



## CHANGES IN LIPID PEROXIDATION, SUPEROXIDE DISMUTASE ACTIVITY, ASCORBIC ACID AND PHOSPHOLIPID CONTENT IN LIVER OF FRESHWATER CATFISH *HETEROPNEUSTES FOSSILIS* EXPOSED TO ELEVATED TEMPERATURE

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**Abstract**—1. Lipid peroxidation, superoxide dismutase (SOD) activity, ascorbic acid (AsA) and individual phospholipid contents in liver of fresh water cat fish *Heteropneustes fossilis* were measured after exposure to different temperatures (25, 27, 32, 37°C) at various times (1–4 h).

2. Lipid peroxidation and superoxide dismutase activity were significantly increased with increases in temperature at various times.

3. Ascorbic acid content was depleted when temperature was increased.

4. After temperature exposure, phosphatidyl inositol was increased while phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine were depleted. Phosphatidic acid level did not change.

5. The findings indicated an increased oxidative stress in liver following increases in temperature at various times. Concurrent with the increase in lipid peroxidation, superoxide dismutase activity and ascorbic acid from the liver of fish varied. It is suggested that depletion of major individual phospholipids following temperature exposure could be due to superoxide created oxidative stress in the liver. Copyright © 1996 Elsevier Science Ltd.

**Key Word Index:** Lipid peroxidation; superoxide dismutase; ascorbic acid; phospholipid; temperature; oxidative damage; *Heteropneustes fossilis*

### INTRODUCTION

Liver is the most important organ in which oxidative reactions involving electron transfer and activation of molecular oxygen can result in the production of reactive oxygen species (ROS) via various unwanted enzymic side-reactions, involving the oxidase activity of cytochrome P<sub>450s</sub> producing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Premereur *et al.*, 1986). There is an increasing use of subcellular biochemical indicators to predict stresses arising from chronic exposure to a given toxin (Larsson *et al.*, 1985). The earliest biological indicators of toxicant-induced stress are usually shifts in metabolite concentrations or changes in other biochemical system (Nriagu, 1988). Changes in superoxide dismutase (SOD; EC 1. 15.1.1), catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GPx; EC 1.11.1.9) protective mechanisms have been identified as early indications of cellular susceptibility to oxidant injury caused by superoxide anion (O<sub>2</sub><sup>-</sup>)

and other oxygen radicals such as hydroxyl radical (OH) (Ardelt *et al.*, 1989).

Superoxide is generated from normal metabolic processes in all oxygen-utilizing organisms. The harmful effects of O<sub>2</sub><sup>-</sup> and other ROS, such as H<sub>2</sub>O<sub>2</sub> and OH include lipid peroxidation, cross-linking and inactivation of proteins, DNA and RNA breaks and cell death (Halliwell and Gutteridge, 1984; Hu *et al.*, 1995; Wiseman and Halliwell, 1996). Phospholipid components of cellular membranes are highly vulnerable targets of ROS due to susceptibility of their polyunsaturated fatty acids (PUFA). Lipid peroxidation has received increasing attention as a mechanism of toxicity for a variety of organic and inorganic environmental pollutants (Gutteridge and Halliwell, 1990). The actual initiating species for the process in membranes is thought to be the 'OH, which arises most often through the interaction of other ROS such as H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> with transition metal ions such as Cu<sup>2+</sup> or Fe<sup>2+</sup> (Gutteridge and Halliwell, 1990; Halliwell and Cross, 1994). However, molecular damage imposed by these ROS is largely dependent on the activity of cellular antioxidants,

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including SOD, GPx and CAT (Misra *et al.*, 1990). The effectiveness of the antioxidant defence system in relation to the lipid peroxidation process is of particular interest in the case of aquatic animals that show seasonal metabolic variations related to fluctuation of environmental factors, including temperature and the physiological status of the animal. SOD works in concert with other antioxidants to remove  $O_2^-$ , a common by-product of oxygen metabolism from cells, that can play a role in membrane degradation (Hassan, 1988). Some nonenzymatic antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E) and carotenoids (vitamin A) have long been linked as essential factors in the amelioration of some of the toxic effects of ROS (Meister and Anderson, 1983; Burton and Ingold, 1989; Krinsky, 1989; Geesin *et al.*, 1990; Aten *et al.*, 1992; Chen and Tappel, 1995).

Similar to SOD and other antioxidant enzymes, the activities of these antioxidants may be altered during oxidative stress. The liver is the major site of uptake and detoxication of natural and pollutant foreign organic chemicals, principally by oxidative reactions followed by conjugation to water-soluble excretable products (Lemaire *et al.*, 1993). The literature on oxidative damage and relationship of antioxidants to temperature exposure in fish liver is scant (Winston and DiGiulio, 1991; Lemaire and Livingstone, 1993). Recently we have demonstrated a temperature dependent increase in lipid peroxidation and decrease in AsA content in respiratory organs of *Heteropneustes fossilis* (Parihar and Dubey, 1995). To establish the variability of lipid peroxidation, SOD activity, AsA and phospholipid contents in relation to temperature exposure for varying periods, the liver from freshwater catfish *Heteropneustes fossilis* was analysed.

## MATERIALS AND METHODS

### Reagents

Thiobarbituric acid (TBA), sodium hydrogen phosphate, ( $Na_2HPO_4$ ) and Pyrogallol were purchased from Loba Chemi (India). Trizma base, Trizma HCl, Triton X-100 and diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma Chemical Company Inc. U.S.A. Other chemicals were of analytical grade.

### Animal and experimental design

Adult freshwater catfish *H. fossilis* (weight  $50 \pm 2.5$  g; length  $19 \pm 1.3$  cm) were obtained from the vicinity of Ujjain, India. The ambient temperature at which the fish were collected was  $24 \pm 1^\circ C$ .

They were acclimatized in laboratory conditions for 2 weeks in dechlorinated tap water (temperature  $25^\circ C$ ). Mixed-sex fish were used for the experiment. Fish were fed with a commercially available pellet containing dried proteins, carbohydrates, vitamins (0.25%) and minerals every day. A photoperiod 12 h light:12 h dark was maintained. After acclimatization, fish were then transferred to separate exposure glass aquaria. Four groups of ten fish were maintained. The first aquarium was kept as a control. The second, third and fourth aquaria were used for the temperature exposure.

### Temperature exposure

The temperature was raised from  $25^\circ C$  (control) to 27, 32 and  $37^\circ C$  using a thermoregulated water heater. The animal was held during this process for periods of 1, 2, 3 and 4 h. The elevated temperature was reached to required levels within 10–15 min and then maintained for varying periods of 1–4 h.

### Tissue homogenization

The animals were killed by a blow to the head, and the liver dissected out. Particular care was taken to minimize disturbance of fish in the other aquaria. The preweighed tissue was homogenized in a Polytron homogenizer at a speed of 13,000 rpm. The buffers for homogenization of tissue were: 50 mM sodium phosphate buffer (pH 7.4) for lipid peroxide assay; chilled 50 mM Tris-HCl (pH 8.2) containing 1 mM DTPA for SOD activity assay and ice cold 0.25 M perchloric acid for AsA assay.

### Lipid peroxide assay

Homogenate was centrifuged at 3000 rpm for 15 min and the supernatant was used for lipid peroxide assay. Lipid peroxide was measured by the thiobarbituric acid test (Ohkawa *et al.*, 1979) in terms of malonaldehyde equivalents (MDA) using a molar extinction coefficient of  $1.56 \times 10^5$  min/cm. Briefly, samples of 0.1 ml homogenate were taken and mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% glacial acetic acid (pH 3.5 adjusted with 1 N NaOH) and 1.5 ml of 0.8% thiobarbituric acid. The mixture was heated at  $95^\circ C$  for 1 h on a water bath and cooled under tap water before mixing 1 ml of distilled water and 5 ml mixture of *n*-butanol and pyridine (15:1 v/v). The mixture was shaken vigorously and centrifuged at 2200 g for 5 min. The amount of MDA formed was measured by the absorbance of upper organic layer at 532 nm using appropriate controls. The results were expressed as nmol MDA/mg protein. Thiobarbituric acid assay is a generally used method for the detection of lipid peroxidation but is limited because other reactive aldehydes may not be



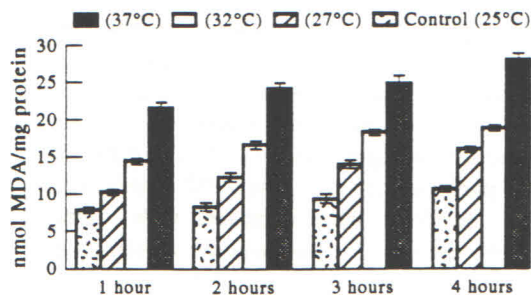


Fig. 1. Changes in lipid peroxidation in liver of *H. fossilis* to temperature exposure from 25 to 37°C at various times. Values are mean  $\pm$  SE.

detected, and the malonaldehyde produced *in vivo* may react with other cellular constituents (Kosugi and Kikugawa, 1989; Janero, 1990; Valenzuela, 1991).

#### Superoxide dismutase (SOD) activity assay

The homogenate for SOD activity assay was treated with triton-X-100 (1% v/v) for 30 min and then centrifuged at 15,000 rpm at 4°C in a Sorvall OTD 65B Ultracentrifuge. The supernatant was subjected to SOD activity assay by the method of Marklund and Marklund (1974), which involves the ability of an enzyme to inhibit autooxidation of pyrogallol. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autooxidation per 3 ml of assay mixture. The enzyme kinetics was carried out at 25°C (room temperature) in a Perkin Elmer U.V. Spectrophotometer. Calculations were made as units per milligram fresh weight of tissue.

#### Ascorbic acid (ASA) assay

The homogenate for AsA assay was centrifuged at 3000 rpm for 15 min and the supernatant was assayed for AsA by the modification of the dinitrophenylhydrazine (DNPH) technique of Terada *et al.* (1978) described by Thomas *et al.* (1982). DNPH was prepared by adding 2 g of 2-4-DNPH in 100 ml of 9 N H<sub>2</sub>SO<sub>4</sub>. O.D. was recorded at 540 nm.

#### Protein assay

Total protein content was determined by the Folin-phenol reaction as described by Lowry *et al.* (1951) using bovine serum albumin as a standard.

#### Thin layer chromatography and phospholipids estimation

Total lipids were extracted from preweighed liver tissue with a mixture of chloroform:methanol (2:1 v/v) by the method of Folch *et al.* (1957). After centrifugation, the phospholipids present in the

bottom chloroform layer were precipitated with cold acetone as described earlier (Parihar, 1987). Individual phospholipids were separated by thin layer chromatography (TLC) on silica gel G (Merk) using solvent chloroform: methanol: ammonia: water (75:30:4:0.5 v/v/v/v). Individual phospholipids were visualized in iodine vapour and identified by comparing their R<sub>f</sub> values with that of authentic reference standard. Separated spots were scraped from the TLC plates and digested in perchloric acid. The charred material was oxidized with 30% H<sub>2</sub>O<sub>2</sub> and neutralized with NaOH. Phospholipid phosphorus was quantified by the phosphorous analysis as described by Fiske and Subbarow (1925). Phospholipid phosphorous value may be converted into phospholipid concentrations by multiplying by a factor 25 (Sunderman and Sunderman, 1960).

#### Statistical analysis

All data are expressed as means  $\pm$  SEM. Statistical comparisons were made relative to the appropriate control group by Student's *t*-test and analysis of variance. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

## RESULTS

Changes in lipid peroxidation, SOD activity and AsA content in liver after temperature exposure are shown in Figs 1–3. Figure 1 shows the levels of lipid peroxidation (in terms of MDA/mg protein) after exposure to temperatures at various times. The MDA levels were significantly increased in liver with the increase in temperature from 25°C (control) to 37°C. Exposure of each temperature was extended for periods of 1–4 h, which resulted in a significant increase in MDA.

Data for SOD activity after temperature exposures at various times are shown in Fig. 2. Mean SOD

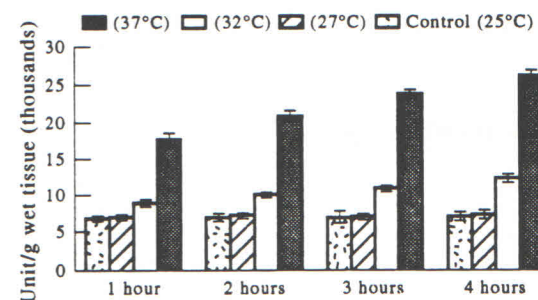


Fig. 2. Changes in superoxide dismutase activity in liver of *H. fossilis* to temperature exposure from 25 to 37°C at various times. Values are mean  $\pm$  SE.

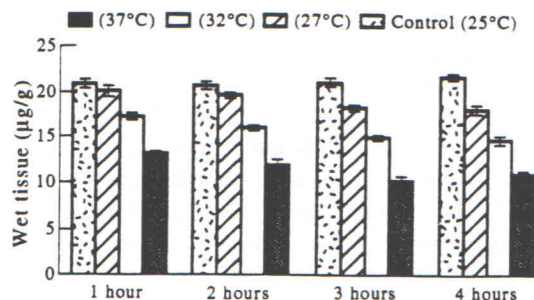


Fig. 3. Changes in ascorbic acid content in liver of *H. fossilis* to temperature exposure from 25 to 37°C at various times. Values are mean  $\pm$  SE.

activity (represented as units per milligram fresh tissue weight) was significantly increased at 32 and 37°C exposure when compared to the control (25°C). The change in SOD activity at 27°C was not significant. When each temperature was extended for periods of 1–4 h the SOD activity did not change significantly.

The effects of temperature increase on the concentrations of AsA in the liver are shown in Fig. 3. AsA content was depleted significantly at 32 and 37°C when compared to 25°C, whereas no change was observed at 27°C temperature exposure. Some similarities in the profiles of SOD activity and AsA content were evident in the liver during extended periods (1–4 h) of each temperature. Neither SOD nor AsA content changed significantly.

Levels of individual phospholipids in liver after temperature exposure are summarized in Table 1. Phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine contents were significantly decreased for varying periods at 37°C. The depletion in these phospholipids at 27 and 32°C temperatures was not significant when compared with control (25°C). Nevertheless, the phosphatidyl inositol level was increased significantly at 32 and 37°C but not at 27°C. Phosphatidic acid level did not change significantly in comparison to the control during any of the temperature exposures.

## DISCUSSION

Lipid peroxidation has been considered to occur because of free radical activity (Cheeseman, 1982). Temperature may mediate the production of ROS capable of inducing lipid peroxidation and oxidative breakdown in membranes (Parihar and Dubey, 1995). Information on the effect of temperature on lipid peroxidation in fish is limited. However, related to this topic is the study of Wilson and Knowles (1987) on temperature adaptation of hemoglobin in fish. An apparent functional adaptation to thermal variations has been implied for at least one locus involved in oxygen activation in poikilothermic vertebrates and similar adaptation can be envisaged for other such loci, i.e. heme-iron proteins in a myriad of poikilotherms throughout the aquatic environ-

Table 1. Changes in individual phospholipids in liver of *H. fossilis* exposed to temperature for varying periods

Phospholipids (mg/g wet tissue)	Treatments	Exposure times			
		1 h	2 h	3 h	4 h
Phosphatidyl inositol	Control (25°C)	0.22 $\pm$ 0.01	0.24 $\pm$ 0.03	0.25 $\pm$ 0.01	0.24 $\pm$ 0.02
	27°C	0.26 $\pm$ 0.03	0.26 $\pm$ 0.04	0.29 $\pm$ 0.03	0.25 $\pm$ 0.01
	32°C	0.35 $\pm$ 0.02*	0.29 $\pm$ 0.02*	0.36 $\pm$ 0.04*	0.31 $\pm$ 0.02*
	37°C	0.49 $\pm$ 0.04*	0.42 $\pm$ 0.03*	0.39 $\pm$ 0.03*	0.47 $\pm$ 0.01*
Phosphatidyl choline	Control (25°C)	0.19 $\pm$ 0.03	0.23 $\pm$ 0.01	0.21 $\pm$ 0.02	0.18 $\pm$ 0.02
	27°C	0.16 $\pm$ 0.01	0.21 $\pm$ 0.02	0.21 $\pm$ 0.03	0.16 $\pm$ 0.01
	32°C	0.15 $\pm$ 0.02	0.20 $\pm$ 0.03	0.28 $\pm$ 0.01	0.15 $\pm$ 0.02
	37°C	0.11 $\pm$ 0.01*	0.12 $\pm$ 0.02*	0.11 $\pm$ 0.11*	0.07 $\pm$ 0.01*
Phosphatidyl serine	Control (25°C)	0.20 $\pm$ 0.02	0.25 $\pm$ 0.02	0.26 $\pm$ 0.02	0.24 $\pm$ 0.01
	27°C	0.18 $\pm$ 0.01	0.24 $\pm$ 0.01	0.24 $\pm$ 0.03	0.24 $\pm$ 0.03
	32°C	0.17 $\pm$ 0.02	0.23 $\pm$ 0.03	0.21 $\pm$ 0.02	0.22 $\pm$ 0.04
	37°C	0.15 $\pm$ 0.01*	0.13 $\pm$ 0.02*	0.15 $\pm$ 0.03*	0.18 $\pm$ 0.02*
Phosphatidyl ethanolamine	Control (25°C)	0.24 $\pm$ 0.02	0.23 $\pm$ 0.01	0.24 $\pm$ 0.02	0.25 $\pm$ 0.02
	27°C	0.20 $\pm$ 0.01	0.21 $\pm$ 0.02	0.23 $\pm$ 0.01	0.26 $\pm$ 0.04
	32°C	0.18 $\pm$ 0.03	0.21 $\pm$ 0.03	0.24 $\pm$ 0.03	0.23 $\pm$ 0.03
	37°C	0.08 $\pm$ 0.01*	0.10 $\pm$ 0.03*	0.15 $\pm$ 0.02*	0.16 $\pm$ 0.01*
Phosphatidic acid	Control (25°C)	0.20 $\pm$ 0.03	0.24 $\pm$ 0.08	0.26 $\pm$ 1.30	0.27 $\pm$ 0.12
	27°C	0.22 $\pm$ 0.02	0.21 $\pm$ 0.13	0.27 $\pm$ 0.81	0.25 $\pm$ 0.08
	32°C	0.21 $\pm$ 0.04	0.23 $\pm$ 0.09	0.24 $\pm$ 0.09	0.29 $\pm$ 0.07
	37°C	0.18 $\pm$ 0.05	0.24 $\pm$ 0.07	0.23 $\pm$ 0.13	0.30 $\pm$ 0.14

Values are expressed as mean  $\pm$  SE ( $N = 10$ ); \*Significant ( $P < 0.05$ ) difference between control (25°C) and treatment groups (27–37°C) at various times (1–4 h).



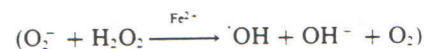
ment. This idea in light of the adaptive nature of membrane lipid composition to temperature variation, e.g. the high content of PUFA found in membranes of cold-acclimated poikilotherms (Winston and DiGiulio, 1991) suggests a broad spectrum of research on production of ROS and oxidative stress in extreme environment.

Our data showed increased levels of lipid peroxidation which, taken together with the SOD changes indicate increased ROS generation and oxidative damage in the liver of *H. fossilis* exposed to temperature for varying periods. A few studies on environmental factors and lipid peroxidation in fish systems have been described. Wofford and Thomas (1985, 1988) have observed elevated lipid peroxidation in fish exposed to carbon tetrachloride and cadmium. Similar findings were also observed in fish exposed with sediment associated organic pollutants (Livingstone *et al.*, 1993).

Concurrent with the increase of ROS, activities of antioxidants may vary. Evidence exists showing variations in activities of antioxidant enzymes for different fish species (Aksnes and Njaa, 1981), tissues (Wdziejczak *et al.*, 1981), seasons (Gabryelak *et al.*, 1983) and for tissues from fish with different feeding habits (Radi *et al.*, 1987). Whereas published data specific to effect of temperature on liver are unavailable, previous reports indicate seasonal and cadmium mediated variability of antioxidant enzyme activities in lake charr (*Salvelinus namaycush*) and white sucker (*Catostomus commersoni*) liver (Palace and Klaverkamp, 1993). Livingstone *et al.* (1992) reported elevation of SOD, GPx and CAT activities in liver of dab (*Limanda limanda*) exposed to organic and inorganic contaminants. An examination of effect of temperature in our study revealed a significant increase of SOD activity at temperatures of 32 and 37°C. The pattern found for SOD was consistent with freshwater fish lake charr (*Salvelinus namaycush*) and pearl dace (*Senotilus margarita*) assaying higher SOD activity during the season of largest ambient temperature variations (Palace and Klaverkamp, 1993). Consistent with a link observed between changing ambient temperature and altered enzyme activity, Kolupaev and Putintseva (1984) reported complex changes of CAT activity in erythrocytes from dace (*Phoxinus phoxinus*) exposed to different water temperatures.

The indicated increases in SOD activity in liver resulting from temperature exposures were paralleled by an increase in lipid peroxidation. Taken together, these events indicate enhanced generation of ROS in the liver. However, at low temperature the response was nonsignificant. Greater increase in SOD activity indicates increased dismutation of  $O_2^-$  to  $H_2O_2$ .

Thus, it can arrest the radical induced damage to cellular membranes by converting more harmful  $O_2^-$  to the less toxic product,  $H_2O_2$  (Farber, 1994). Although the excessive production of  $H_2O_2$  in cells may be considered toxic, it is not the toxicity of  $H_2O_2$  *per se* that creates the major problem. Rather,  $H_2O_2$  in the presence of  $Fe^{2+}$  (or  $Cu^{2+}$ ) is readily converted via the Fenton reaction to the highly oxidizing molecule,  $\cdot OH$ :



In most cells there are two antioxidant enzymes that detoxify the  $H_2O_2$  to nontoxic products include CAT and GPx. Enhanced CAT activity quickly decomposes  $H_2O_2$  to water and  $O_2$  in tissues (Reiter, 1995). A depletion of CAT or GPx could lead to a  $H_2O_2$ -induced oxidative stress.

Extending the period of exposure to each temperature however did not change SOD activity significantly. Assuming temperature exposure resulted in increased production of ROS in liver of fish, the relatively small response of the inducible antioxidant enzymes may have been accounted for by an otherwise adequate supply of nonenzymatic cellular antioxidants. Such cellular antioxidants include AsA (Miyazawa *et al.*, 1986) and tocopherol (Burton and Traber, 1990). The present work showed maintenance of a fairly constant level of AsA in liver when exposure time was increased from 1 to 4 h. Relative maintenance of constant levels of AsA in liver at various times could be due to its role in acclimatization in response to temperature exposure. Whether this indicates a cellular ability to substitute enzymatic antioxidant with nonenzymatic antioxidant is not very clear.

AsA is the most effective aqueous phase antioxidant (Frei *et al.*, 1989). In the common mussel (*Mytilus edulis*), Ribera *et al.* (1991) have observed the effective role of several free radical scavengers including AsA in detoxication of damaging ROS. In fish, the present study indicated a significant decline in AsA in liver at 32 and 37°C temperature exposure for varying periods (1–4 h) when compared to the control. As the temperature exposure resulted in significantly higher activity in lipid peroxidation, the depletion of AsA was probably due to the acute ROS generation. Henning *et al.* (1991) reported a similar disappearance of AsA in mammals which was accompanied by lipid peroxidation. The reducing power of AsA for organic peroxide is very evident (Jenkins *et al.*, 1988) which can be helpful in preventing oxidative stress. Major antioxidant activities of AsA *in vivo* are its recycling of



$\alpha$ -tocopherol in cell membranes and its ability to scavenge some pollutants (Halliwell, 1994). One of the reasons why AsA disappears is to preserve  $\alpha$ -tocopherol as has been reported by Bendich *et al.* (1986). Nevertheless, the study of other small molecular scavengers such as GSH, carotenoids and  $\alpha$ -tocopherol, which has not been measured in the present study, may be important in understanding the process of detoxication of ROS. An examination of the balance between enzymatic and nonenzymatic cellular antioxidants would further strengthen our understanding of cellular defence mechanism against temperature exposure.

The intracellular membranes contain a high proportion of individual phospholipids which are particularly sensitive to oxidative stress (Vliet and Bast, 1992). It has been demonstrated in many diseases that the breakdown of membrane phospholipids and lipid peroxidation are very common, and this breakdown process is mediated by free radicals (Halliwell and Gutteridge, 1989). Temperature may cause changes in individual lipid classes by altering the degree of unsaturation in tissue lipids. Chain length and degree of unsaturation determine the fluidity and permeability of membranes. In the present study, major phospholipids e.g. phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine were decreased, phosphatidyl inositol level was increased and phosphatidic acid did not change after temperature exposures. The indicated decreases in major individual phospholipids in our study were paralleled by an increase in lipid peroxidation. The very marked decline in phospholipid with exposure to temperature is almost certainly due to the loss of lipid material in membranes during temperature exposures. These metabolic modifications in fish affected by temperature appear to cause damage to the liver of fish.

In conclusion, the results indicate an increase in oxidative stress in liver of *H. fossilis* exposed to temperature for varying periods. Increase in lipid peroxidation is indicative of increased  $O_2^-$  generation which has upset the endogenous balances of enzymatic antioxidant SOD and non-enzymatic antioxidant AsA in liver. A high level of SOD activity and depletion of AsA suggest that fish liver SOD and AsA act as a very effective oxyradical scavenging sink during temperature exposure. The decline in major phospholipids could be due to  $O_2^-$  mediated destruction of membranal lipids.

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