific Activity (% activity in sub-cellular fraction/ total protein in that fraction (Ueno et al 1986)

Appendix 2: cont.

Species	Tissue	Method	Activity	Units and Reference
carp (*Hypopthalmichthyes molitrix*)	liver	decrease of H_2O_2 at 240nm	0.66 (±0.05)	Bergmeyer Units/gm wet tissue
	gill		0.012 (±0.001)	1 B.U. decomposes 1000mg H_2O_2 in one minute
	muscle		0.003 (±0.0002)	25°C, pH=7.0
	heart		0.105 (±0.0011)	(Matkovics et al 1984)
toadfish (*Opsanus tau*)	gill	decrease of H_2O_2 at 240nm	19.8 (±1)	Units/mg of protein
	kidney		147 (±11)	1 unit degrades 1 µmol of H_2O_2
	heart		15.9 (±1)	in one minute 25°C, pH=7.0
	blood		2.3 (±0.7)	(Morris and Albright 1981)
	gas gland		8.3 (±0.6)	
	rete mirabile		19.1 (±1.5)	
carp (*Cyprinus carpio*)	erythrocytes	luminescent technique	341 (±8)	Given as µgm of catalase/gm of Hb (Mazeaud et al 1979)
perch (*Perca flavescens*)	swim bladder	manometric	7	Given as O_2 evolved in cu mm/mg wet tissue/minute 10-12°C, pH=6.8 (Black 1946)
	liver		108	
	muscle		1.3	
	brain		4.0	
tilapia (*Tilapia nilotica*)	erythrocytes	reaction of residual H_2O_2 (after enzyme incubation) with $KMnO_4$ at 480nm	0.25	Given as the rate constant K 0-4°C, pH=7.0 (Rabie et al 1972)
clarius (*Clarius lazera*)			0.015	
synodontis (*Synodontis shall*)			0.01	

Appendix 2: cont.

Species	Tissue	Method	Activity	Units and Reference
saithe (*Pollachius virens*)	liver	decrease in H_2O_2 at 240nm	6135	Given as µmoles H_2O_2 degraded/gm wet tissue (Aksnes and Njaa 1981)
	kidney		1747	
	gill		711	
	white muscle		717	
	red muscle		725	
	spleen		2529	
	heart		1608	
	intestine		1251	
	brain		376	
	swim bladder		703	
	roe		1155	
mackerel (*Scomber scomber*)	liver		9003	
	kidney		2314	
	white muscle		930	
	red muscle		788	
	spleen		3052	
	heart		1744	
	intestine		1166	
	roe		299	
	milt		1767	
cod (*Gadus morhua*)	whole body		386	
capelin (*Mallotus villosus*)			424	
rainbow trout (*Salmo gairdneri*)			592	

Appendix 2: cont.

Species	Tissue	Method	Activity	Units and Reference
sprat (Sprattus sprattus)	continued from previous page		616	
Norway pout (Boreogadus esmarkii)			1057	
blue whiting (Micromesistius poutassau)			801	
silver smelt (Argentina silus)			501	
tench (Tinca tinca)	erythrocytes	decrease of H_2O_2 at 240nm	6.1	Bergmeyer Units/gm of Hb 1 B.U. decomposes 1000mg of H_2O_2 in one minute 25°C, pH=7.0 (Wdzieczak et al 1981)
pike (Esox lucius)			7.1	
crucian carp (Carassius carassius)			8.6	
carp (Cyprinus carpio)	liver	decrease of H_2O_2 at 240nm	0.008 (±0.0001)	Bergmeyer Units/mg of protein 1 B.U. decomposes 1000mg of H_2O_2 in one minute 25°C, pH=7.0 (Radi et al 1987)
	kidney		0.001 (±0.0001)	
	gill		0.0002	
silver carp (Carpiodes cyprinus)	liver		0.0028 (±0.0003)	
	kidney		0.0005	
	gill		0.0005	
wels (Siluris glanis)	liver		0.081 (±0.01)	
	kidney		0.0065 (±0.001)	
	gill		0.0057 (±0.0001)	

APPENDIX 3: GLUTATHIONE PEROXIDASE ACTIVITY IN FISH TISSUES

Species	Tissue	Method	Activity	Units and Reference
carp (*Hypophthalmichthys molitrix*)	liver	H_2O_2 innitiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	0.047 (±0.002)	Given as µmoles GSH consumed/min/gm of wet tissue 20°C, pH=7.4 (Matkovics et al 1984)
	gill		0.059 (±0.004)	
	muscle		0.014 (±0.001)	
	heart		0.021 (±0.003)	
rainbow trout (*Salmo gairdneri*)	plasma	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	57.35 (±4.74)	Given as nanomoles of NADPH oxidized/min/ mg of protein or ml of plasma 20°C; pH=7.4 (Bell et al 1986)
	liver		2.50 (±0.68)	
toadfish (*Opsanus tau*)	gill	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	32.4 (±2.9)	Given as nanomoles of NADPH oxidized/min/ mg of protein 25°C, pH=7.0 (Morris and Albright 1981)
	kidney		33.4 (±2.4)	
	heart		9.4 (±1.4)	
	blood		1.9 (±0.2)	
	gas gland		9.8 (±0.4)	
	rete mirabile		2.6 (±0.7)	
mackerel (*Scomber scombrus*)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	1.02	Given as µmoles of NADPH oxidized/min/gm of wet tissue 20°C, pH=7.4 Aksnes and Njaa 1981
	kidney		4.10	
	gill		0.46	
	white muscle		<0.03	

Appendix 3: cont.

Species	Tissue	Method	Activity	Units and Reference
scup (*Stonotomus crysops*)	gas gland and rete mirabile	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	12.9	Given as nanomoles of NADPH oxidized/min/mg of protein 20°C, pH=7.0 (Morris and Albright 1984)
sea bass (*Centropristes striatus*)			10.1	
tautog (*Tautoga onitis*)			7.5	
sea robin (*Prionotus carolinus*)			19.9 (±2.5)	
toadfish (*Opsanus tau*)			11.3 (±0.6)	
eel (*Anguilla rostrata*)			18.2 (±2.8)	
trout (*Percosidae* ?)	blood	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	0.0	Given as µmoles of NADPH oxidized/min/mg of protein 20°C, pH=7.4 (Smith and Shrift 1979)
	muscle		0.046	
	liver		0.032	
goldfish (*Carassius auratus*)	blood		0.067	
killifish (*Fundulus* ?)	blood		0.044	
sunfish (*Centrarchidae*)	blood		0.046	
	muscle		0.010	
	liver		0.280	
blue gourami (*Trichogaster trichopterus*)	blood		0.115	
	muscle		0.080	
	ovary		0.121	

Appendix 3: cont.

Species	Tissue	Method	Activity	Units and Reference
carp (*Cyprinus carpio*)	erythrocytes	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	225 (±6)	Given as µmoles of NADPH oxidized/min/gm of wet tissue or gm of Hb 20°C, pH=7.0 (Mazeaud et al 1979)
	red muscle		2	
	white muscle		22	
	liver		22	
	spleen		16	
	kidney		5	
	swim bladder		0	
	heart		4	
	gill		4	
	stomach		5	
	intestine		4.5	
	brain		1	
rainbow trout (*Salmo gairdneri*)	blood	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	1.13 (±0.25)	Given as nanomoles of NADPH oxidized/min/mg of protein 20°C, pH=7.4 (Hilton et al 1980)
catfish (*Ictalurus punctatus*)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	214.40 (±20.00)	Given as units/mg of protein 1 Unit liberates half the peroxide O_2 from H_2O_2 20°C, pH=7.4 (Mather-Mihaich and DiGiulio 1986)
	gill		4.76 (±0.28)	

Appendix 3: cont.

Species	Tissue	Method	Activity	Units and Reference
carp (Cyprinus carpio)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	0.63 (±0.07)	Given as µmoles of NADPH oxidized/min/mg of protein 20°C, pH=7.4 (Radi et al 1987)
	kidney		0.094 (±0.005)	
	gill		1.16 (±0.23)	
silver carp (Carpiodes cyprinus)	liver		3.02 (±0.19)	
	kidney		0.54 (±0.032)	
	gill		1.95 (±0.20)	
wels (Siluris glanis)	liver		2.71 (±0.48)	
	kidney		1.05 (±0.10)	
	gill		2.47 (±0.22)	
rainbow trout (Salmo gairdneri)	liver	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	19	Given as µmoles of NADPH oxidized/min/mg of protein 20°C, pH=7.4 (Walton et al 1982)
Atlantic salmon (Salmo salar)	plasma	colorimetric degradation of GSH reacted with DTNB at 412nm	7.09	Units/ml of plasma 1 Unit decreases the concentration of reduced glutathione by 0.1 log unit in one minute 25°C (Poston et al 1976)
catfish (Ictalurus punctatus)	plasma	H_2O_2 initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	0.2	Given as µmoles of NADPH oxidized/min/ml room temp., pH=7.0 (Lance and Elsey 1983)
	erythrocytes		200	

Apppendix 3: cont.

Species	Tissue	Method	Activity	Units and Reference
black bullhead (Ictalurus melas)	blood	colorimetric degradation of GSH reacted with DTNB at 412nm	31.98 (±6.44)	Units/mg of protein or mg of Hb
	liver		17.30 (±4.30)	1 Unit =1/time (log 100/X) where X = % of GSH remaining after enzymatic action 25°C (Heisinger and Dawson 1983)
Atlantic salmon (Salmop salar)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	20.51 (±2.23)	Given as nanomoles of NADPH oxidized/min/mg of protein or ml of plasma 20°C, pH=7.4 (Bell et al 1987)
	plasma		235.77 (±40.56)	
rainbow trout (Salmo gairdneri)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	0.9	Given as nanomoles of NADPH oxidized/min/mg of protein 20°C, pH=7.4 (Tappel et al 1982)
	kidney		2.3	
	heart		3.2	
	gill		1.7	
	intestine		0.0	
	stomach		0.0	
brook trout (Salvelinus fontinalis)	liver		0.9	
	kidney		0.9	
	heart		2.3	
	gill		2.7	
	intestine		0.0	
	stomach		0.0	

Appendix 3: cont.

Species	Tissue	Method	Activity	Units and Reference
bluegill sunfish (Lepomis macrochirus)	liver	continued from previous page	3.4	
	heart		0.6	
	gill		1.6	
	intestine		3.4	
	stomach		4.7	
carp (Cyprinus carpio)	liver		143	
	heart		8	
	gill		4	
	intestine		30	
	stomach		14	
plaice (Pleuronectes platessa)	liver	cumene hydroperoxide initiated reaction of EDTA, NADPH, GSH and glutathione reductase at 340nm	2.7 to 3.6	Given as µmoles of NADPH oxidized/min/gm of wet tissue 20°C, pH=7.4 (George and Young 1986)

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