T463

INFLUENCE OF SELECTED DIETARY MICRONUTRIENT ELEMENTS ON GROWTH AND BIOCHEMISTRY OF FARMED PRAWNS

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By

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CERTIFICATE

This is to Certify that this thesis entitled "INFLUENCE OF SELECTED DIETARY MICRONUTRIENT ELEMENTS ON GROWTH AND BIOCHEMISTRY OF FARMED PRAWNS" is the bonafide work by KAVITHA, O carried out at Biochemistry and Nutrition Division, Central Institute of Fisheries Technology, Kochi – 682 029 under my guidance and supervision. This work has not been submitted anywhere for the award of any degree, diploma, associateship, fellowship or any other similar titles or recognition.

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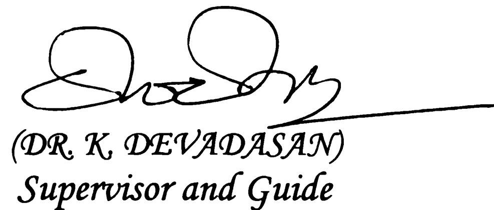

(DR. K. DEVADASAN)
Supervisor and Guide

DECLARATION

I hereby declare that this thesis entitled **"INFLUENCE OF SELECTED DIETARY MICRONUTRIENT ELEMENTS ON GROWTH AND BIOCHEMISTRY OF FARMED PRAWNS"** is a research work carried out by me in Biochemistry and Nutrition Division, Central Institute of Fishery Technology, Kochi- 682 029. This work has not been submitted else where for the award of any degree, diploma, associateship, fellowship or any such titles.

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LIST OF ABBREVIATIONS

AAS	-	Atomic absorption spectrophotometer
Ala	-	Alanine
ANSA	-	Amino naphtholsulphonic acid
AOAC	-	Association of Analytical Chemists
APS	-	Ammonium persulfate
AR	-	Analar
Arg	-	Arginine
Asp	-	Aspartic acid
b.p	-	Boiling point
BHT	-	Butylated hydroxy toluene
BSA	-	Bovine serum albumin
CAT	-	Catalase
CDNB	-	1-chloro-2,4-dinitrobenzene
cm	-	centimeters
CNS	-	Central Nervous System
CoA	-	Coenzyme A
CP	-	Chrompack
Cu-TM	-	Copperthiomolybdate
Cys	-	Cystine
DHA	-	Docosahexaenoic acid
DNA	-	Deoxyribonucleicacid
DTNB	-	5,5'-dithiobis 2-nitrobenzoic acid
EDTA	-	Ethylene diamine tetra acetic acid
EFA	-	Essential fatty acids
EPA	-	Eicosapentaenoic acid
FAO	-	Food and Agriculture Organization
FCE	-	Feed Conversion Efficiency
FCR	-	Feed Conversion Ratio
Fig	-	Figure
g	-	grams
GLC	-	Gas liquid chromatography
Glu	-	Glutamic acid
GLUT	-	Glucose transporter

Gly	- Glycine
GPx	- Glutathione peroxidase
GSH	- Reduced Glutathione
GS-Se-SG	- Selenodiglutathione
GSSG	- Oxidized glutathione
GST	- Glutathione-s-transferase
His	- Histidine
HPLC	- High performance liquid chromatography
hrs	- Hours
Ile	- Isoleucine
ILL	- Incipient lethal level
INT	- 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoleum chloride
Kg	- Kilogram
LC	- Lethal concentration
Leu	- Leucine
L	- Levo
ln	- Natural logarithm
ltr	- Liter
Lys	- Lysine
M	- Molar
Met	- Methionine
μ	- Micro
mg	- milligrams
ml	- milli liter
min	- minutes
MMA	- Methyl malonic acid
mRNA	- messenger Ribonucleicacid
mt	- million tones
n mole	- Nano mole
N	- Normal
N_2	- nitrogen
NADPH	- Reduced Nicotinamide Adenine Dinucleotide Phosphate
nm	- Nano meter

No	- Number
^{o}C	- Degree celcius
^{o}E	- Degree East
^{o}N	- Degree North
OPA	- O-Phthalaldehyde
^{o}S	- Degree South
P.	- *Penaeus*
PAGE	- Polyacrylamide gel electrophoresis
PAPS	- 3'-Phospho adenosine 5'-phosphosulfate
PER	- Protein Efficiency Ratio
Phe	- Phenyl alanine
PMS	- Phenazine methosulfate
ppm	- Parts per million
ppt	- Parts per thousand
Pro	- Proline
PUFA	- Polyunsaturated fatty acid
RBC	- Red blood cells
ROS	- Reactive oxygen species
RSH	- Thiol compound
SAM	- S-adenosyl methionine
SDS	- Sodium dodecyl sulfate
SE	- Standard error
SeMC	- Selenomethylcystein
SeMet	- Selenomethionine
SeP	- Selenoprotein P
Ser	- Serine
SGR	- Specific Growth Rate
SH	- Sulfhydryl
SOD	- Superoxide dismutase
SO	- Sulfite oxidase
t	- Time
TBA	- Thiobarbituric acid
TCA	- Trichloroacetic acid
TEMED	- N,N,N^{1},N^{1}-tetra ethyl methylene diamine
Thr	- Threonine

Trp	-	Tryptophan
TSH	-	Total sulfhydryl content
TTM	-	Tetrathiomolybdate
Tyr	-	Tyrosine
UI	-	unidentified
UV	-	Ultra violet
v/v	-	Volume/volume
Val	-	Valine
W	-	Weight
XO	-	Xanthine oxidase

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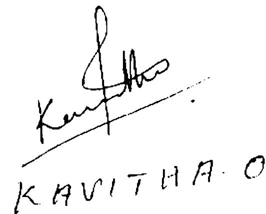

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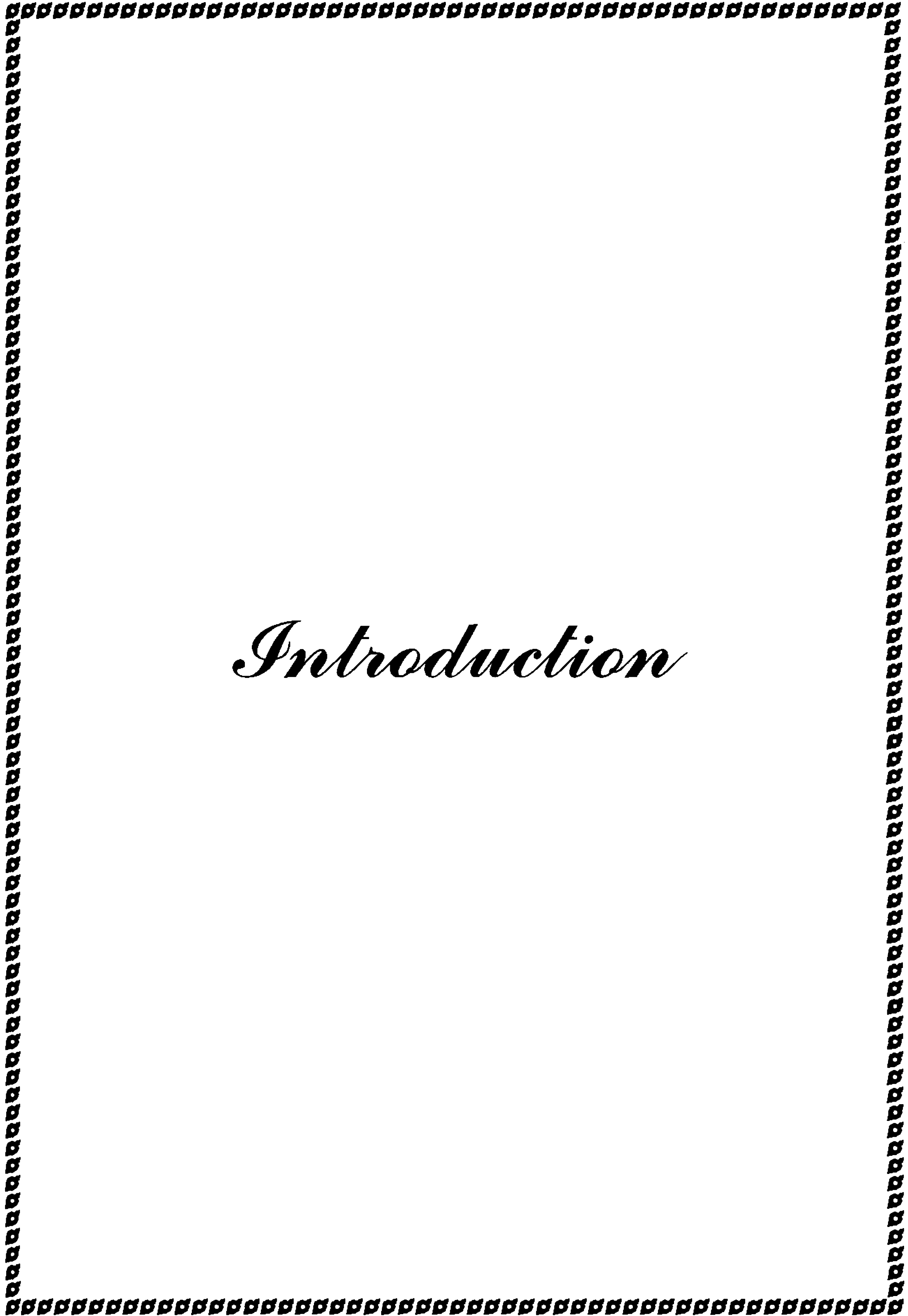


Introduction

1. INTRODUCTION

Global aquaculture has registered a phenomenal growth in a short span of time, increasing from 7.4 million tons in 1980 to more than 42 million tones in 1999. In this sector production is increasing at an average rate of more than 10% per year. The contribution of aquaculture to world food fish landings is more than doubled since 1984. Asian aquaculture farmers contribute about 90% of world aquaculture production (FAO, 2001). As the most lucrative and sought after item, shrimp has been the preferred species for culture. Shrimp culture was practiced, though not in an organized scale, centuries ago in South-East Asia. Global production of farmed shrimp increased from 576,453 mt in 1988 to 941,814 mt in 1997. Today 58% of the world shrimp production is from culture. In the case of India, the farmed shrimp production was 62000 mt in 1993-1994. The production increased to 82,000 mt during the year 1998-1999. Processed shrimp is a very important export item, earning valuable foreign exchange for the country. Shrimp processing provides direct and indirect employment to millions, especially the rural poor belonging to the weaker sections of the society. It is one of the sectors where large number of women gets employment also.

Processed marine products are today India's largest foreign exchange earning export item from the agriculture sector. Till

recently, this industry was depending on catches landed from the sea, which often showed erratic fluctuations. Demand for processed marine products is increasing in importing countries. Though a variety of food fishes are landed all along the coast-line of India, prawns which constitute only about 10% of the total landings account for almost 75% of the export earnings from the sector even today.

Under intensive fish culture conditions, the natural food available from the environment will not meet the needs of the growing fish biomass, necessitating supplementation with externally supplied nutritious diet. Artificial diet becomes important as stocking density of shrimp biomass increases. In intensive culture system, the farmed shrimp has to rely almost exclusively on supplemented feed for its nutritional requirements. Among the favorite species for culture, *Penaeus monodon* and *Macrobrachium rosenbergii* are the most important. Scientific aquaculture of any species of fish/prawns demands a clear knowledge of the detailed nutritional requirements of the concerned species. Nutritional requirements must be considered as approximations based upon the average individual and perhaps under average conditions, as we understand them today. Recommended allowances, as distinct from requirement, are always set somewhat above estimated requirements to provide a safe margin. As information on the exact nutritional requirements of *P.monodon* was inadequate, the formulation of compound diet for this species was

primarily based on the known nutritional requirements of *P. japonicus* (Pascal, 1989). Carbohydrates are the most abundant and least expensive source of energy. For man and terrestrial farm animals, dietary carbohydrates serve as the principal source of metabolic energy. For fish and shrimp, carbohydrates are nonessential dietary nutrients but they have protein-sparing function. Moreover certain carbohydrate sources serve to increase the palatability of the compounded feed.

Proteins represent the largest chemical group in the animal body, next only to water (Lehninger *et al.*, 1993). They are involved in tissue repair and maintenance, growth, as source of energy, as substrate for formation of carbohydrates or lipids and also for the formation of a wide variety of biologically important substances including various enzymes (Paulraj, 1997). The nutritive value of protein largely depends upon its amino acid composition (Nair, 2001) and the requirement of the consuming organism. Lipids form the nutrient group with more energy per unit weight than any other biochemical constituent. They are important as a source of energy, essential fatty acids, sterol, phospholipids, as carriers of fat soluble vitamins and in lipid transport (Paulraj, 1997).

Many studies on the requirements for various nutrients during different life stages of *P. monodon* have been conducted. But these

have been mostly on the requirements of the macronutrients. Though reports are available on the requirements of micronutrients (minerals and vitamins) for fish, the requirements of different trace elements and the effect of supplementing the feed with important trace elements, on the growth and general biochemistry have not yet been fully studied.

Dietary need for 22 minerals has been demonstrated in fish (Schmittou *et al.*, 1998). Minerals are divided into two groups depending on their requirements in the diet. The macro or major elements are those required at levels of 100 mg or more per day and the micro or trace elements are those required in trace amounts, a few mg or μg per day (Mayes, 1996). The important macro elements are calcium, phosphorus, magnesium, sodium, potassium and chlorine. The important trace elements include, iodine, manganese, copper, cobalt, zinc, selenium, molybdenum, fluorine, aluminum, nickel, vanadium, silicon, tin and chromium (Paulraj, 1997). These minerals serve a wide variety of functions in the biological system, which include formation of the structural component of the tissue, maintenance of osmotic balance, cofactor for important enzymes etc (Paulraj, 1997). Trace elements may also hold the key to growth as well as disease resistance in farmed fish and shrimp. Fixing the optimum dietary concentrations of various trace elements for aquatic animals is very difficult as the requirements vary from species to species. Moreover the minerals absorbed through the gills and

imbibed from drinking water also make fixing optimum dosage difficult. Feeds in use contain various minerals incorporated at different levels without a scientific knowledge of the requirements. Indiscriminate use of these minerals can have deleterious biochemical effects in the organisms. But reports available on these aspects are scanty. This study is an attempt to provide such information in the case of some of the important microelements.

The essentiality of dietary selenium was established as recently as 1973 when it was shown to be a part of the enzyme glutathione peroxidase. Together with catalase, this enzyme serves to protect red blood cells from damage by peroxides. This explains why selenium is regarded as an essential dietary supplement. At the same time when in excess, selenium is a potentially toxic trace element also. Its toxic effects in cattle and sheep have been reported from several countries (Linder, 1985). Selenium-induced toxicity has been reported in rats and pigs also (Orville and Philip, 1996; Kim and Mahan, 2001). Lemy (1997) has examined the selenium induced teratogenic effects in fish. The long-term ingestion of selenium at high levels is reported to cause liver damage in Carp (Chen *et al.*, 1997). The effect of dietary selenium in the feeds used for aquaculture is an area demanding attention but is one of the least studied.

Molybdenum is an important trace element involved in the metabolism of carbon, nitrogen and sulfur (Kisker *et al.*, 1997). The biochemical role of this element in an animal is as two molybdenum containing enzymes, xanthine oxidase (XO) and sulfite oxidase (SO) (Kisker *et al.*, 1997; Moriwaki *et al.*, 1997). XO is essential for the metabolic degradation of purines to uric acid and SO is important in the terminal degradation of sulfur containing amino acids (Rajagopalan, 1988). Increased activities of XO release excess super oxides to the system resulting in peroxidative damage (Shaw, 1989; Maytin *et al.*, 1999). The deficiency of XO results in xanthinuria (Sumi and Wada, 1996) and SO deficiency results in sulfite toxicity (Mudd *et al.*, 1967; Calabrese *et al.*, 1981). The important aspect regarding molybdenum toxicity is that it will lead to impaired copper absorption (Ward and Spears, 1997).

Cobalt is also an important trace element, which is essential for normal metabolic processes of the prawn. It acts as an important coenzyme forming an integral part of vitamin B_{12}. The role of cobalt in lipogenesis is well known (Kennedy *et al.*, 1991). It is also involved in the synthesis of amino acids (Kennedy *et al.*, 1992). Alteration in the antioxidant status by administration of cobalt in rats has been reported (Southern and Baker, 1981).

Though these three elements are of biological importance, their importance in shrimp culture has received very little attention. The

practical difficulties in determining and differentiating the environmental and nutritional impact of trace elements in aquatic living organisms (unlike the terrestrial organisms) may be the reason for the scanty documentary evidence available in this regard. The influence of these elements on tissue antioxidant status of shrimp is also yet to be explored in detail. The exact biochemical mechanism involved in toxicity of trace elements in aquatic animals also has not been established. Indiscriminate and excessive use of trace elements may lead to their accumulation to a toxic level in the environment. Extrapolation of the qualitative or quantitative requirements of land animals to aquatic organisms can lead to erroneous conclusions. Hence an attempt to study the influence of three such important trace elements on the growth and biochemistry of *P. monodon* was taken up.

The main objective of the work is to study the effects of selenium, molybdenum and cobalt on growth and metabolism of *P. monodon* by determining average weight gain, feed conversion ratio (FCR), feed efficiency (FCE), specific growth rate (SGR), protein efficiency ratio (PER), mortality, proximate composition, amino acid composition, fatty acid profile, mineral content ant tissue antioxidant status.

Review of Literature

2. REVIEW OF LITERATURE

TRACE ELEMENTS

With the rapid growth of shrimp culture, management techniques, disease control and feed formulations have become important. Fundamental studies on the nutritional requirements of the species cultured are required, for the development and commercial production of formulated feeds. Studies on nutritional requirements have always been centered on the macronutrients. The nutritional importance of trace elements in feed is not fully understood, as very little work is reported on this topic. Trace metals may hold the key to immunity as well as growth.

The physiological importance of minerals is well documented in the case of humans and some animals. Not all the trace elements essential for higher animals have been described in the case of fish. Information on the nutritional requirement of trace elements for fish is also fragmentary, particularly because many are needed in very small amounts. Although trace elements are required in very small quantity, they are absolutely essential for the growth of the animal. At the same time if excess amount of these elements are ingested and assimilated, it may result in toxicity. Therefore animal maintains a delicate balance of the body levels of the trace metals by integrating the various parameters of uptake, storage and excretion (Watanabe *et al.*, 1997).

Fish may derive these inorganic elements from diet or from ambient water. There are a few reported works on the nutritional importance of some trace elements (Deshimaru and Yone, 1978; Davis and Robinson, 1987). Unlike in other animals, researchers in this area face many problems. The exchange of ions from the surrounding water across the gills and skin of fish complicates the measurement of actual mineral requirements.

Disorders related to essential micronutrients arise from inadequate intake, genetic defects, excess exposure and impaired elimination. Severe deficiency is rare, but specific symptoms and reduced protection against risk factors have been attributed to sub optimal intake of trace elements (Schmittou *et al.*, 1998). The prevention, diagnosis and monitoring of pathological condition related to trace elements as well as pharmacokinetic studies of metallo drugs and therapy monitoring relate mainly to the dosage of metal made available in appropriate body systems compartments such as blood, tissues etc. However appropriate composition of the elements may not always be amenable for sampling. In such condition whole body concentration is the only dependable factor.

Health risks are also associated with excessive exposure to potentially toxic elements. Impaired elimination may be a cause for the accumulation of toxic metals in the animals. Environmental

exposure to toxic metals may occur even before birth, Developing organisms are at a greater risk of suffering permanent damages from exposure to toxins, and metals are wide spread and persistent environmental pollutants with the potential for developmental and reproductive toxicity, even at low levels of exposure (Kim, 1999).

PENAEUS MONODON

P. monodon also known as giant tiger shrimp, is one of the largest penaeid shrimps in the world. When fully grown it can come up to 34 cm in body length and upto 250g in weight. Its coloration is variable. The body is gray to green; carapace and abdomen are transversely banded black and yellow. Pleopod appendices are blue and yellow. Shrimps from brackish water or ponds are often brown or blackish. It is distributed in the Indo West Pacific; (East and South East Africa and Pakistan to Japan, in Malay archipelago and Northern Australia) generally from 30^0 E to 155^0 E in longitude and from 35^0 N to 35^0 S in latitude. The adults inhabit the benthic zone of the outer littoral area. The longevity of *P. monodon* is estimated to be about one and half years for males and two years for females.

Shrimps and prawn culture has made significant advances during the last decade in many parts of the world. The incentive for such development was often the profit motive; the most favored

species were those, which commanded the highest prices when sold as luxury foods. Tiger shrimp is one such species in demand

Aquaculture, as an organized production activity is of relatively recent origin. For the same reason, it can be treated comparatively as a virgin field calling for more research. Though fish in nutrition has been studied extensively, fish nutrition, toxicology, growth of cultured organisms as influenced by different parameters including diet composition etc is yet to be studied in detail. Shrimp, being the main commercially cultured seafood deserves special attention in this context. Till recently, especially in India, shrimp culture was not done on a commercial scale. But the last two decades have seen a phenomenal increase in production from this sector. This has necessitated considerable research in this area to understand and solve the problems. Nutritional requirements, susceptibility to diseases, toxicology and mortality are all aspects to be studied in detail. Trace metals present in feed and their role in health and growth of cultured shrimp needs to be studied in detail as these trace constituents may hold the key to many of the problems in the field. As very little work is reported in this area, there is not much information to fall back upon and any work in such a virgin field of production, is of vital commercial importance to the country's economy. The current work is an attempt in this direction.

SELENIUM

Selenium was discovered in 1817 by Swedish chemist Jons Jacob Berzelius while analyzing a red deposit on the wall of lead chambers used in the production of sulfuric acid (Tinggi, 2003). Selenium is a semi metal (or metalloid), with properties of both metals and nonmetals. It is closely related to sulfur in its structure and chemical properties. It forms inorganic compounds such as selenite and selenate, and organic compounds such as selenoaminoacids, which corresponds to sulfites and sulfates and S-amino acids (Reilly, 1998). It is present chiefly in Cretaceous rocks, volcanic materials, some sea floor deposits and glacial drifts in the form of metallic selenides. The selenides are often associated with sulfides. Selenium exists in soil as basic ferric selenite, calcium selenite, elemental selenium and organic compounds derived from plant tissue.

BIOLOGICAL REQUIREMENTS

In the past two decades there had been much progress in our knowledge and understanding of the biological role of selenium and its importance in human and animal nutrition. The recent interest in selenium has been further stimulated by new findings of several selenoproteins in animals. Even though the functional roles of these selenoproteins are not fully understood, there is increasing evidence that these proteins and other selenium metabolites are important in

immune function and reduces the cancer risk (Tinggi, 2003). Selenium has three levels of biological activity. 1) Trace concentrations are required for normal growth and development, 2) moderate concentrations can be stored and homeostatic functions maintained and 3) elevated concentrations can result in toxicity (Hamilton, 2004).

The source of naturally incorporated selenium is an important consideration for dietary selenium supplementation studies. The first evidence that selenium was an essential element involved the discovery that it would prevent liver necrosis in rats and exudative diathesis in chicken. Before this, scientists were aware of the toxicity symptoms caused by the selenium. By this discovery, instead of a primary concern with the toxicity of selenium, nutritionists turned their attention to the metabolic functions of this element and consequences of its deficiency. The important biochemical functions of selenium in higher animals were defined by Rotruck *et al.* (1973), who discovered that selenium was an integral part of the enzyme Glutathione peroxidase. This enzyme destroys lipid peroxides and thus functions in protecting cell membrane against peroxidative damages. In the 1980s further selenoproteins were discovered which indicated that the biological activity of selenium is not restricted to its role in antioxidant activity alone and that it is involved in many other aspects of metabolism (Tinggi, 2003). More recently selenium has been found to be essential for the activity of thyroid gland also. The

iodothyronine deiodinase enzyme, which converts thyroxine to active tri iodothyronine, is a selenium containing protein (Yu *et al.*, 2002). Other biological roles of selenium include the activity of prostaglandins (Lee *et al.*, 2002), glutathione (Hoffman, 2002) and heme (Padmaja *et al.,* 1996). Selenium is also important in spermatogenesis (Marin-Guzman *et al.*, 2000). It is a component of proteins other than glutathione peroxidase also. Dietary selenium status plays an important role in the regulation of arachidonic acid metabolism and subsequently the activation of phospholipase D (Cao *et al.,* 2002). According to Stadtman (1990), selenium has a property to bind to proteins, as yet unidentified, as evidenced by dissociation of selenium from the respective proteins in the liver of animals. A significant amount of selenium in the rat plasma is in the form of serum selenoprotein P (Burk *et al.*, 2003).

Tissue level of selenium depends upon the dietary level of selenium. Yeh *et al.* (1997) had shown the effect of selenium supplementation on the level of selenium and selenoprotein w in sheep tissue. Maximum level of selenium was observed in liver tissues and it was also established that the selenium level increased with the supplementation. But selenoprotein w was found to be highest in muscle tissue, which was also sensitive to dietary selenium level.

DEFICIENCY

Diseases associated with selenium deficiency have been a serious problem in farm animals in many parts of the world. For instance, hepatosis dietetica, a liver necrosis caused by selenium deficiency can cause death to pigs within hours after the symptoms appeared. Exudative diathesis again due to selenium deficiency can cause death to poultry within a few days as a result of edema of body tissues (Tinggi, 2003). The most common selenium deficiency disease is the white muscle disease, which is a nutritional muscular dystrophy (Tinggi, 2003). The symptoms of sub clinical selenium deficiencies are more difficult to detect, but usually these are associated with depressed growth and production, and impaired immune response (Tinggi, 2003). The direct effect of selenium deficiency in human health was reported first from China in the 1970s when an endemic cardiac myopathy, called Keshan disease, and an osteoartropathy, known as Kashin–Beck disease, a generative articular disease caused by oxidative damage to cartilage leading to deformation of bone structure (Tan *et al.,* 2002) were identified as selenium deficiency problems.

Foster and Zhang (1995) compared the spatial distribution of the elderly in two countries with the prevalence of Kashin-beck syndrom and Keshan disease, both of which involve extreme selenium

deficiency. They could observe that human life span was shortened by severe selenium deficiency even if there is no direct evidence of an associated endemic disease. According to them, extreme selenium deficiency promotes the free radical damage at cellular level and thus accelerates the aging process. But effects of selenium deficiency or toxic effects when in excess have not been studied in aquatic organisms.

The effect of selenium deficiency has been postulated to result from the destruction of cellular membrane or of critical cellular proteins, thus affecting cellular integrity (Suzuki *et al.*, 1988). The basis for this postulate is the biochemical role of selenium as an antioxidant. Since selenium is very important in maintaining the normal antioxidant functions of the body, antioxidant system is the primary target affected by selenium deficiency (Avanzo *et al.,* 2001). The activity of selenium containing glutathione peroxidase (GPx), the most important anti oxidant enzyme, decreases in selenium deficient condition (Altimira *et al.*, 2000). Selenium deficient mice were more susceptible to Central Nervous System (CNS) listeriosis produced by oral infection (Altimira *et al.*, 2000). The complex effects of selenium deficiency also result in pulmonary disorders resulting in lethal hyperoxia (Coursin and Cihla, 1996).

Dietary selenium affects GPx expression at mRNA level (Cheng *et al.,* 1997). Selenium deficiency increases the reactive oxygen metabolite release (Altimira *et al.,* 2000). Peroxidation damage caused by selenium deficiency will subsequently lead to other pathological condition such as degenerative myopathy as observed in cattle (Walsh *et al.,* 1993), increased pulmonary oxidant stress (Coursin and Cihla, 1996) and chronic renal failure (Makropoulos *et al.,* 1997). It was also reported that selenium deficiency resulted in damage in cellular lipids and proteins in liver (Muller *et al.,* 2002). Bates *et al.* (2002) reported that the level of serum selenium level was very low in smokers and that they are more susceptible to selenium deficiency diseases.

In a review, Tinggi (2003) points out that in Australia selenium deficiency has caused health problems to live stock; however the problems were eliminated after the introduction of selenium supplementation.

There are selenium-containing proteins other than GPx, present in tissues. Such selenoproteins are of particular importance in sheep as it is involved in sheep nutritional myopathy and white muscle disease. Failure to incorporate Se in this protein in selenium deficient lamb is responsible for the myopathy observed in them (Yeh *et al.*, 1997).

It was also reported that selenium has an important role in maintaining the immune system in active and potent condition. In the deficiency of selenium, tissues were found to be more susceptible to viral infection, due to the decreased immunocompetency (Sher, 2001). This may be significant in susceptibility to viral disease in farmed shrimps.

TOXICOSIS

Selenium toxicity in animals results in the development of the disorders known as "alkali disease" and "blind staggers". The alkali disease is a chronic poisoning of horses and cattle from continuous ingestion of plants containing high selenium. The disease is characterized by dystrophic changes in hooves and rough hair coats. The blind staggers is also a chronic poisoning in animals, which again is associated with feeding on plant species with high selenium content. The selenium accumulating plant species were found to contain selenocystathionine, an organic form of selenium that is toxic to animals. Selenocystathionine will finally be metabolized and converted to selenomethionine, which is the predominant form of selenium compounds in tissues of plants, algae and yeast (Tinggi, 2003). The toxicity of selenium not only depends on the chemical form and quantity of the element consumed, but also on a variety of

other factors that include species, age, physiological state, nutrition, dietary interaction, and the route of administration (Tinggi, 2003).

Selenium toxicity has also been reported in some regions of Australia, as a result of live stock feeding on plant species which accumulate selenium. The major source of selenium is the diet, and in many regions of the world, the level of selenium in the soils generally reflects the selenium status in human population.

Selenium expresses its toxicity in several manners; (1) It forms CH_3Se which either generate super oxides and cause subsequent oxidative stress, or forces formation of free radicals, that bind to and inhibit important enzymes (Spallholz and Hoffman, 2002). (2) Excess selenium, in the form of seleno cysteine results in inhibition of selenium methylation metabolism. As a consequence, hydrogen selenide, an intermediate metabolite accumulates in the animal, which is hepatotoxic, possibly causing other selenium related problems (Spallholz and Hoffman, 2002). The presence of excess selenium analogues of sulfur-containing enzymes and structural proteins is suspected to play a role in avian teratogenesis (Hoffman, 2002). L-selenomethionine, the major dietary form of selenium in aquatic birds was found to accumulate selenium in tissue protein of adult birds and in the protein of egg white as would be expected to occur in animals. Injection of selenomethionine was more toxic than dietary administration. L-seleno methionine present in the eggwhite protein

serves as a source of selenium for GPx synthesis in the developing aquatic chicks (Spallholz and Hoffman, 2002).

High content of selenium in the environment often causes pollution of the aquatic environment leading to, increased mortality, impaired reproduction with teratogenesis, reduced growth, histopathological lesions and alteration in hepatic GSH metabolism (Hoffman, 2002). As dietary and tissue concentration of selenium increases, increase in plasma and hepatic GPx activities occur. This is followed by dose dependent increase in the ratio of oxidized to reduced GSH in liver and alternately hepatic lipid peroxidation. This is known to be responsible for various toxicological problems associated with selenium toxicity in aquatic birds (Hoffman, 2002)

Various neurological abnormalities known as selenoses result due to selenium toxicity. This is mainly due to the inhibition of squalene mono oxygenase, an enzyme involved in cholesterol biosynthesis, by selenite (Gupta and Porter, 2002). Inhibition of this enzyme results in peripheral demyelinating neuropathy and the inhibition of this enzyme by selenite and methyl selenonium iodide was found to be slow and irreversible. These compounds react with the SH-group of the (cysteine) squalenemonooxygenase. Selenite was more toxic in the presence of dithiols as it gets reduced to selenide, which is having high affinity to the enzyme (Gupta and Porter, 2002).

METABOLISM

The absorption of selenium from selenomethionine (SeMet) is not different from the absorption from selenate, another form of selenium. But true absorption, after adjustment for urinary excretion, is higher in the case of SeMet than selenate (Finley, 1999). Retention in the tissue also depends on the form in which selenium is present. SeMet is often retained in the body, as it can substitute for methionine. Thus it accumulates within the tissues. But selenomethyl cysteine (SeMC) tends to accumulate less in body tissue. This selenium can be metabolized into a form, which can be retained within the body for use when dietary selenium is deficient. However when dietary selenium is high, retention within the tissue may be difficult (Finley, 1998). SeMC is initially converted into methyl selenol, which can then be metabolized by several pathways. Some could go into the pool that appears to be active against certain cancer. Another possible metabolic route for the methyl selenol is methylation and excretion through urine (Foster *et al.*, 1986). It is also possible that another specific lyase could convert the methyl selenol to selenide thus allowing selenium to get incorporated into selenoprotein (Finley, 1999).

Selenium from salts such as sodium selenite follows a pathway intermediate between SeMC and SeMet (Butler *et al.*, 1990). Such selenium can be more quickly converted into the hydrogen selenide

pool than SeMet, but like SeMC, it cannot be incorporated into SeMet and can only randomly substitute for methionine. Within the body, selenomethionine is considered as a component of albumin where as selenocysteine is the sole component of GPx and selenoprotein P (SeP). Irrespective of the form in which it is present in the body, its catabolism leads to excretion through urine following the same pathway as that of selenite (Janghorbani *et al.,* 1999).

The body can utilize both organic and inorganic forms of selenium and selenium consumed through foods and supplements exists in a number of forms, including selenomethionine (SeMet) and selenite. SeMeth is non specifically incorporated in to the general tissue protein pool in place of methionine (Thomson *et al.*, 1993; Burk and Hill, 1993) and does not have a selenium related redox function in proteins like selenocysteine (Behn *et al.*, 1995). SeMet has been found to be metabolized into selenocysteine by the trans sulfuration pathway (Burk and Hill, 1993; Behn *et al.*, 1995; Burk, 1991). Selenite is readily reduced to selenide, which is a precursor of selenophosphate, the universal selenium donor *in vivo* (Allan *et al.*, 1999).

One of the most important differences in the metabolism of selenite and SeMet is that the former cannot be stored, but is used directly in selenoprotein synthesis (Swanson *et al.*, 1991).

Selenite reacts with thiols as follows.

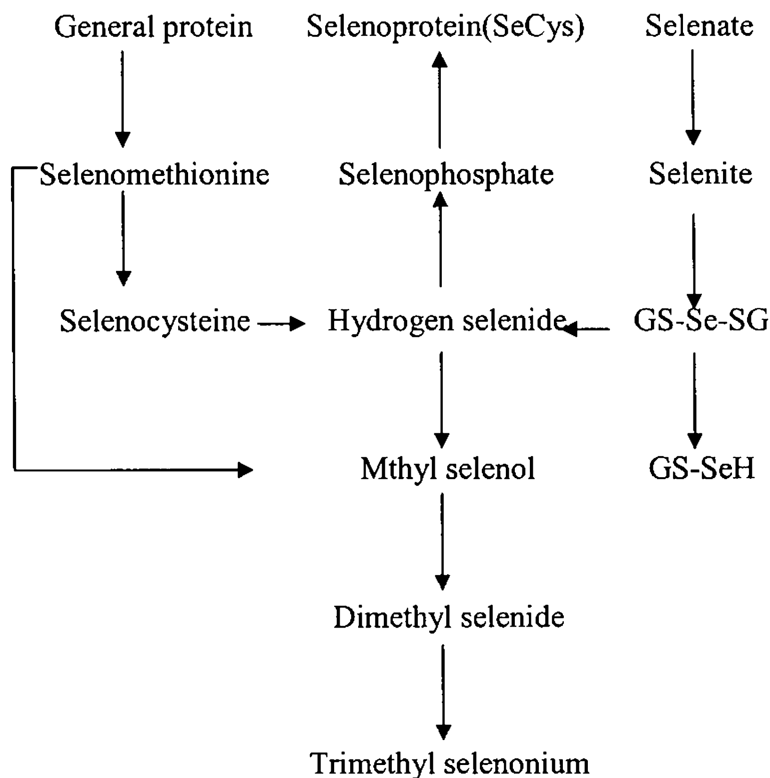
$$4RSH + H_2SeO_3 \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 3\ H_2O$$

Reduced Glutathione is the main component in the series of reactions, which convert selenite to selenide by the action of selenodiglutathione (GS-Se-SG) through reduction by thiols and NADPH-dependent reduction. It was demonstrated that GS-Se-SG was an active intermediate in GSH oxidation by selenite (Tsen and Tappel, 1958). There are contradictory reports as to how selenite affects the content of GSH. Selenite has been shown to increase the GSH content in canine mammary tumor cells (Kuchen and Milner, 1991), whereas it was found that selenite decreased, while SeMet had no effect on the intracellular glutathione concentration in human mammary tumor cells (Yan *et al.*, 1991).

Not all of the selenium ingested by an organism is absorbed. Insoluble selenium and indigestible selenium proteins are excreted in the feces. Absorbed selenium may also be lost through excretion via the urinary tract as trimethyl selenonium ion, or via respiratory tract after methylation to dimethyl selenide (Alarcon and Martinez, 2000). The remaining selenium is metabolized to selenium analogs of sulfur containing amino acids, which form the basis for selenium containing polypeptides and proteins. Selenium has the ability to substitute for sulfur in both organic and inorganic molecules due to similar ionic

radii of the two elements. However selenium analogs of sulfur containing proteins do not necessarily behave in the same manner in the biological system. Selenium is ultimately reduced to selenide in the living system where as sulfur containing proteins are reduced to sulfite for excretion.

Metabolism of selenium in animals (Tinggi, 2003)



ROLE IN CARCINOGENESIS

The relationship of selenium and cancer has been reviewed by Alarcon and Martinez, (2000). Selenium dependent enzymes have specific carcinostatic action by destroying reactive oxygen. Several

mechanisms involving selenium have been proposed for its inhibiting effect on cancer, namely modulation of cellular division rate, the metabolic alteration of some carcinogens, metabolic or cellular protection by an antioxidant system against severe oxidation and even by the stimulation of the immune system (Alarcon and Martinez, 2000). These authors have reported that in cancer patients, the plasma or serum selenium levels were significantly lower than in healthy controls. This resulted in a decrease in efficiency of the organism's defense against oxidative stress and consequent genesis and development of cancer.

Selenium is well recognized as a protective factor against cancer and cardio vascular diseases (Miyazaki *et al.*, 2001). Drinking water supplemented with selenium was effective in reducing tumor incidence and 2-acetyl aminofluorene induced hepato carcinogenesis in male rats (Mukherjee *et al.,* 1996). Selenium has been reported to inhibit the development of a variety of experimental tumors of gastro intestinal tract (Davis *et al.*, 2002). The preventive role of selenium in colon cancer in rats was reported by Finley *et al.* (2000). Selenium supplementation was found to be effective in preventing liver cancer in populations living with high risk of cancer (Li *et al.*, 2000). Various mechanisms have been proposed for the inhibitory effect of selenium on carcinogenesis. These include the inhibition of mutagenesity, effects on carcinogen metabolism, protection against oxidative cellular

damage or augmentation of host immune mechanism. The effect of selenium on the delivery of copper, zinc and other trace elements, which are required by the tumor cells, may also be responsible for its anticarcinogenic effects. Selenium can influence iron metabolism also. Adequate iron, copper and zinc are required for optimal function. Selenium maintains immune system active by maintaining the levels of these elements.

According to Davis *et al.* (2000) diet deficient in selenium causes global hypomethylation of liver and colon DNA in experimental animals. Thus alteration in DNA methylations may be a potential mechanism by which deficiency of dietary selenium increases tumor genesis.

SELENIUM IN AQUATIC ANIMALS

The major source of selenium is diet and in many regions of the world, the levels of selenium in the soil generally reflect the selenium status in animals. The bioavailability and toxicity of selenium depends on its chemical form. Generally organic form of selenium is more bioavailable and less toxic than inorganic forms (Tinggi, 2003). Uptake of selenium can be from water or from diet. Uptake of water soluble selenium by fish can be either through gills, epidermis or gut. Bioaccumulation of selenium in food chain components such as aquatic plants and invertebrates and the resulting effect in fish from

aquatic ecosystems containing selenium is well documented (Lemly, 2002). Selenium accumulation from dietary sources such as phytoplankton and zooplanktons is greater than from water in fish. Parents can pass on selenium to their offspring through eggs (Lemly, 1997).

Lemly, (2002) had described about the selenium toxicity in fish in Belews lake, North Carolina. Symptoms included the swelling of gill lamella, elevated lymphocytes, reduced hematocrite and hemoglobin, corneal cataract, exophthalmis and pathological alteration of liver, kidney, heart and ovary. Reproductive failure and teratogenic deformities of spine, head, mouth and fin were also observed. According to him the primary target of selenium toxicity is the egg, which will adversely affect the reproductive capability of the fish.

Selenium is toxic to aquatic life at relatively low concentrations. The occurrence of selenium in surface water is extensive resulting from a variety of natural and anthropogenic sources. These sources include natural weathering and irrigation-induced leaching of selenium containing rocks and soils, mobilization and discharge from mining and smelting activities, flue gas emission from fuel oil and coal combustion and fly-ash disposal practices. Selenium contamination in aquatic ecosystem has been linked to adverse

ecological effects in several field settings that include reproductive and developmental impairment of aquatic birds and fish. A hypothesis has also been extended that selenium contamination may have contributed to the historical decline of some endangered fish species in Colorado River Basin (Hamilton, 1998).

Selenium is an essential trace element for many aquatic and terrestrial species, with required level approaching just one order of magnitude below those causing toxicological effects in fish. Therefore when setting aquatic toxicological benchmarks for selenium, consideration must be given to ensure that both sufficiency and toxicity concerns are appropriately balanced. Besides sufficiency versus toxicity concern, several other factors complicate the establishment of aquatic toxicological threshold for selenium. These factors are associated with a complex biogeochemistry of selenium in aquatic ecosystems. Selenium occurs in several different oxidation states in aquatic environment that include oxidized selenates and selenites, elemental selenium and reduced selenides. Each form is known to differ in bioavailability and toxicity to aquatic organisms. Selenium can undergo biotransformations between inorganic and organic forms as a result of biotic and abiotic processes, which are not well characterized. Selenium also has been found to bioaccumulate in aquatic food webs to the extent that dietary exposure to selenium

becomes a critical exposure pathway for top predatory aquatic and aquatic dependent organisms.

Selenium interacts with several trace elements in fish, mammals and birds. These interactions could be additive, antagonistic or synergistic. Selenium partially diminishes the effect of cadmium cardio toxicity (Zikic *et al.*, 1998). The potential of selenium to improve the antioxidant system to prevent cadmium induced nephrotoxicity was also reported (Sarkar *et al.*, 1997; Ognjanovic *et al.*, 1995). Apart from this it was also reported that sulfate significantly reduces the acute toxicity caused by selenium in fresh water aquatic life (Brix *et al.*, 2001).

Even though reports are available on the adverse effects of selenium on aquatic organisms, the positive and negative impact of this metal in aquaculture has yet to be explored, especially in the case of shrimps. Literatures concerning the pressure of dietary as well as environmental selenium on shrimps are scanty. Any attempt in this area will be of crucial commercial importance to the field of aquaculture.

MOLYBDENUM

Molybdenum is now recognized as a ubiquitous element in earth surface and living matter. The essentiality of molybdenum for plant growth and the functional role-played by this metal in several

bacterial, plant and animal enzymes have long been recognized (Rajagopalan, 1988). However indisputable evidence for the importance of this element in living beings has been difficult to adduce owing to the absence of any documented instance of nutritional deficiency of the metal or of any pathological condition attributable to a dysfunctional molybdenum containing enzyme (Rajagopalan, 1988).

THERAPEUTIC USES OF MOLYBDENUM COMPOUNDS

Molybdenum is used in a variety of applications. Magnesium molybdate is used in treatment of anemia and for restoring appetite after convalescence (Vignoli and Defretin, 1963). A sustained release preparation of a molybdenised iron sulfate is capable of promptly correcting iron deficiency anemia (Stevenson, 1962; Rudolf *et al.*, 1963). Molybdenum has been shown to reduce the solubility of teeth in acid and also reduce the acid out put by the salivary glands. There is an additive effect between the benefit of fluoride and molybdenum though fluoride is undoubtedly the more important one. Molybdenum increased the absorption of fluoride from the stomach (Kambara *et al.*, 1989). It is also reported that higher intake of molybdenum is associated with lower incidence of dental caries by enhancing the remineralizing activity of fluorine in subsurface bovine enamel lesions *in vitro* (Kambara *et al.*, 1989). Another important effect of

molybdenum is in reducing the blood glucose and free fatty acid levels. Diabetic rats treated with molybdate showed significant improvement in postischemic cardiac function (Lord *et al.*, 1999). Ammonium tetrathiomolybdate has been regarded as the treatment choice for copper poisoning in sheep, promoting rapid clearance of the chealated complex copperthiomolybdate (Cu-TM) by biliary feacal route. This drug has also been used in the treatment of Wilsons disease in clinical trials. Tetrathio molybdate was found to be effective against Wilson's disease. It removes copper, accumulating in the form of bound metallothionein in the liver of the patients (Ogra *et al.*, 1999). Metallothionein bound copper in the liver of Long-Evans rats with a cinnamon like coat colour (an animal model for Wilson's disease) was removed by treatment with tetra thiomolybdate. Ammonium tetra thiomolybdate enhances the urinary excretion of copper and molybdenum (Komatsu *et al.*, 2000). The anticancerous effect of molybdenum is also well known. Brewer *et al.* (2000) developed a strategy for the treatment of cancer based on the modulation of total body copper status by using tetra thiomolybdate.

BIOLOGICAL REQUIREMENTS

Molybdenum is an essential trace element for animals as a compontent of metallo enzyme xanthine oxidase and a cofactor for at least seven metallo enzymes (Kisker *et al.*, 1997). The biological

requirement for molybdenum was first demonstrated by Bortels (1930), who found molybdenum to be an essential nutrient for the growth of *Azobacter* sp. Further works on the metabolic significance of molybdenum in animals reveal that molybdenum is an essential trace element for the normal functioning of animal systems (Wang *et al.*, 1992). The severity of pathology associated with simple sulfite oxidase deficiency, first discovered in 1967 (Mudd *et al.,* 1967) and the subsequent characterization of the enzyme as a molybdenum containing protein (Cohen *et al.,* 1971) established the essentiality of molybdenum for normal growth of living beings.

The major biochemical role of this element in animals is in the form of two molybdenum-containing enzymes, xanthine oxidase and sulfite oxidase. Xanthine oxidase is essential for the metabolic degradation of purines to uric acid and sulfite oxidase for the terminal degradation of sulfur containing amino acids. Molybdenum containing enzymes catalyze basic metabolic reactions in the nitrogen, sulfur and carbon cycles. Molybdenum is incorporated into protein as the molybdenum co-factor that contains a mononuclear molybdenum atom coordinated to the S-atom of the pterin derivative named molybdopterin (Kisker *et al.,* 1997). Low molybdenum concentration has beneficial effects on the growth of the animals (Probst, 1971). In blood, molybdenum binds to α-2 macroglobulins in the form of molybdate (Barceloux, 1999).

Reports of several works revealed the copper-molybdenum interrelationship in experimental animals (Ward *et al.,* 1993; Frank *et al.,* 2000). Molybdenum supplementation alleviates the chronic copper toxicity and in experimental animals copper deficiency can be induced by excessive supplementation of molybdenum. Tetrathiomolybdate (TTM) injection also releases the copper from metallothionines to raise bile and blood copper levels (Komatsu *et al*., 2000).

Earlier studies on the effect of molybdenum on lead-induced plumbism revealed the protective nature of molybdenum against heavy metal toxicity (Flora *et al*., 1993). The work carried out by Lai and Sugawara, (1997) by giving intra venous injection of tetrathiomolybdate in rats showed an increased biliary excretion of cadmium.

According to Sugawara *et al.* (1999), molybdenum affects the iron metabolism also. In his experiment, TTM treatment increased the hepatic iron concentration from 120ug/g to 250ug/g tissue.

NUTRITIONAL ASPECTS

Dietary supplementation of molybdate has been reported to affect the tissue concentration of molybdenum in rats (Ogra and Suzuki, 1998). The magnitude of tissue response to elevation in dietary molybdenum depends on concomitant inorganic sulfate, iron and copper levels (Lener and Bibr, 1984). Major part of tissue

molybdenum is presumably associated with molybdeno enzymes. Tissue levels of molybdenum can be altered by ingestion of foodstuffs grown in soil of high molybdenum content or experimentally supplemented diet (Rajagopalan, 1988). According to Chan *et al.* (1998), the blood concentration of molybdenum depends on the concentration and duration of exposure to molybdenum in rats and mice.

DEFICIENCY

Molybdenum form all food sources are not absorbed as well as molybdenum added into the diet. But molybdenum in some food is as available as extrinsically added molybdenum. Despite this difference, molybdenum is better absorbed than many other minerals and inadequate dietary molybdenum is unlikely (Turnlund *et al.*, 1999). True molybdenum deficiency has not been achieved in experimental animals. Early efforts to produce molybdenum deficiency using low molybdenum diets caused attenuation of rat liver xanthine oxidase activity to 10% of the normal level, without affecting the health of the animal much or changing the excretion of uric acid or allantoin. But increased excretion of xanthine and sulfite were also reported in man due to deficiency (Sumi and Wada, 1996).

There is also evidence indicating the importance of molybdenum in normal neurological functions. Sumi and Wada,

(1996) reported neurological symptoms such as severe convulsions in patients with molybdenum cofactor deficiency. Sardesai, (1993) reported neurological problems and mental retardation in molybdenum deficient conditions. Dandy-Walker malformation characterized by neurological abnormalities such as diffuse atrophy in cerebral hemispheres, delayed myelinisation and cerebral maturation hypoplasia of corpus callosum, multiple cystic cavities in the cerebral white matter, hypoplasia of cerebellum etc. is a condition associated with molybdenum cofactor deficiency (Arslanoglu *et al.*, 2001).

Deficiency of sulfite oxidase is mainly due to the deficiency of molybdenum, resulting in numerous adverse effects such as respiratory impairment, interference with immunological response and oxygen transport and platelet aggregation inhibition. It may also lead to alteration in DNA during replication, chromosomal aberrations and depression of DNA synthesis (Calabrese *et al.*, 1981).

Accumulation of iron in liver was also reported under molybdenum deficiency (Kelley and Amy, 1984). Amino acid intolerance, irritability, elevated urinary xanthine and sulfite and reduced uric acid and sulfate were other consequences of molybdenum deficiency (Abumrad *et al.*, 1981).

TOXICOSIS

Molybdenosis in Washington, Idaho, Montana, Wyoming and Colorado is a wide spread soil-related nutritional problem in grazing animals (Joe Kubota, 1975). The effect of excessive molybdenum intake on animals appears to depend on a number of variables, including the age, and species, the relative amount of molybdenum, copper and sulfite in the diet etc. Molybdenum toxicity is closely related to the soil level of molybdenum. Molybdenum concentration increases in the soil as the soil pH increases. A level of 10-20 ppm or more of molybdenum in legumes was used as an indicator of molybdenum toxicity and was a nutritional problem in cattle (Joe Kubota, 1975). The symptoms vary among species but may include failure to thrive, anemia, dermatogenic changes, anorexia and diarrhea. Ruminants such as cow and sheep show extreme sensitivity to dietary molybdenum levels as low as 2-30 ppm while horses and pigs can show tolerance to levels that are much higher (1000 ppm). Cattle grazed on forage plants with 10-20 ppm or more of molybdenum exhibit typical symptoms of molybdenum toxicity, and they respond to copper supplementation (Joe Kubota, 1975). In many ways symptoms resemble that of copper deficiency and treatment with supplemented copper usually reverses them. However symptoms may be produced where the dietary copper is normal but molybdenum content is considerably higher than normal (Rajagopalan, 1988).

It has been suggested that underlying biochemical events leading to molybdenum toxicosis takes place within the gastro intestinal tract, where the interaction among dietary copper molybdenum and sulfur is maximal (Rajagopalan, 1988). According to Huisingh *et al.* (1973) copper becomes unavailable when it interacts with molybdate to form the biologically inactive cupric molybdate. An alternate hypothesis proposed by Dick *et al.* (1975) stated that toxicity is the consequence of gut flora reducing sulfate or sulfur compounds to sulfides, followed by the sulfide reacting with molybdate to form thiomolybdate. Thiomolybdates are more toxic than molybdenum oxides when administered to animals that lack rumen. The protective action of copper against molybdenum toxicity in ruminants was explained by its forming a complex with thiomolybdate to render the molybdenum complex nutritionally ineffective. When the diet is low in copper, intestinal absorption of free thiomolybdate would occur followed by a reaction of the absorbed thiomolybdate with intracellular copper and other metals or proteins to produce the toxic effects. This theory would explain the differences in susceptibility between ruminants and non-ruminants subjected to the same dose of molybdenum in the diet. The lack of a rumen and associated flora would prevent the toxicity in rats except high concentration of molybdenum, while the presence of sulfide producing organism in the cow rumen would render the animal extremely susceptible to

thiomolybadte production. Variation in the copper intake would affect the level of thiomolybdates absorbed and determine the extent of observed toxicity.

Inhalation of molybdenum caused dose dependent increase in the degeneration of hyaline in the nasal olfactory epithelium and metaplasia of epithelial lining at the base of the epiglottis (Chan *et al.*, 1998). Molybdenum can affect the balance of other trace elements. Harmful effect of this element is more prominent in the kidneys and liver. Bones are also susceptible to the damage (Oguro *et al.*, 1996).

The genotoxicity at relatively high doses of molybdenum was reported both in vitro in human beings and *in vivo* in rats (Titenko-Holland *et al.*, 1998)

A negative effect of molybdenum was observed in male reproductive system of rats. Oral administration of molybdenum decreased organ to body weight ratios of testes epididymis, seminal vesicle and ventral prostate. Sperm abnormality associated with decreased sperm motility and sperm count was observed. In addition, accumulation of molybdenum in testes and epididymis was also reported (Pandey and Singh, 2002).

Molybdate is able to impair the sulfation of chemicals; because as an analogue of sulfate, it decreases the availability of sulfate thereby decreasing the synthesis of PAPS (3'- Phospho adenosine 5'-

phosphosulfate), the activated form of sulfate and the obligate co-substrate required for the sulfation of the chemicals (Boles and Klaassen, 2000).

Very few molybdenum toxicity studies have been reported in which sufficient molybdenum was used to cause death of the animals. One of the important mechanisms in molybdenum induced toxicity is the capillary trophic insufficiency syndrome caused by narrowing of capillaries. Significant, 30-55% narrowing of capillary diameter was observed in rats administered with large doses of molybdenum (Markarian and Meliksetian, 1998).

METABOLISM

Absorption: Little is known about the actual uptake of molybdenum in fresh water fish or other vertebrates. It has been demonstrated that *Escherichia coli* possesses three pathways that transport molybdenum with varying affinities. These include high affinity molybdenum transport system that is preferred and two alternative methods. 1) A sulfur transport system and, 2) non specific anion transport system. It is believed that molybdenum is taken up in these modes and transported in the form of simple molybdate ion Mo_4^{2-}. Freshwater fish showed a dose dependent accumulation of molybdenum in both gills and liver (Reid, 2002). According to him the route of uptake was most likely the gill. Molybdenum moves into

and across the gill, as molybdate, via the chloride bicarbonate exchanger. Absorption of hexavalent molybdenum compounds from the gastro intestinal tract was reported to be over a range of 25-75% of total molybdenum in humans (Vyskocil and Viau, 1999).

Body distribution: Molybdenum occurs in low concentrations in all tissue fluids of the body. Studies on the distribution of molybdenum in guinea pigs and rats, after a single oral administration of molybdenum trioxide show that there was an immediate accumulation in kidneys, liver, adrenal glands and bone (Lener and Bibr, 1984). Highest level was found in kidneys. In plasma, molybdenum is found in the form of molybdate, specifically bound to α-2macro globulins (Lener and Bibr, 1984). Binding of molybdenum to the protein, spectrin also occurs on the erythrocyte membrane.

Excretion: molybdenum is primarily excreted in the urine and to some extent in bile in humans. Normally small amount is excreted in feces. In a recent review Vyskocil and Viau, (1999) reported that in humans, urinary excretion is 17-80% of the total dose. Animal data indicate low retention and more or less complete excretion of molybdenum, primarily via urine (36-90%) in guinea pigs, rats, goats and swine (Fairhall, 1945; Neilands *et al.*, 1948; Vyskocil and Viau, 1999). The main route of excretion is via kidney, although fecal excretion and excretion through bile, milk and hair has been reported in ruminants (Friberg and Lener, 1986).

ROLE IN CARCINOGENESIS

Role of molybdenum in carcinogenesis is still not very clear. There are contradictory reports regarding this property of the element. According to Komada *et al.* (1990), the tumor incidence and tumor development in the esophagus were significantly lower in the rats supplemented with high levels of molybdenum (2 ppm). In these groups, high concentration of molybdenum was observed in esophagus together with an increase in the activity of the enzyme xanthine oxidase. Brewer *et al.* (2000) also showed that tetrathiomolybdate is an effective and suitable agent to impair neovascularisation in metastatic solid tumors and this ability of tetrathiomolybdate is due to its ability to achieve and maintain mild copper deficiency. Tetrathiomolybdate together with other antiangiogenic therapies was found to be effective against kidney cancer (Redman *et al.*, 2003).

But in contrast to the above reports, the incidence of alveolar/bronchiolar adenoma or carcinoma was marginally increased by inhalation of molybdenum trioxide in rats and mice (Chan *et al.*, 1998). Inhalation also caused increased incidence of lung lesion (Ozaki *et al.*, 2002). There is report indicating the increased concentration of molybdenum in squamous epithelial carcinoma cells (Tang *et al.*, 2000).

MOLYBDENUM IN AQUATIC ANIMALS

As for all other animals, molybdenum is an essential element for fish also. But the nutritional importance of this element in aquaculture has received least attention. However, there are many reports on the effects of molybdenum in fish. Less is known about the physiological impact of molybdenum in fish. It has been demonstrated that this metal is nontoxic for fish. Toxicity estimates of molybdenum to fresh water fish, based on 96 hour LC 50s ranges from 70-2000 mg per liter depending on size, species and test conditions (Easterday and Miller, 1963; Hamilton and Buhl, 1990). Clearly however exposure to sufficiently elevated levels of molybdenum can lead to fish mortality and yet little is known about the mechanism of toxicity and the physiological impact of sub lethal levels of molybdenum in fish. Natural sources of molybdenum in aquatic environment include the weathering of ores from igneous and sedimentary rocks and subsequent run off to streams and lakes. Important anthropogenic sources of molybdenum include the leaching process at or near molybdenum mines, contamination of water used for manufacturing process and the use of fertilizers containing molybdenum as growth promoters. Areas exposed to water drainage from agricultural subsurface were found to have higher molybdenum concentrations. Molybdenum concentrations of blue gills and common carp exposed to such drainage water had a whole body concentration of 2.8 and 3.6

ug/g dry weight of the tissue respectively (Saiki and May, 1988). But molybdenum is considered to be a relatively non-toxic element for aquatic organisms (Hamilton and Buhl, 1990). Despite the nontoxic nature of molybdenum, acute sub lethal exposure to this metal had physiological consequences to those fish exposed even for only a brief period (Reid, 2002). Further studies are needed to more fully elucidate the metabolism and mode of action of this metal in fish.

Molybdenum readily forms organometallic complexes in aquatic systems. Although valence states of +2 to +6 exist, the dominant form in water is tetra valent molybdenum sulfide (MoS_2) and hexavalent anions (Molybdate $MoO_4^{2-}$and bimolybdate $HMoO_4^-$) (Jarrel *et al.*, 1980).

COBALT

Cobalt (Co) is an element that occurs naturally in many different chemical forms throughout the environment. It makes up only 0.0025% of the earth's crust and usually occurs with nickel or sulfide or arsenic ores. Small amounts are present in rocks, soil surface, underground water, plants and animals. Cobalt is also released to the environment from burning coal and oil, from motor vehicle exhausts and also from industrial processes that use the metal or its compounds. Cobalt is required by living organisms in small quantities for good health.

BIOLOGICAL REQUIREMENTS

Cobalt is an essential trace element for ruminants. It is required for the rumen synthesis of vitamin B_{12} (Smith and Marston, 1970). Vitamin B_{12} is the physiologically active form of cobalamine. The natural diet of herbivores is devoid of vitamin B_{12}, so that ruminants are entirely dependent on the maintenance of a steady supply of cobalt for microbial synthesis of this vitamin in the rumen. Vitamin B_{12} acts as the cofactor for two enzymes namely methyl malonyl CoA mutase and methionine synthase (Kennedy *et al.*, 1992). Both of these enzymes have been linked to changes in the lipid metabolism. Impairment of methyl malonyl CoA mutase results in the derangement of fatty acid synthesis in cobalamine deficient animals (Kennedy *et al.*, 1992). Impairment of methionine synthase results in decreased methylation of ethanolamine phosphoglyceride and has been shown to alter tissue lipid levels (Kennedy *et al.,* 1992). Functions of Vitamin B_{12} such as synthesis of DNA, production of RBC, maintenance of nerve function and detoxification of cyanide are mediated through cobalt.

Methionine synthase is necessary for the transfer of methyl group from methyl tetra hydrofolic acid to homocysteine, in order to from methionine. Deficiency of cobalt or vitamin B_{12} will result in

reduced methionine synthase activity, which can lead to neuropathological changes (Frank *et al.,* 2004).

Extensive investigations have been carried out on the effect of dietary Co on ruminants. Tomlinson *et al.* (2003) suggested at least 3 possible modes of action for Co on ruminants. 1) Supplemented dietary Co could increase ruminal synthesis of Vitamin B_{12} 2) Enhance the activity of fiber digesting bacteria and therefore increases ruminal cellulose digestion or 3) there may be a metabolic role for Co that is yet to be recognized. They reported that milk production efficiency increased with increased Co supplementation.

Cobalt has a protective role against diabetes. The work conducted by Ybarra *et al.* (1997) explained that glycemia lowering effect was observed in rats treated with Co (II), which is not mediated by the regulation of insulin, but by enhanced expression of GLUTI (glucose transporter) mRNA expression.

Tissue level of Co depends on area, age and gender of the animal (Webb *et al.*, 2001). The accumulation of this element in myocardium, sarcoplasmic reticulam and mitochondrial membrane was reported (Clyne *et al.*, 1990). Persson *et al.* (1992) reported that cobalt binds with the Ca-ATPase in sarcoplasmic reticulumof myocardium. Van Rysson *et al.* (1987) and Brown and Southern, (1985) found out a direct correlation with tissue level and

supplementary Co levels. Co also has a tendency to accumulate in Calcium storing organelles, which would affect the Ca flux in myocardial cells and secondarily tension development in cardiac muscles (Clyne *et al.*, 1990). The other major accumulation sites of Co are liver and kidney (Southern and Baker, 1981). According to Persson *et al.* (2003), cobalt entered into the body by inhalation is found to be highest in the kidney, followed by the liver and pancreas.

Increase in cobalt supply increased tissue concentration of cobalt in different plant parts. Total cobalt content was also increased. Roots had the maximum concentration of cobalt (Tewari *et al.*, 2002)

METABOLISM

***Absorption*:** Cobalt is mainly absorbed from the pulmonary and the gastrointestinal tracts. Absorption through the skin can occur but is low. Concomitant exposure to tungsten carbide increases the pulmonary absorption rate of cobalt metal (Lauwerys and Lison, 1994). The degree of gastro intestinal absorption depends on the dose. Very small doses are absorbed almost completely; where as larger doses are absorbed to lesser extent. Soluble forms are found to be absorbed readily. Iron deficiency led to increased absorption of Co from the gastrointestinal tract, relative to iron-sufficient animals (Flanagan et al., 1980).

Cobalt injected subcutaneously was transferred very rapidly to kidneys via blood and excreted into urine without accumulating in renal tissues (Horiguchi *et al.*, 2004). The metal entering into the olfactory mucosa is taken up into the primary olfactory neurons and transferred in these neurons to the olfactory bulbs (Persson *et al.*, 2003). Cobalt is absorbed by a mechanism similar to that of calcium or it can be a non-specific or adsorptive fluid-phase endocytosis (Persson *et al.*, 2003).

Body distribution: Liver has been found to be the organ containing highest level of cobalt. This may be attributed to the storage of vitamin B_{12} in liver. In addition to liver, cobalt was found distributed in bile, kidney and tibia in chicks (Brown and Southern, 1985).

Excretion: Co is excreted through both urine and the feces, with absorbed Co being excreted largely in the urine. Fecal excretion is more important for homeostatic regulation at a higher dietary cobalt concentration. Nevertheless the elimination of excess cobalt mainly takes place by adjusting urinary excretion (Kirchgessner *et al.*, 1994).

DEFICIENCY

Various deficiency symptoms have been reported in animals due to insufficient quantity of Co in the diet. Most important factor affected by Co deficiency is Vitamin B_{12} (Mburu *et al.*, 1993). The deficiency symptoms observed in Co deficiency is characterized by

pernicious anemia, nerve disorders and abnormalities in cell formation as observed in Vitamin B_{12} deficiency.

Proteins are the other biomolecule affected by Co deficiency. Co deficiency causes decreased protein synthesis and relatively impaired rate of secretion of protein from liver to serum (Mallik, 1989). The synthesis of methionine was affected by cobalt deficiency as the activity of the enzyme methionine synthase was affected in Co deficient condition (Kennedy *et al.*, 1992).

Lipid metabolism was also affected by Co deficiency. The activity of S-adenosyl methionine (SAM) dependent methyl transferase was affected by Co deficiency (Kennedy *et al.*, 1992). This enzyme is involved in methyl group transfer reaction, essential in phospholipid synthesis. As a result, cobalt deficiency leads to lower levels of phospholipids in the tissues.

Co deficiency affects thyroid hormone status also by reducing the activity of type I thyroxine de-iodinase enzyme, bringing out a significant reduction in tri iodothyronine level in serum. Low folate level and reduced levels of heme-dependent parameters are also observed during Co deficiency. Deficiency also causes dramatic accumulation of the trace elements iron and nickel in livers.

It has been established that the immune status of the animals depends on the availability of cobalt. Cattle treated with diet low in

cobalt showed a significant reduction in their immune status with in 10 weeks (Paterson and Mac Pherson, 1990).

Abnormal fermentation was reported by McDonald and Suttle (1986) in continuous cultures of rumen microorganisms given Co deficient food substrate. Production of cobalamine and Vitamin B_{12} analogue decreased significantly in these groups.

Deficiency of caobalt in goats reduces the rumen microorganisms, decreases the intestinal absorption of nutrients, and reduces the synthesis of vitamin B_{12}-dependent methyl malonyl coenzymeA mutase and methionine synthase, the two enzymes essential for protein and energy metabolism. All these factors ultimately results in lowering of the apparent digestibility coefficients in goats (Kadim *et al.*, 2003).

The moose sickness observed in nova Scotia moose is due to cobalt deficiency. Reduction of supplementation will eventually restrict the synthesis of vitamin B_{12} and consequently metabolic disturbances such as increase in plasma methylmalonic acid (MMA) and homocysteine levels (Frank *et al.*, 2004)

TOXICITY

Under practical conditions cobalt deficiency is more likely than cobalt toxicities. Nevertheless, in supplementation to prevent a

deficiency, accidental over supplementation is possible, which will produce deleterious effects. Toxicity can also occur from excess inorganic Co found as a food contaminant. Co introduced into the beer also causes toxicity. Co released into the environment by various anthropogenic activities also contributes to the toxicity. Domingo, (1989) has reviewed the toxological implication of Co in the environment.

The early toxic effect of Co was primarily related to the decreased efficiency of the light reactions of photosynthesis in green alga *Chlorella pyrenoidosa*, which further reduced the alga productivity (Plekhanov and Chemeris, 2003).

Excess concentration of the cobalt in the growth medium of plant produced visual symptoms of toxicity that intensified with increasing level and duration of metal supply. The tissue concentration of cobalt increases with increasing level of supply. There was a strong induction of oxidative stress due to excess cobalt in the growth medium. Cobalt reduces the activity of catalase, an iron enzyme by interfering in iron metabolism of plants (Tewari *et al.*, 2002). They have observed that high concentrations of cobalt (in the range of 300-400μM) will enhance generation of reactive oxygen species (ROS). The potential mechanism for Co toxicity is thought to be through oxidant based and free radical based processes (Leonard *et al.*, 1998;

Tewari *et al.*, 2002). Exposure to soluble Co increases indices of oxidative stress including diminished levels of GSH (Christova *et al.*, 2003) and free radical mediated DNA damage (Baldwin *et al.*, 2004). Co metal particles and Co ions were found to react with H_2O_2 to form reactive species under physiological condition, which can hydroxylate aromatic compounds and degrade deoxyribose to TBA reactive material (Moorhouse *et al.*, 1985; Lison and Lawverys, 1993).

There are conflicting reports regarding the production of reactive oxygen species by Co particles (Lison and Lavwerys, 1993). But it is evident in many experiments conducted in animals that Co administration produced a much higher degree of lipid peroxidation (Christova *et al.*, 2003; Christova *et al.*, 2001). The intra hepatic GSH content and anti oxidant enzymes were found to be altered by Co administration in guinea pigs. Acute Co loading caused decreased activities of the enzymatic scavengers of free radicals such as SOD, CAT and GPx (Christova *et al.*, 2002). The toxicity of Cobalt observed in alveolar macrophages also points towards oxidant-mediated toxicity (Hoet *et al.*, 2002).

Co also affects the Cu assimilation at elevated levels. Excess Co produces goiter and reduced thyroid activity (Chang *et al.*, 2001). Exposure to Co may also result in an allergic contact dermatitis and

occupational asthma (Meier *et al.,* 1999). This may also lead to severely impaired lung function and cardiovascular effects.

Elevated level of Co had a negative impact on the reproductive performance of cow (Olson *et al.*, 1999). Mollenhauer *et al.* (1985) worked out the effect of dietary Co on testicular structure. They observed a progressive deterioration of cell architecture and decrease in testicular volume. But according to them testicular degeneration is not a primary response to Co, but due to hypoxicity of testes due to both blockage of veins and arteries by RBCs and due to changes in permeability caused by thickening of basal lamina and basement membrane. Consistent degenerative and necrotic lesions in the seminiferous tubules were also reported in rats by Corrier *et al.* (1985).

Intoxication with cobalt caused reduced feed intake, body weight gain, gain/feed, followed by a dose dependant increase in mortality in chicks (Diaz *et al.*, 1994). Polycythemia is another abnormality observed with elevated cobalt levels (Corrier *et al.*, 1985).

In plants (tomato) excess cobalt induced iron deficiency by developing chlorosis on young leaves and decreasing the biomass, concentration of iron, chlorophyll a and b, photosynthesis and activity of catalase (Chatterjee and Chatterjee, 2003). The inhibitory role of

excess cobalt was discernible on elevated activity of peroxidase, acid phosphatase, ribonuclease and accumulation of cobalt, carbohydrate and phosphorus fractions (Chatterjee and Chatterjee, 2003).

Acute exposure to cobalt can cause hemolysis in glomerular capillaries of rats, leading to gross methemoglobinuria (Horiguchi *et al.*, 2004). Cobalt injection induces acute and transient hemoconcentration resulting from an increase in vascular permeability and decrease in plasma volume and, protein or alteration of fibrinogen confirmation to form jelly like plasma. This will cause the stagnation of RBCs in glomerular capillaries after cobalt exposure, leading to mechanical degradation of RBCs (Horiguchi *et al.*, 2004).

Toxic effect of excess cobalt results in failure of emergence and unfolding of young emerging leaves in plants, chlorosis of subtending younger leaves, restricted expansion of laminar and decreasing growth and dry matter production (Tewari *et al.,* 2002). Excess cobalt despite producing metabolic lesions including induction of oxidative stress produced visible toxicity effects at 300 to 400 uM cobalt supply, also decreased function of iron in plants due to its precipitation as phosphate or sequestration in ferritin (Tewari *et al.,* 2002).

Cobalt is a neurotoxic metal, which will cause memory deficiency among workers exposed to hard metals via inhalation (Persson *et al.*, 2003). The hard metal deposited in the nasal passages

will release cobalt which will be taken in to the brain via the olfactory path way may cause neurotoxicity (Persson *et al.*, 2003). Treatment of cells with cobalt generates DNA strand breaks and covalent protein-DNA complexes in cultured cells (Baldwin *et al.*, 2004).

ROLE IN CARCINOGENESIS

Co (II) ions are genotoxic in vitro and in vivo, and carcinogenic in rodents. Hard metal dust, of which occupational exposure is linked to an increased lung cancer risk, is proven to be genotoxic in vitro and in vivo. Possibly, production of active oxygen species and/or DNA repair inhibition are mechanisms involved (De Boeck *et al.*, 2004).

COBALT IN AQUATIC ANIMALS

Little work has been done regarding the importance of cobalt in aquaculture. But vitamin B_{12} is required for normal maturation and development of erythrocytes, synthesis of methyl compounds, normal cholesterol metabolism and in purine and pyrimidine synthesis (Paulraj, 1997). Since cobalt is essential for the synthesis of this vitamin, it is necessary to supplement Co through diet. Dietary supplementation of cobalt as cobalt chloride increased the growth rate, FCR and PER in Tilapia (Anadu *et al.,* 1990). This element can enter into the body by translocation through gill epithelium (Comhaire *et al.,* 1994). The kidney was the most contaminated organ, in cobalt exposure in Traut, followed by the viscera, head, gills, and liver. Co

concentration was by far the lowest in the muscle, which accounted for about 45% of the total body weight (Baudin *et al.,* 1997). Hence, both survival and behavior were changed in fishes by the excessive concentration of Co2+ (Janssen, 2000).

Cobalt was a slower acting and less potent toxicant. It was recorded that the estimated incipient lethal level of cobalt was 346 ug/ltr for 50% mortality of rainbow trout. (Marr *et al*., 1998). Cobalt is lethal to rainbow trout fry at concentration> 50ug/ltr (Marr *et al*., 1998).

Hyperlacticemic effect was observed in freshwater tropical teleost *Colisa fasciatus* by cobalt intoxication at the sub lethal dose of 232.8 ppm or 9.743 × 10 4 moles/l (Nath and Kumar, 1988). Accumulation of cobalt by fish eggs was described by Kunz *et al.* (1978). The cobalt level of the eggs was found to be directly proportional to the cobalt content of the aquatic medium, and its uptake decreased with increasing calcium ion concentration in the water. The accumulation of bivalent cobalt ions was found to take place on the surface of the eggs, where they are reversibly bound by the binding force of chorion-mucopolysaccharide Kunz *et al.* (1978).

A 14-day toxicity test with rainbow trout, conducted by Marr *et al.* (1998) to evaluate, (1) the acute toxicity of Co and Cu, and (2) the effects of selected Co concentrations on the toxicity of Cu in Co/Cu

mixtures showed that Co was a slower-acting and less potent toxicant than Cu. For Co, the incipient lethal levels (ILL) for 50% mortality were 346μg per liter; for Cu, the ILL for 50% mortality was 14μg per liter. Moreover, in Co/Cu mixtures, Co acted as an antagonist during the first 48–96 h, but later acted as an additive or slightly synergistic toxicant—making it difficult to predict short-term mortality of fish in Co/Cu mixtures.

SCOPE OF THE PRESENT INVESTIGATION

The most important factor in successful aquaculture is the supply of nutritionally balanced feed. This is often found to be difficult as aquatic environment has its own limitations and problems. But the constant efforts of researchers had lead to considerable advances in this field. But almost all the works were concentrating on the macronutrients. Micronutrients research is still in its infant stage. Natural occurrence of trace elements in the aquatic environment together with the ability of fish to imbibe and absorb these elements from the surrounding environment which makes it difficult to fix an optimum dosage for each element. Anthropogenic activities also contribute to the mineral content of the environment. However the additional supplementation of trace element through diet is found to be worthy of consideration. The biochemical impact of these elements and their toxic limits are also important for formulating the diet.

Selenium, molybdenum and cobalt are three trace elements, which are found to be essential for living beings. But, considering the case farmed aquatic animals, the roles of these elements are still not clear. Although some work has been reported on the impact of selenium on aquatic animals, in the case of molybdenum and cobalt this is almost a virgin area.

Selenium is the component of the important antioxidant enzyme GPx. Molybdenum also forms co-factor for certain important metabolic enzymes such as xanthine oxidase and sulfite oxidase. The only one vitamin, which contains a metal, is vitamin B_{12}. Here cobalt forms the metal portion and this vitamin plays important roles in metabolism of various macromolecules.

Fish may require these elements for their healthy growth. Dietary supply of these minerals may enhance the growth performance of the species under culture. Similarly, elevated levels may be deleterious to the animal. So an attempt has been made by the present study to analyze the impact of these three elements on growth and biochemistry of the shrimp *P. monodon*.

Materials and Methods

3. MATERIALS AND METHODS

3.1 ANIMALS. *Penaeus monodon*, the most important farmed species of prawns in India was selected for the study. The post larval forms of the shrimps were maintained in large tanks and allowed a normal larval diet obtained from Higashimaru Feeds India Limited, Cochin, a commercial source, *ad libitum* till attaining a weight of 1-1.5 g.

3.2 DIETS. A control feed was formulated from normal fish feed ingredients (Table1) After analyzing the composition of the formulated diet, the different experimental diets were prepared from the basal diet by supplementing the specified trace elements (selenium, molybdenum and cobalt) in inorganic form (Sodium selenite, ammonium molybdate and cobalt sulfate) as per the required levels.

3.3 EXPERIMENTAL PROTOCOL. Separate experiments were conducted for all the three elements. Three different concentrations of each element were selected for the study: one lower concentration (0.5 ppm), one medium concentration (2.5 ppm) and one higher concentration (5.0 ppm).

Shrimps weighing 1-1.5 g were transferred from the main tank to 100 liter capacity tanks. Initially the animals were allowed to acclimatize for a period of 10 days. The temperature, pH and salinity

of the water were maintained as 27°C, 7 and 15 ppt respectively. A continuous circulation of the water was also maintained.

Table 1: Ingredients of the experimental diet

Ingredients	Quantity (g/kg)
Fish meal Danish	250
Fish meal Indian	100
Shrimp head meal	50
Maida	350
Soya flour	160
Wheat gluten	20
Yeast	10
Fish oil	12
Lecithin	25
Mineral premix*	1.0
Vitamin premix**	2.9
Calciumpropionate (Antifungal agent)	4.0
BHT (Antioxidant)	0. 1
Disodium phosphate	15

* Composition given in table 2

** Composition given in table 3

Table 2: Components of Mineral Mix Used in Experimental Diet

Mineral	Quantity (mg/Kg feed)
Copper	3
Iron	50
Magnesium	100
Manganese	10
Iodine	1
Zinc	15
Cobalt	2
Phosphorus	1.65

Table 3: Components of vitamin mix used in experimental diet

Vitamin	Quantity (per Kg feed)
Vitamin A	8000 IU/Kg
Vitamin D	1600 IU/Kg
Vitamin E	160 mg/Kg
Vitamin K	16 mg/Kg
Thiamine	32 mg/Kg
Roboflavine	16 mg/Kg
Pyridoxine	32 mg/Kg
Pantothenic acid	60 mg/Kg
Niacin	160 mg/Kg
Biotin	0.6 mg/Kg
Folic acid	6.0 mg/Kg
Vitamin B_{12}	0.04 mg/Kg
Inositol	240 mg/Kg
Vitamin C	250 mg/Kg

At the start of the experiment the initial weight and length of the animals were taken and the animals were divided in to four groups for experimental purpose. Group I animals were the controls and received the control feed. Groups II, III and IV were experimental animals and received the experimental diet supplemented with the specified trace elements at 0.5 ppm, 2.5 ppm and 5.0 ppm levels respectively. The number of animal was restricted to 6 per tank and three tanks were kept for each group. The animals were provided with a ration equivalent to 10% of their body weight from four equal feedings every day. The fecal matter and left over feed were removed every day by siphonation and were dried separately in hot air oven to check the feed efficiency and growth parameters. The animals were weighed once in every 10 days.

Feeding trial was conducted for a period of 60 days. At the end of the experimental period, the animals were killed by decapitation. Hepatopancreas and muscle tissue were immediately excised and washed in ice-cold saline. Accurately weighed tissues were homogenized in 0.1M Tris-HCl buffer, with 7.4 pH, in Kinematics homogenizer and centrifuged using REMI, R4C Laboratory centrifuge and the supernatant was used for the assay of enzyme activities and estimation of cellular macromolecules. Parts of the tissues were also taken to determine the proximate composition, amino acid composition, fatty acid profile and metal content.

3.4 METHODS

3.4.1 BODY WEIGHT GAIN (%)

The average weight gain percent was calculated according to the following formula.

$$\text{Body weight gain } \% = \frac{Wt\text{-}Wo}{Wo} \times 100$$

Where, *Wo* is the weight at the start of the experiment and *Wt* is the weight at the end of the experiment

3.4.2 FEED CONVERSION RATIO (FCR)

Feed conversion ratio was calculated according to the following formula (Pratap, 1999)

$$FCR = \frac{\text{Feed consumed}}{\text{weight gain}}$$

Where, feed consumed = Total feed given – leftover feed; weight gain = Wt-Wo

3.4.3 FEED EFFICIENCY RATIO

Feed efficiency ratio was calculated by the formula (Pratap, 1999),

$$FCE = \frac{\text{Weight gain}}{\text{Feed consumed}}$$

Where, feed consumed = Total feed given – leftover feed; weight gain = Wt-Wo

3.4.4 SPECIFIC GROWTH RATE (SGR)

Specific growth rate is given as, $SGR = \frac{\ln W_t - \ln W_o \times 100}{t}$

Where, $\ln W_o$ is the natural log of initial weight; $\ln W_t$ is the final weight and t is the time in days (Pratap, 1999)

3.4.5 PROTEIN EFFICIENCY RATIO (PER)

Protein efficiency ratio was calculated according to the formula (Murthy, 2001),

$$PER = \frac{\text{Weight gain}}{\text{Protein consumed}}$$

Where weight gain = Wt-Wo; Protein consumed = protein content of the feed given – protein content of the leftover feed and fecal matter

3.4.6 MORTALITY

Mortality was calculated according to the following formula

$$\text{Mortality} = \frac{\text{No of animals dead} \times 100}{\text{Total No of animals}}$$

3.4.7 DETERMINATION OF MOISTURE CONTENT

Moisture content was determined by standard AOAC procedure (1975)

Weighed 4-5 g of the sample in a covered, flat, dish. Dried to constant weight at 100-105°C in a drying oven.

$$\text{Moisture content (\%)} = \frac{\text{Weight of fresh sample} - \text{weight of dry sample} \times 100}{\text{Weight of the sample}}$$

3.4.8 DETERMINATION OF CRUDE PROTEIN

Crude protein was determined by AOAC procedure (1975)

3.4.8.1 Reagents

1. Sulfuric acid (98%), nitrogen free,
2. Digestion mixture
3. Distilled water
4. Mixed indicator
5. Boric acid 2%
6. Sodium hydroxide 40%
8. Standard sulfuric acid solution (0.01N).

3.4.8.2 Procedure

Weighed out 0.1 g of the tissue in to the Kjeldahl flask. 10 ml concentrated sulfuricacid together with 1 g of digestion mixture were added. Some beads were introduced into this to prevent bumping out of the sample and digested continuously by heating till the sample became colourless. Made up the sample solution in to a known volume with distilled water. 5 ml of the made up solution was distilled in microkjeldahl apparatus and the distillate was collected into 2% boric acid solution containing one drop of the mixed indictor. It was

then titrated against 0.01N sulfuric acid. The protein content of the sample was calculated assuming that total nitrogen content will constitute about 16% of total protein.

3.4.9 DETERMINATION OF CRUDE FAT

Crude fat content was determined by AOAC procedure (1975)

3.4.9.1 Reagents

1. Petroleum ether (b.p. 60-80oC),

3.4.9.2 Procedure

Two g of the dried sample was weighed in to an extraction thimble, (residue from dry matter was used). The thimble was placed inside the Soxhlet apparatus. A dried pre-weighed solvent flask was connected beneath the apparatus. After adding the required quantity of solvent, the apparatus was connected to the condenser. Extraction was done for 16 hrs. On completion, thimble was removed and removed the ether completely. The flask was dried at 105°C for 30 min. It was then cooled in a desiccator and weighed.

Calculation

$$\text{Crude fat (\%)} = \frac{\text{Weight of fat x 100}}{\text{Weight of sample}}$$

3.4.10 ASH

Ash content was determined by standard AOAC procedure (1975)

3.4.10.1 Procedure

2 g of the sample was weighed into a dry, porcelain dish and then placed in a muffle furnace at 600°C for 6 hrs. It was then cooled and weighed.

Calculation

$$\text{Ash (\%)} = \frac{\text{Weight of ash x 100}}{\text{Weight of the sample}}$$

3.4.11 ESTIMATION OF TOTAL PROTEIN

The protein content in the muscle tissue and hepatopancreas was determined by the method of Lowry *et al.* (1951)

3.4.11.1 Reagents

1. Alkaline copper reagent

   Solution A: 2% sodium carbonate in 0.1N sodium hydroxide solution.

   Solution B: 0.5% copper sulfate in water.

   Solution C: 1% sodium potassium tartrate in water. 50 ml of solution A was mixed with 0.5 ml of solution B and 1 ml of solution C just before use.

2. Folin's phenol reagent: 100 g sodium molybdate, 700 ml water, 50 ml 85% O-phosphoric acid and 100 ml concentrated hydrochloric acid, taken in a 500 ml round bottomed flask were refluxed gently for 10 hrs. 150 g lithium sulfate, 50 ml water and a few drops of bromine were added to this mixture. It was then cooled and diluted to 1 liter and filtered. Prior to use, the solution was diluted in 1:2 with double distilled water.

3. Standard Bovine serum albumin (BSA): Dissolved 100 mg of BSA in distilled water and made the volume to 100 ml using distilled water. From the stock, 10 ml was diluted to 100 ml to get a working standard of 100 μg/ml.

3.4.11.2 Procedure

Pipetted out 0.2 ml of tissue homogenate and standard BSA in the range of 20-100 μg in to test tubes and made the volume up to 0.5 ml with distilled water. A blank was run by adding 0.5 ml distilled water. 2.5 ml of alkaline copper reagent was added to this tubes and left for 10 minutes. Added 0.25 ml of Folin's phenol reagent into the tubes and kept for 30 minutes to develop the colour. The absorbance was read at 640 nm against the reagent blank in Shimadzu-UV spectrophotometer.

Protein values are expressed as mg/g (tissue).

3.4.12 DETERMINATION OF AMINOACID CONTENT

Amino acid analysis was done using Shimadzu High-Performance Liquid Chromatograph (LC-4A) "Amino Acid Analysis System" (Ammu *et al.*, 1994).

3.4.12.1 Separation

Styrene-divinyl benzene copolymer with sulfinic-group i.e. strongly acidic cation exchange resin was used for the separation of the amino acids.

3.4.12.2 Detection

O-phthaladehyde reacts with compound with –NH2- functional group (primary amino acids) into strongly fluorescent substance in the presence of 2-mercaptoethanol. In addition, when sodium hypochlorite is added, imino acids such as proline react with OPA. These reactions were used for detection in this system.

3.4.12.3 Reagents

1. Hydrochloric acid- 6N
2. Hydrochloric acid- 0.05N

4. Buffer A: Sodium citrate, 58.8g dissolved in 2.5 liter distilled water and then 210 ml of ethanol and 50 ml perchloric acid were added. This was made up to 3 liter with distilled water.

5. Buffer B: Sodium citrate tribasic, 58.8 g and boric acid 12.4 g were dissolved in 800 ml distilled water and then 45 ml of 4N NaOH were added. This was made up to 2 liter with distilled water.

3.4.12.4 *Procedure*

100 mg of the muscle tissue was weighed into a test tube. In to this added 10 ml of 6N HCl. Evacuated and sealed the test tube under nitrogen. Hydrolyzed the sample at 110°C for 24 hrs. Filtered the hydrolyzate quantitatively into a round bottom flask using Whatman No 42 filter paper and evaporated in a vacuum flash evaporator. Repeated the evaporation 3 times with distilled water to remove the acid completely. Made up the sample to 10 ml with 0.05M HCl. Before injecting into the HPLC, the sample was filtered using Whatman membrane filter (0.45μm, nylon filters). Calibration was done using authentic standards.

3.4.13 DETERMINATION OF TRYPTOPHAN

Tryptophan was estimated by colorimetric method according to Sastry and Tammuru, (1985)

3.4.13.1 Reagents

1. Sodium hydroxide (NaOH) 5%
2. Phenolphthalein indicator 0.1%

3. Sucrose 2.5 %
4. Thioglycolic acid 0.6% in distilled water
5. Sulfuric acid 50 %
6. Hydrochloric acid 0.1 N

3.4.13.2 Procedure

Weighed 0.3 g of the tissue into a test tube and added 10 ml of 5% NaOH into this. Evacuated and sealed the tube under nitrogen. Hydrolyzed at 110°C for 24h in a hot air oven. Transferred the content in to a beaker and neutralized with 0.1N HCl using phenolphthalein as indicator followed and made up to 100 ml. The made up sample was filtered through Whatman No 42 filter paper. Heated a mixture of 4 ml of 50% sulfuric acid, 0.1 ml of thioglycolic acid and 0.1 ml of sucrose solution, in a water bath at 47°C for 7 minutes. After cooling, 0.2 ml of the sample was added into this and made-up to 5 ml using 0.1N HCl. The reaction mixture was kept for an incubation period of 10 minutes and read the colour developed against the blank at 500 nm in a Shimadzu UV-1601 spectrophotometer. A series of standards was also done along with the samples.

3.4.14 LIPID EXTRACTION

Lipid extraction was done according to the method of Bligh and Dyer (1959).

3.4.14.1 Reagents

1. Chloroform methanol mixture 2:1 v/v

3.4.14.2 Procedure

10g of the tissue was weighed and minced well. The minced tissue was homogenized in chloroform methanol mixture. Filtered through Whatman No1 filter paper. Repeated the whole procedure for 2 times with 50 ml of chloroform methanol mixture. Transferred the filtrate into a separating funnel and 20% (v/v) distilled water was added to this filtrate. The mixture was allowed to stand overnight. The lower phase was dried using anhydrous sodium sulfate and filtered quantitatively. The filtrate was evaporated under vacuum and made up the content of the flask to a 10 ml using chloroform.

3.4.15 DETERMINATION OF THE FATTY ACID PROFILE

Methyl esters were prepared by the method of Metcalfe *et al.*, (1966)

3.4.15.1 Reagents

1. Alcoholic KOH 5%
2. BF3 Methanol
3. Petroleum ether (b.p 60-80)
4. Purified dry anhydrous sodium sulfite

3.4.15.2 Procedure

Weighed and transferred 0.2 g of lipid into a round bottom flask. Added 5 ml of alcoholic KOH and refluxed for 5mts. This was followed by addition of 6 ml BF3 methanol and refluxing for 5 minutes. The sample was cooled and transferred to 100 ml separating funnel. 25 ml of the petroleum ether was added into this. After mixing with water, 20% (v/v) the sample was kept for some times to get the two layers separated. The upper layer was collected. Lower layer was extracted again with petroleum ether 3 times with 25 ml each. The extract was then washed in distilled water and evaporated to almost dryness. All procedures were done in an inert atmosphere of nitrogen. The evaporated sample was collected into small vials and filled with nitrogen and kept at $0^{o}C$ till analysis.

The analysis was done using Chrompack CP 9001, Gas Liquid Chromatograph (GLC), using packed column, 2mx1/8 , packed with 15% OV 275, on chromosorb w, flame ionization detector and carrier nitrogen. Identification and quantification of the fatty acids were done using authentic standards.

3.4.16 ESTIMATION OF THE EXTENT LIPID PEROXIDATION IN MUSCLE AND HEPATOPANCREAS

The extent of lipid peroxidation was determined by the method developed by Ohkawa *et al.*, (1979).

3.4.16.1 Reagents

1. Acetic acid: 20%, pH 3.5
2. Sodium dodecyl sulphate: 8.1% in distilled water
3. Thiobarbituric acid: 0.8% in 20% acetic acid
4. n-butanol-pyridine mixture : 15:1,v/v
5. Standard solution: 16 mg of 1,1',3,3'-tetramethoxy propane was accurately weighed and dissolved in 100 ml of distilled water. Further dilution was made, so as to contain 2 n moles per ml.

3.4.16.2 Procedure

In to 0.2 ml of the sample, added 1.5 ml acetic acid, 0.2 ml SDS, 1.5 ml TBA and made the volume up to 4 ml with double distilled water. A series of standards and a reagent blank by adding distilled water instead of sample was run along with this. The tubes were heated for 60 minutes in a boiling water bath. After cooling, 4.0 ml of n-butanol-pyridine mixture was added and shaken well. Centrifuged the sample at 3000 x g for 10 minutes. The organic layer was taken and read at 532 nm.

The lipid peroxide content was expressed as n mols of malondialdehyde per mg of protein.

3.4.17 DETERMINATION OF TOTAL REDUCED GLUTATHIONE CONTENT

The method of Ellman (1959) was adopted to determine the reduced glutathione (GSH) in the muscle tissue and hepatopancreas.

3.4.17.1 Reagents

1. DTNB (5,5'-dithiobis 2-nitrobenzoic acid): 0.6 mM in 0.2 M phosphate buffer, pH 8.0,
2. Phosphate buffer, 0.2 M, pH 8.0: 4.08g potassium dihydrogen phosphate and 1.1g sodium hydroxides were dissolved in 300ml distilled water.
3. Trichloroacetic acid: 5%
4. Standard solution: A standard solution of GSH was prepared by dissolving 10 mg GSH in double distilled water and making up the volume to 100 ml using double distilled water. This solution was further diluted to get a concentration of 10 µg/ml, which was used as the working standard.

3.4.17.2 Procedure

Protein form of the tissue was precipitated by adding (0.5 ml) 5% TCA to tissue homogenate. The contents were mixed well for

complete precipitation and centrifuged. 1.8 ml of phosphate buffer and 2.0 ml DTNB were added to 0.2 ml of the sample (supernatant). Adding 0.2 ml TCA ran a reagent blank. The absorbance was read at 412 nm against the blank. A series of standards were run to determine the concentration of the GSH.

The amount of GSH was expressed as n moles/mg protein.

3.4.18 ESTIMATION OF *VITAMIN B_{12}* IN THE HEPATOPANCREAS

Vitamin B_{12} was determined according to the method of De Carneri (1955)

3.4.18.1 Reagents

1. Sodium cyanide
2. Sodium sulfite
3. Benzyl alcohol
4. Chloroform
5. Potassium dihydrogen phosphate

3.4.18.2 Procedure

1 mg of crystalline sodium cyanide was added into 2 ml of the tissue homogenate. It was allowed to stand for a period of 5 hrs at pH 10. Added 0.4 g of sodium sulfite into this, and extracted with 15 ml of benzyl alcohol in three times (5mlx3). The extract was mixed with

7.5 ml of chloroform. Extraction was repeated with total 12.5 ml distilled water 3 times. The aqueous extract was made to 12.5 ml with distilled water. Into 5 ml of the extract added 1 ml of 10% sodium cyanide and mixed another 5 ml with 1 ml of 12.5% potassium dihydrogen phosphate. A seies of standards were also run. The amount of Vitamin B_{12} present can be determined from the difference in the absorbance 582 nm.

3.4.19 DETERMINATION OF TOTAL SULFHYDRYL (THIOL) CONTENT

Total sulfhydryl content was determined by the method of Sedlak and Lindsay (1968).

3.4.19.1 Reagents

1. Tris-HCl buffer 0.2 M, pH 8.2 with 2 mM EDTA
2. Tris-HCl buffer 0.4 M, pH 8.9
3. DTNB 99mg/25 ml methanol
4. Reduced glutathione

3.4.19.2 Procedure

0.5 ml of the tissue homogenates were mixed with 1.5 ml of 0.2M Tris buffer, pH 8.2, and 0.1 ml of DTNB. This was brought to 10 ml with 7.9 ml of absolute methanol. Prepared a reagent blank (without sample) and sample blank (without DTNB) in a similar

manner. Stoppered the tubes with rubber caps and allowed to stand, with occasional shaking, for 30 minute. It was then centrifuged. The absorbance of clear supernatant was read at 412 nm.

3.4.20 ASSAY OF SELENIUM DEPENDENT GLUTATHIONE PEROXIDASE (EC 1.11.1.9)

Selenium dependent Glutathione peroxidase was assayed by the method of Pagila and Valentine (1967).

3.4.20.1 Reagents

1. Phosphate buffer, 0.1 M, pH 7.0: 5.4g potassium dihydrogen phosphate and 931.2mg sodium hydroxide were dissolved in 100ml distilled water
2. EDTA: 1.0 mM,
3. Glutathione reductase: Prepared from commercial suspension 2.4 units/ml of 0.1M phosphate buffer, pH 7.0,
4. GSH: 10 mM in distilled water,
5. NADPH: 1.5 mM in 0.1% sodium bicarbonate,
6. Sodium azide: 1 mM,
7. 1.5 mM hydrogen peroxide (H_2O_2) prepared from commercial 30% solution

3.4.20.2 Procedure

In to 0.2 ml of the tissue homogenate was added 0.4 ml phosphate buffer and 0.1 ml of Glutathione reductase, 0.1 ml of reduced glutathione and 0.1 ml of sodium azide. The reaction mixture was incubated for 10 minutes at 37°C. 0.1 ml of NADPH and 0.1 ml of H_2O_2 were added to the test cuvetts and the change in absorbance was monitored at 340 nm for 5 minutes at 30 seconds intervals against a reference cuvette containing the reaction mixture except NADPH and hydrogen peroxide.

The enzyme activity was expressed as n moles of glutathione oxidized per minute per mg of protein.

3.4.21 ASSAY OF GLUTATHIONE-S-TRANSFERASE

The method developed by Habig *et al.,* (1974) was used to determine the activity of the enzyme GST.

3.4.21.1 Reagents

1. Phosphate buffer, 0.3 M, pH 6.5: 10.2g potassium dihydrogen phosphate and 3g sodium hydroxide were dissolved in 250ml distilled water.
2. 1-chloro-2, 4-dinitrobenzene (CDNB): 30 mM in 10% methanol
3. Reduced glutathione: 30mM in distilled water.

3.4.21.2 Procedure

The reaction mixture containing 1.0 ml of buffer, 0.1 ml CDNB and 0.1 ml tissue homogenate was made up to 2.5 ml with distilled water. It was then incubated at 37°C for 5 minutes and 0.1 ml of GSH was added and the change in absorbance was measured at 340 nm for 3 minutes at 30 seconds interval.

The enzyme activity was expressed as μ moles CDNB conjugate formed/min/mg protein.

3.4.22 ASSAY OF CATALASE (EC 1.11.1.6)

Catalase was assayed according to the method of Takahara *et al.*, (1960).

3.4.22.1 Reagents

1. Phosphate buffer, 50mM, pH 7.0: Prepared by mixing 3.4g potassium dihydrogen phosphate and 580mg sodium hydroxide in 500ml distilled water. From this solution, one ml was taken and diluted to 100ml

2. Hydrogen peroxide: 30 mM solution in the above buffer.

3.4.22.2 Procedure

1.2 ml of phosphate buffer was added to 0.2 ml of tissue homogenate and the enzyme reaction was initiated by the addition of 1.0 ml of H_2O_2 solution. The decrease in absorbance was monitored at 240 nm at 30 seconds intervals for three minutes. Enzyme blank was run simultaneously with 1.0 ml distilled water instead of hydrogen peroxide.

The enzyme activity was expressed as n moles of hydrogen peroxide decomposed per minute per mg protein.

3.4.23 ASSAY OF SUPEROXIDE DISMUTASE (EC 1.15.1.1)

The method developed by Misra and Fridovich (1972) was adopted to determine the activity of superoxide dismutase.

3.4.23.1 Reagents

1. Carbonate-bicarbonate buffer: 0.1M, pH 10.2, containing 57 mg/dl EDTA,
2. Epinephrine: 3.0 mM in carbonate-bicarbonate buffer,
3. Chloroform: AR,
4. Ethanol: AR.

3.4.23.2 Procedure

Diluted 0.5 ml of the homogenate with 0.5 ml water. To this, added 0.25 ml ethanol and 0.15 ml chloroform (all reagents chilled in ice) and centrifuged. The supernatant was used for the assay. 1.5 ml of buffer was added to 0.5 ml of the supernatant. The reaction was initiated by the addition of 0.4 ml of epinephrine and the change in optical density per minute was measured at 480nm in Shimadzu-UV spectrophotometer.

One unit of enzyme activity was the amount of protein required to give 50% inhibition of epinephrine autoxidation.

3.4.24 ASSAY OF XANTHINE OXIDASE

The activity of xanthine oxidase was estimated by the method developed by Snell and Snell (1971)

3.4.24.1 Reagents

1. 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazoleum chloride (INT): 8% in distilled water
2. Xanthine: 228μg/100 ml distilled water
3. Ethylenediaminetetra acetate (EDTA): 26μg/100 ml distilled water
4. Gelatin solution: 0.1% in distilled water
5. Phosphate buffer, pH 7.8, 0.1 M

6. Phenazine methosulfate solution (PMS): 0.02% in distilled water
7. HCl: 1:30 (HCl: water)

3.4.24.2 Procedure

To 0.5 ml INT was added 0.1 ml xanthine, 0.5 ml EDTA, and 1ml gelatin and the volume was made up to 3.5 ml with phosphate buffer. Incubated the tubes at 37°C for 5 minutes and 0.1 ml of PMS was added in to this mixture. 0.2 ml of the sample was added the reaction mixture was incubated at 37°C for 15 minutes with shaking. The reaction was then arrested by adding 0.2 ml of 1:30 hydrochloric acid and the absorbance was measured at 540 nm.

One unit of enzyme activity was n moles of xanthine disappearing/min/mg protein

3.4.25 ASSAY OF SULFITE OXIDASE

Sulfite oxidase was assayed according to the procedure of Cohen and Fridovich (1971).

3.4.25.1 Reagents

1. Sodium sulfite: 1.26 mg/ ml in distilled water
2. Potassium ferricyanide: 3.29 mg/ml in distilled water
3. Tris-HCl: 0.10 M, pH 7.8 with 0.05 M Potassium phosphate
4. EDTA : 0.4 mM in Tris-HCl

3.4.25.2 Procedure

In to 0.5 ml of the enzyme solution was added 0.1 ml of each sodium sulfite, potassium ferricyanide and EDTA solutions and made up the volume to 2.5 ml with Tris-HCl. The reduction of ferricyanide was followed by a decrease in the absorbance at 420 nm.

Enzyme activity was expressed as n moles of sulfite oxidized/min/mg protein.

3.4.26 EXTACTION OF TOTAL LIPIDS FOR THE ESTIMATION OF CHOLESTEROL AND PHOSPHOLIPIDS

The total lipid was extracted by the method of Bligh and Dyer (1959) as given in 3.4.14.

The lipid was resuspended in 5 ml of chloroform-methanol mixture. This was used for the estimation of cholesterol and phospholipid content.

3.4.27 ESTIMATION OF TOTAL CHOLESTEROL

Total cholesterol was estimated by the method of Parekh and Jung (1970)

3.4.27.1 Reagents

1. Ferric acetate-uranyl acetate reagent: 10 ml of water and 3 ml of concentrated ammonia were added to 500 mg of

crystalline ferric chloride. The precipitate was washed several times with distilled water and dissolved in glacial acetic acid. This was made up to 1 liter with acetic acid. 100 mg of uranyl acetate was added and shaken well and kept overnight in an amber coloured bottle.

2. Sulfuric acid-ferrous sulfate reagent: 100 mg of anhydrous ferrous sulfate was added to 100 ml of glacial acetic acid and shaken well. 100 ml of concentrated sulfuric acid was added to this mixture. After cooling, the volume was made up to 1 liter with concentrated sulfuric acid.

3. Cholesterol standard: The stock was made by dissolving 100mg of cholesterol in 100 ml of chloroform.

3.4.27.2 Procedure

From the lipid extract, 0.1 ml was taken and made up to 3 ml with ferric acetate uranyl acetate reagent. The solution was mixed well and allowed to stand for 10 minutes and then centrifuged. 2.0 ml of ferrous sulfate-sulfuric acid reagent was added to 3.0 ml supernatant, and mixed well. After 20 minutes incubation, the optical density was measured at 560 nm. A reagent blank and a series of standards were also run along with the sample.

3.4.28 ESTIMATION OF PHOSPHOLIPIDS

Phospholipid content of the tissues was estimated by the method of Fiske and Subbarow (1925) as inorganic phosphorus liberated after Bartlette's perchloric acid digestion (1959)

3.4.28.1 Reagents

1. Ammoniummolybdate reagent:
2. 2.5 g ammonium molybdate in 100 ml water
3. Aminonaphthosulphonic acid: 0.5 g of 1,2,4 aminonaphthosulfonic acid was dissolved in 195 ml of 15% sodium metabisulfite and 50 ml of 20% sodium sulfite was added for complete solubilisation. The solution was filtered and stored in brown bottle.

Standard solution: Standard solution was prepared by dissolving potassium dihydrogen phosphate in distilled water so that the solution would contain 80 mg phosphorus per ml.

The working standard was prepared by diluting 0.1 ml from the above solution to 100 ml, to concentration of 80 microgram per ml.

3.4.28.2 Procedure

To 0.1 ml of the tissue homogenae was added 0.4 ml of perchloric acid and made up to 3 ml with double distilled water. Then

0.5 ml of ammonium molybdate reagent was added followed by the addition of 0.2 ml of ANSA. The standard and blank were treated in the same manner.

The absorbance was read at 620 nm, after an incubation period of 20 minutes.

3.4.29 ELECTROPHORETIC SEPARATION OF PROTEINS IN THE MUSCLE AND HEPATOPANCREAS

Proteins were separated by SDS-PAGE, the method developed by Laemmli, (1970)

3.4.29.1 Reagents

1. Acrylamide-bis acrylamide solution: 30 g of acrylamide and 0.8 g of bis acrylamide in 100 ml double distilled water.
2. Separating gel buffer: 1.5 M Tris-HCl, pH 8.8.
3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8.
4. SDS solution: 10% in distilled water
5. Ammonium persulphate: 10% in distilled water
6. Electrode buffer: Stock solution- Dissolved Tris base, 9 g; Glycine, 43.2 g and SDS, 3 g in 600 ml deionized water. From this solution, 100 ml was diluted to 500 ml using double distilled water, to prepare the working solution.

7. Sample buffer: Glycerol, 0.8 ml; 10% SDS, 1.6 ml; 2-mercaptoethanol, 0.4 ml and 1% bromophenol blue and Tris-HCl, 1.0 ml (0.5 M, pH 6.8) were mixed and made up to 8 ml with deionized water.
8. Staining solution: 1.2 g of Comassie blue R-250 was dissolved in 500 ml of methanol and mixed with 200 ml of glacial acetic acid and 500 ml distilled water. It was then filtered through Whatman No.1 filter paper.
9. Destaining solution: 300 ml of methanol and 100 ml of glacial acetic acid were mixed with 1000 ml of distilled water.

3.4.29.2 Procedure

Separating gel was prepared by mixing 2.5 ml Tris-HCl (1.5M), 100µl 10% SDS, 3.3 ml of acrylamide-bis acrylamide stock solution, 50 µl ammonium persulphate (APS) (10%), 5 µl N,N,N^1,N^1-tetra ethyl methylene diamine (TEMED) and 4.05 ml distilled water. this solution was poured between a mould formed by two glass plate, separated by 0.75 mm thickness spacers. It was overlaid by 10 µl of distilled water. After polymerization was completed, the overlay was removed and washed with distilled water. Then a comb was inserted over this. The stacking gel was prepared by mixing 6.01 ml distilled water, 2.5 ml Tris-HCl (0.5 M), 100 µl SDS (10%), 1.33 ml

acrylamide-bis acrylamide, 50 µl of APS (10%) and 10 µl of TEMED. This solution was poured over the separating gel.

The comb was removed after the complete polymerization of the gel. Supernatant of tissue homogenate was diluted with sample buffer in the ratio of 1:4. It was then heated at 95°C for 4 minutes. The sample was loaded (10µl) in to the well and the electrophoresis was carried out at 200 volts. After completion of the run the gel was removed from the plates and stained with coomassie brilliant blue solution for half an hour and then destained using the destaining solution.

3.4.30 ANALYSIS OF METAL CONTENT

3.4.30.1 Reagents

1. Sulfuric acid
2. Nitric acid
3. Metal standards

3.4.30.2 Procedure

3.4.30.2.1 Digestion procedure: Metals were determined in tissue samples by wet oxidation method (AOAC, 1990) using concentrated nitric acid and sulfuric acid in the ratio of 6:1 (v/v). The homogenized samples were into a Bethge's flask and subjected to predigestion by adding 15-20 ml concentrated nitric acid and keeping it overnight.

After this the samples were heated gently and continuously first, until the first vigorous reaction subsided, continued heating until the organic matter got completely destroyed as indicated by slight yellow colour. This clear solution was cooled, filtered and made up to a known volume and analyzed by AAS.

A blank was also prepared in the same manner.

4. RESULTS AND DISCUSSION

4.1 *Diets*

Many kinds of formulated fish feeds are in use in aquaculture. The primary objective of feed formulation is to provide the species under culture with an acceptable diet, which meets all its nutritional requirements at different stages of life, so as to yield optimum production at minimum cost (Reddy and Rao, 1999).

A formulated feed must fulfill a complex set of demands in order to be functional. The most important factor is that it should be nutritionally adequate and at the same time economically viable in commercial practice. Nutritionally balanced compounded diet must include an energy source with sufficient essential amino acids, essential fatty acids and non-energy nutrients such as vitamins and minerals for the health and growth promotion of the fish (Halver, 1976). Proper utilization of macronutrients depends on the availability of adequate supply of micronutrients. With growth, there can be subtle progressive changes in the nutritional requirements also, which is yet another variable calling for detailed study in the case of each candidate species. Requirements of each trace metal at different stages of growth in each species cultured is to be studied further, which is beyond the scope of the present study.

4.1.1 *Proximate Composition*

Table 4.1.1 shows the proximate composition of the basal diet used in the present study. Moisture content does not affect the nutritive value of the feed and low moisture content is important for the storage of feeds. Moisture content of the feed was in the range observed in various formulated feeds for *P.monodon* (Kavitha *et al.*, 2003). The studies conducted on the requirements of protein showed that fishes have high dietary requirement for protein, ranging from 35%-55% (Hafedh, 1999; El-Sayed and Teshima, 1991), which is equivalent to 45-77% of the gross energy content of the diet. In the present experiment the protein content was 39.9%, which was sufficient to meet the requirements of the shrimp. In the case of *P. monodon,* a diet containing 35% protein was found to give satisfactory performance (Ali, 2003). The fat content and mineral content of the diet were also in the optimal range for the growth of the shrimp as per presently accepted standards and in the commercially available shrimp feeds (Kavitha *et al.*, 2003).

4.1.2 *Amino acid composition*

Amino acid composition of the formulated feed is given in the table 4.1.2. The amino acid composition of the feed is of particular importance to fish as they are utilized for the synthesis of specific protein of the tissues. *P. monodon* requires 10 essential amino acids,

which must be given through the diet, as the shrimp is incapable of synthesizing the same (Ali, 2003). For prawn, tissue amino acid profiles are used as guidelines for balancing amino acids in feeds (Deshimaru and Shigenok, 1972). The formulated feed used in the present study was found to be adequate in essential amino acids required by the shrimp, *P. monodon.*

4.1.3 *Fatty Acid Profile*

Lipids are important in the feed as a source of energy as well as essential fatty acids. The fatty acid profile of the feed used is given in the table 4.1.3. Some fatty acids are essential in the diet of crustaceans as they cannot be biosynthesized by these animals. These are linoleic acid (18:2 n-6), linolenic acid (18:3 n-3), arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3) and docasahexaenoic acid (22:6 n-3) (Paulraj, 1997). So diet should provide optimum levels of these fatty acids. Glencross and Smith (2001) had studied the optimum requirements of EFA, DHA and EPA in the diet of *P.monodon.* According to Glencross and Smith, (1999), linoleic acid has higher growth promoting effect than linolenic acid. More over, linolenic acid appeared to have detrimental effect on the digestibility of other fatty acids. Accordingly, in the present study diet was formulated such that it contains linoleic acid in higher percentage than other essential fatty acids.

Table 4.1.1 Proximate Composition of basal diet (%)

Moisture	Protein	Crude fat	Ash
7.3	39.9	7.6	9.9

Table 4.1.2 Amino acid composition (% of total protein)

Amino acid (%)	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Trp
	13.6	4.0	6.2	31.6	6.9	7.0	7.8	0.8	3.0	4.2	3.8	7.3	3.4	5.3	3.4	2.1	6.7	2.9

Table 4.1.3 Fatty Acid Profile (% of total fatty acid)

Fatty acid (%)	C14:0	C16:0	C18:0	C16:1	C18:1	C18:2	C18:3	C20:5	C22:6	UI*
	5.99	22.6	4.7	7.5	19.7	23.4	2.2	5.1	2.7	6.2

* Un identified

Table 4.1.4 selenium, molybdenum and cobalt levels of the basal diet

Element	Quantity
Selenium	0.30μg/g feed
Molybdenum	17.6μg/100g feed
Cobalt	9.2 μg/100g feed

4.1.4 Trace elements (Selenium, Molybdenum and Cobalt)

Crustaceans are capable of absorbing minerals directly from the water in which they inhabit, through the gills and body surfaces. But diet is the major source of minerals. Nonavailability of adequate quantities of minerals affects growth moulting and many irrecoverable deficiency diseases (Paulraj, 1997). So artificial diet is supplemented with minerals in various levels. Selenium, molybdenum and cobalt levels in the experimental diet are given in the table 4.1.4.

4.2 EFFECT OF SELENIUM ON GROWTH AND OTHER BIOCHEMICAL PARAMETERS

4.2.1 *Growth of P. monodon*

Selenium is an important trace element with antioxidant property and was found to be essential for plants and animals. It has a reported growth promoting effect in plants (Santhosh *et al.,* 1999). The present study confirms this observation in shrimp also, as the weight gain increased ($p<0.05$) in groups fed Se supplemented diets (Fig.4.2.1.1). The effect of Se in feed conversion ratio (FCR) and specific growth rate (SGR) is given in Fig.4.2.1.2. Groups supplemented with the diet containing 0.5 ppm and 2.5 ppm levels of selenium showed a significant increase ($p<0.05$) in feed conversion efficiency (FCE) (Fig. 4.2.1.3) and a better FCR. A significant

increase ($p<0.05$) in SGR was observed in all groups of prawns supplemented with selenium, when compared to control group (Fig.4.2.1.2). The increased PER (Fig. 4.2.1.3) might be responsible for the observed increase in growth of prawns fed diet supplemented with selenium at low levels. A significant reduction in mortality was also observed in group II and III when compared to control groups. But in groups of shrimps fed diet supplemented with higher level (5 ppm) of Se, the mortality showed no significant difference with control group (Fig. 4.2.1.4).

4.2.2 *Effect of selenium on the biochemistry of P. monodon*

4.2.2.1 *Proximate composition*

Proximate composition of meat from the control shrimps and groups supplemented with Se are given in Table 4.2.2.1. Shrimps did not exhibit significant changes in moisture content, protein, or mineral content when compared to that of control group. But slight variation was observed in the fat content.

4.2.2.2 *Amino acid composition*

Table 4.2.2.2 gives the amino acid composition of the control and selenium supplemented groups of *P. monodon.* Amino acids are the building blocks of proteins. Some free amino acids are also found in the system. Non-essential amino acids showed significant changes in their levels in selenium supplemented experimental shrimps. A

Fig.4.2.1.1 Effect of dietary selenium on growth of control and experimental shrimps

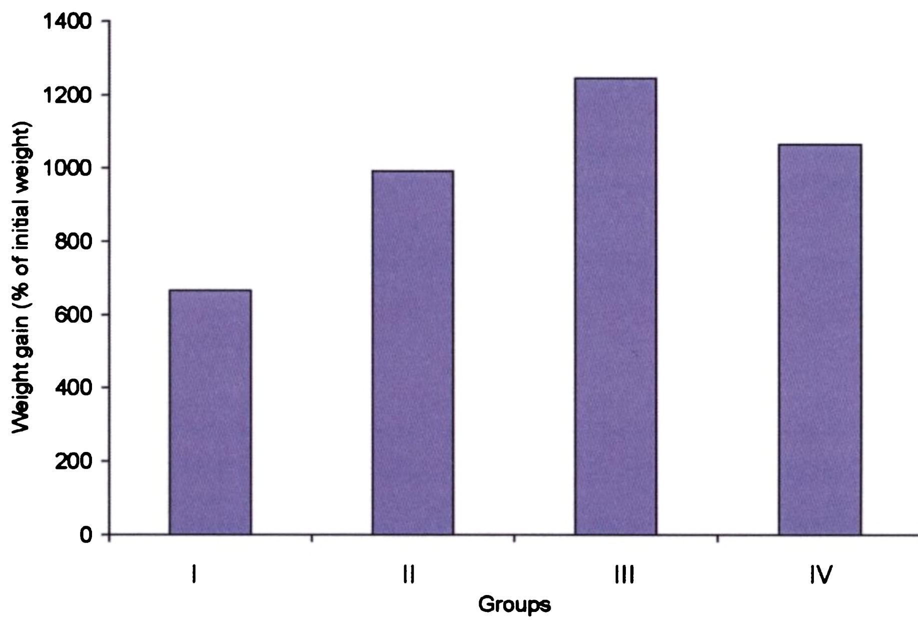
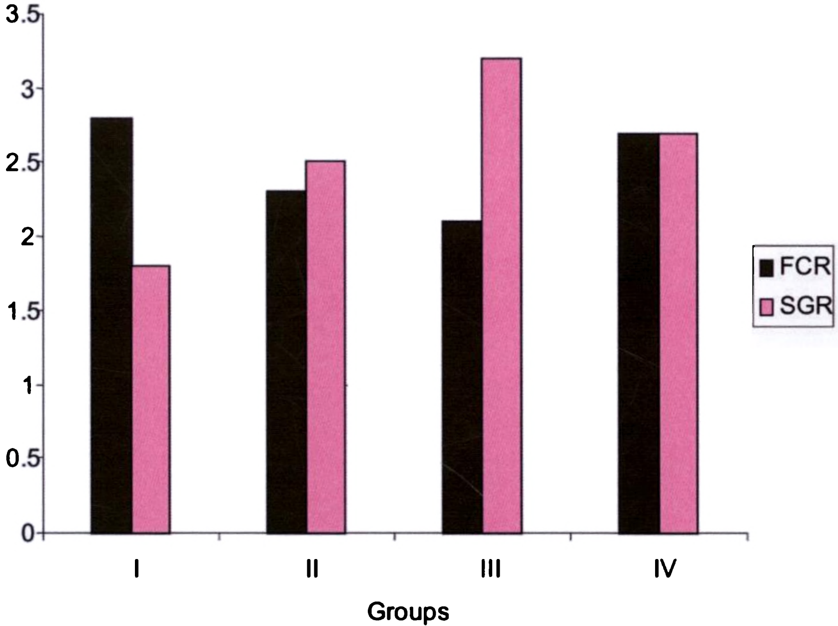


Fig.4.2.1.2 Effect of dietary selenium on FCR and SGR of control and experimental shrimps



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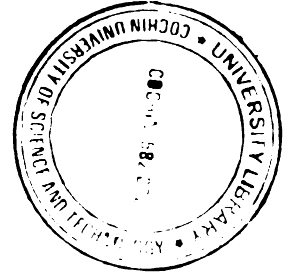


Fig. 4.2.1.3 Effect of dietary selenium on FCE and PER of control and experimental shrimps

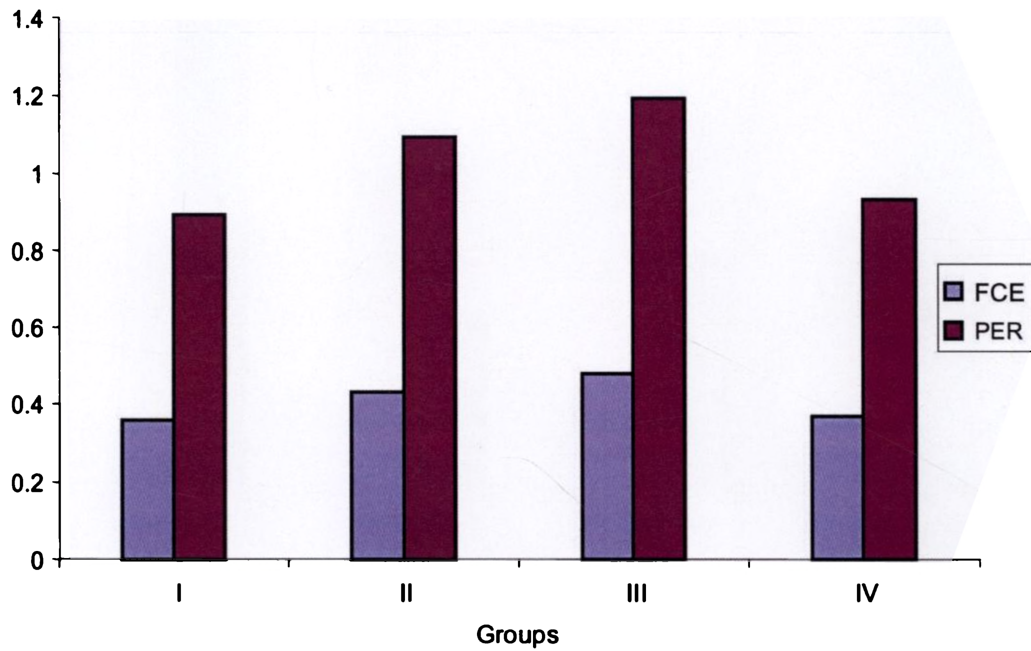


Fig. 4.2.1.4 Effect of dietary selenium on mortality of control and experimental shrimps

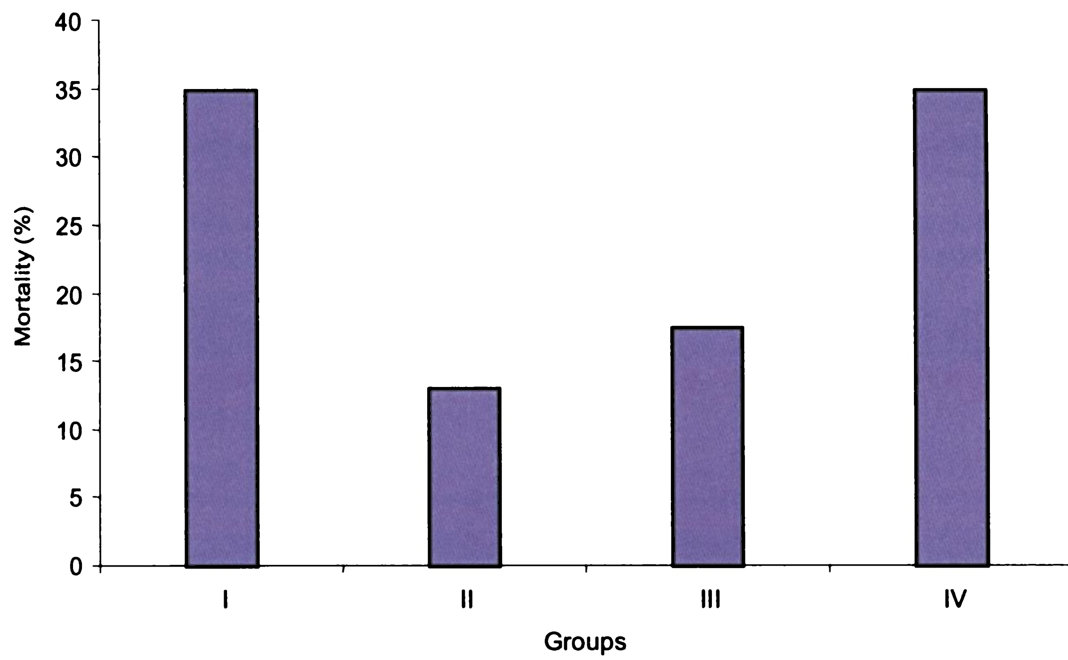


Table 4.2.2.1 Proximate composition of normal and selenium supplemented experimental *P. monodon*

	Group I (Control)	Group II	Group III	Group IV
Moisture	74.2±0.19	74.0±0.29	74.7±0.39	74.4±0.26
Total protein	21.8±0.12	21.9±0.11	21.9±0.09	22.0±0.09
Crude fat	1.8±0.02	1.6±0.04^{a}	1.8±0.01^{b}	2.0±0.05^{ab}
Ash	1.5±0.02	1.4±0.01	1.4±0.01	1.5±0.02

Values are expressed as Mean ± SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals.

Table 4.2.2.2 Amino acids of the control and selenium supplemented *P. monodon*

Amino acid ($g/16g\ N_2$)	I	II	III	IV
Asp	9.9±0.34	11.8±0.34[a]	11.3±0.28[a]	11.2±0.20[a]
Thr	3.4±0.20	4.2±0.17[a]	3.6±0.17	3.6±0.20
Ser	3.9±0.14	4.5±0.11	4.2±0.16	4.3±0.17
Glu	16.4±0.36	17.6±0.38	17.8±0.31	17.9±0.35
Pro	5.6±0.17	5.0±0.20	5.2±0.23	5.0±0.26
Gly	8.5±0.26	10.3±0.17[a]	9.4±0.11[a]	9.8±0.20[a]
Ala	8.3±0.23	8.8±0.08	8.4±0.17	8.9±0.14
Cys	0.6±0.05	0.93±0.04[a]	0.7±0.05[a]	0.8±0.04[a]
Val	3.1±0.18	3.5±0.14	3.1±0.12	3.1±0.13
Met	1.9±0.05	2.0±0.06	1.8±0.05	2.1±0.08
Ile	2.1±0.08	2.4±0.03	2.3±0.08	2.6±0.03[a]
Leu	7.1±0.15	7.1±0.21	7.6±0.17	7.6±0.11
Tyr	1.5±0.15	1.8±0.21	2.4±0.06[ab]	2.4±0.03[ab]
Phe	3.1±0.05	3.4±0.06	3.4±0.12	3.1±0.38
His	2.2±0.15	2.3±0.08	2.5±0.03[a]	2.2±0.12
Lys	2.4±0.1	2.7±0.03[a]	2.4±0.1	2.4±0.03
Trp	1.4±0.03	1.3±0.03	1.4±0.03	1.5±0.03

Values are expressed as Mean±SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals.

Table 4.2.2.3 Fatty acid profile (%) of control and selenium supplemented *P. monodon*

FATTY ACIDS (%)	Group I	Group II	Group III	Group IV
C14:0	1.4±0.08	1.6±0.08	1.7+0.05	1.6±0.06
C16:0	24.1 ±0.6	24.11+0.1	24.1+0.4	24.2±0.3
C16:1	3.0+0.11	3.3+0.23	3.6+0.34	3.1±0.17
C17:0	1.7+0.03	1.7+0.05	1.7+0.00	1.2±0.08
C18:0	8.9+0.15	I0.3±0.24^{a}	11.7±0.20^{ab}	I2.5±0.29^{abc}
C18:1	15.93±0.17	13.97±0.37^{a}	14.84±0.40^{a}	14.23±0.20^{a}
C18:2	18.0+0.31	18.8+0.23	19.0±0.23^{a}	18.2±0.30
C18:3	0.8±0.00	0.8±0.08	1.3±0.05^{a}	1.2±0.11^{a}
C18:4	1.3±0.08	1.03+0.03	1.3±0.15	1.2±0.09
C20:2	1.0±0.11	1.0±0.03	1.1±0.09	1.02+0.08
C20:4	1.6+0.08	1.6±0.17	1.6±0.11	1.6±0.08
C20:5	9.3±0.11	9.5±0.20	9.97±0.17	9.31+0.20^{c}
C22:6	7.7±0.25	9.9±0.17^{a}	8.3+0. 15^{b}	8.29±0.20^{b}

Values are expressed as Mean±SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals

significant increase in the level of aspartic acid was observed in shrimps fed diet supplemented with selenium. Glutamic acid, Glycine and cystein also increased significantly in selenium supplemented groups of shrimps. These three amino acids are the building blocks of the cellular tri peptide glutathione. Phenyl alanine to tyrosine conversion was also higher in selenium supplemented groups of shrimps. Since levels of some of the essential amino acid also increased in the selenium supplemented groups, their dietary absorption also might have been influenced by the selenium. Scarcity of literature regarding the effect of selenium on amino acid metabolism delimits a solid and proper explanation for these variations in amino acid levels. Further investigations are required to confirm the role of selenium in amino acid metabolism.

4.2.2.3 *Fatty acid composition*

Fatty acid profiles of the control and selenium supplemented experimental shrimps are presented in table 4.2.2.3. C18:0 was found increased in all groups supplemented with selenium when compared to that of control. This fatty acid is a non essential fatty acid which could be synthesized within the body of the shrimp. Dietary selenium might have increased their synthesis within the body. A reduction in the levels of C18:1 was also noted in selenium supplemented groups. But the percentage of some of the other polyunsaturated fatty acids

showed an increase in selenium supplemented groups. Since such an erratic behavior is obtained, it is difficult to draw a conclusion about the effect of dietary selenium on fatty acid composition of the shrimp. Further more, literatures available in this regard also are scanty.

4.2.3 *Effect of selenium on Tissue Defense System of P. monodon*

The ubiquitous presence of free radicals in aerobic organisms has necessitated the development of some efficient defense systems. Both enzymatic and non enzymatic anti oxidants have evolved and together these provide efficient protection under normal circumstances. Excessive exposures to free radicals can of course, overwhelm these systems causing injury (Mutlu-Turkoglu *et al.,* 2000). Numerous studies have demonstrated enhanced toxicity when endogenous anti oxidant systems is individually impaired (Mutlu-Turkoglu *et al.,* 2000; Orhan *et al.,* 1999; Spolar *et al.*, 1999).

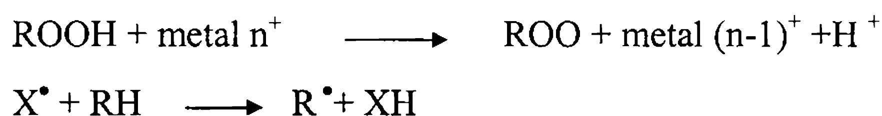
The enzymatic method of tissue defense involves the direct method in which the enzymes Glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) are involved and indirect method in which the enzyme glutathione S-transferase (GST) is involved. Glutathione together with other thiol groups and the vitamins, ascorbic acid and α-tocopherol plays important role in non-enzymatic method of tissue defense.

Glulathione family provides us with vitally important intra cellular defense against disease causing invaders and chemicals. Among their critical biological effects are vital roles they play in fighting off damaging free radicals (Mutlu-Turkoglu *et al.*, 2000), the neutralization of chemical toxins and the inhibition of carcinogenic mutagenic compounds (Spolar *et al.*, 1999).

4.2.3.1 *Lipid Peroxidation*

Peroxidation of lipids exposed to oxygen is responsible for damage to tissues where it may cause cancer, inflammatory diseases, aging etc. The deleterious effects are initiated by free radicals. Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation. The whole process can be represented as follows (Mayes, 1996).

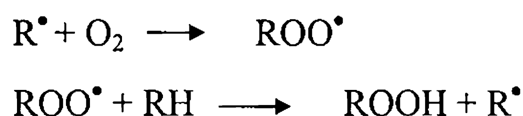
1) Initiation



$$ROOH + metal\ n^{+} \longrightarrow ROO + metal\ (n\text{-}1)^{+} + H^{+}$$

$$X^{\bullet} + RH \longrightarrow R^{\bullet} + XH$$

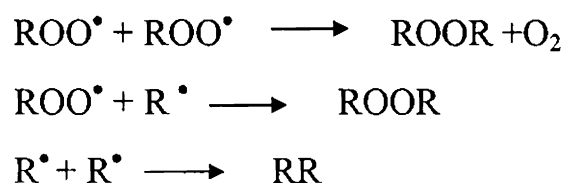
2) Propagation



$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$$

3) Termination



$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2$$

$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR$$

$$R^{\bullet} + R^{\bullet} \longrightarrow RR$$

Selenium is well known for its ability to protect cells or tissues from various types of oxidative damages (Mukherjee *et al.*, 1996). Feeding diets depleted of selenium resulted in increased peroxidative challenges in the muscle tissues of calves (Walsh *et al.*, 1993). In the present study, shrimps, fed on diets containing selenium at 0.5 ppm level showed significantly decreased level of lipid peroxidation in hepatopancreas when compared to that of control animals (Fig. 4.2.3.1). But the trend was different in the animals given diets containing higher concentrations of Se (5 ppm). At 5 ppm level the malondialdeyde released increased by about 10% in hepatopancreas and 13 % in muscle tissue. The decrease in lipid peroxidation at 0.5 ppm level could be attributed to the counteraction of free radical, probably by Se dependent antioxidant systems. It was already reported that in rats, selenium deficiency impaired the ability of both liver and colon to mount an induced detoxification response to acute oxidative stress (Pence, 1991). But higher levels of Se supplementation (5 ppm) might have resulted in excessive accumulation of the element in the tissues. Increased level of lipid peroxidation in Se toxicity has been reported earlier in experimental rats (El demerdash, 2001). There are several mechanisms by which selenium expresses its toxicity, one of which is the formation of CH_3Se, which enters a redox cycle and generates superoxide and oxidative stress (Spallholz and Hoffman, 2002) as is evident from the present study.

4.2.3.2 *Reduced glutathione content (GSH)*

The GSH content of the muscle tissue and hepatopancreas of control group shrimps and shrimps supplemented with Se are given in fig 4.2.3.2.1 and 4.2.3.2.2. There was a significant ($P<0.05$) increase in the level of GSH in the hepatopancreas and muscle tissue of *P.monodon* as a result of feeding Se-supplemented diet. Similar increase in GSH was reported earlier also (Bansal and Sood, 2001).

Glutathione and the enzymes that it forms such as GPx are essential to life and are present in all cells of both plants and animals. GSH plays several important roles in the body. GSH is an important inhibitor of free radical mediated lipid peroxidation (Meister, 1994). Depletion of GSH can lead to increased susceptibility of cells to reactive oxygen metabolites and free radical mediated membrane damage (Jain *et al.*, 1992). GSH, as the chief intra cellular non-protein thiol compound functions as a cellular storage pool of reduced thiols. Since selenium is the cofactor for the enzyme glutathione peroxidase, dietary levels of selenium is supposed to have a role in maintaining normal level of glutathione in tissues (Santhosh *et al.*, 1999).

The trend was however different in the muscle tissues of shrimps fed diet containing Se at 5 ppm level. Depletion in GSH content by high Se supplementation has also been reported in rats

(Kristina *et al.*, 1987) and it has been explained as due to increased activity of the GSH dependent enzyme GPx.

4.2.3.3 *Total sulfhydryl content (TSH)*

TSH has a critical role in protecting cells from peroxidative damage. Free radical scavengers were reported to generate TSH (Selvam and Ravichandran, 1993). It includes both protein bound sulfhydryl groups and non protein sulfhydryl groups. Selenium was found as a sulfhydrilic inductor in plants by Hawrylak and Szymanska (2004). The TSH content of the hepatopancreas and muscle tissue of the control and experimental shrimps are given in table 4.2.3.3. In hepatopancreas, TSH level increased significantly in all groups of shrimp fed diet supplemented with selenium. Muscle tissue also showed a similar trend. But here the TSH level in shrimps supplemented with higher level of selenium (5.0 ppm) was less than that present in groups supplemented with 2.5 ppm of selenium. The elevated level of lipid peroxidation observed in 5.0 ppm selenium supplemented groups might have resulted in increased utilization of TSH in this group, which exceeded the rate of their regeneration.

4.2.3.4 *Glutathione peroxidase (GPx)*

GPx is a Se-dependent enzyme found in the cytoplasm and also in mitochondria, requiring 4 Se atoms per active molecules. It is the primary mechanism for degrading low levels of H_2O_2 in cells.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

Since GPx also acts on hydro peroxides of unsaturated fatty acids, the enzyme plays an important role in protecting membrane lipids and thus protecting the cells from oxidative disintegration. Failure of peroxide destruction can explain the hemolysis *in vitro* and oxidative damage of hemoglobin and possibly the wide variety of degenerative changes that occur in Se deficiency (Hill *et al*., 2001; Zachara *et al*., 2001). The role of Se in GPx accounts for the apparent anti oxidant effect of dietary Se and there exists a direct relation between the dietary selenium and GPx activity (Yu *et al.,* 2002). Measurement of GPx is a useful means for defining Se requirements and for identifying Se deficiency in animals and humans (Rotruck *et al*., 1973). Fig 4.2.3.4.1 and fig. 4.2.3.4.2 show the GPx activities in the hepatopancreas and muscle of control group shrimps and shrimps supplemented with selenium. A significant elevation in the activity of GPx was noticed in the muscle tissue of shrimps fed diets supplemented with selenium. The increase in activity of GPx was about four-fold at supplementation level of 5.0 ppm. A marked decline in activity was observed in the hepatopancreas of animals supplemented at 2.5 and 5.0 ppm levels of selenium. Formation of CH_3Se results in the formation of free radicals that bind to and inactivate important enzymes and proteins, which is considered as a toxic effect of selenium (Spallholz and Hoffman, 2002). This might be the reason for the observed decrease in GPx activity in the

hepatopancreas of shrimps supplemented with 2.5 and 5.0 ppm levels of Se. Elevated levels of selenium in the diet might have caused more accumulation of selenium in hepatopancreas than that of muscle tissue, which is not a specialized organ for detoxification. Accumulation of selenium in hepatopancreas might have resulted in excess production of CH_3Se and subsequent inhibition of the enzyme.

4.2.3.5 *Glutahione-S- transferase (GST)*

GST is an enzyme, which plays a critical role in detoxification of chemicals. This enzyme catalyzes the conjugation of glutathione to electrophilic substrates and possesses selenium independent glutathione peroxidase activity (Wang *et al.*, 2000). Glutathione conjugates are thought to be metabolized further by cleavage to glutamate and glycine residues, followed by acetylation of the resultant free amino group of cystenyl residues to produce a final product, the mercapturic acid, which is then excreted. Present study shows that activity of GST increased significantly in both hepatopancreas and muscle tissue at 5.0 ppm levels (fig.4.2.3.5.1 and 4.2.3.5.2). Such elevated activity of GST had been reported earlier upon supplementation of selenium (Orhan *et al.,* 1999). A marked decline in the activity of GST was noticed in the muscle tissue at 0.5 and 2.5 ppm levels and in hepatopancreas at 0.5 ppm level. Since the other antioxidants were functioning in an efficient manner, the necessity for the higher function of GST ultimately might have

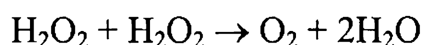
reduced in the hepatopancreas of 0.5 ppm group and muscle tissue of 0.5 and 2.5 ppm groups.

4.2.3.6 *Anti peroxidative enzymes Superoxide dismutase (SOD) & catalase (CAT)*

SOD and CAT, responsible for the destruction of peroxides have specific roles in protecting tissues against oxidative damage (Wohaieb *and Godin*, 1987; Poonam *et al.*, 1994). SOD is a family of metallo enzymes that catalyzes reaction of 2 anions with each other yielding triplet oxygen and H_2O_2.

$2O^{2-} + 2O^{2-} + 4H^+ \rightarrow 2O_2 + 2H_2O$ One or more of SOD enzymes are found in virtually all aerobic organisms.

Catalase is a dismutase enzyme. It takes 2 H_2O_2 molecules degrading them to oxygen and water.



$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$$

The activities of catalase were found significantly higher in the muscle tissue of shrimp treated with 2.5 ppm and 5.0 ppm Se (Fig 4.2.3.6.1) which is in line with previous reports in rats (Zikic *et al.*, 1998) and in carp (Jovanovic *et al.,* 1997). In the case of hepatopancreas, all the groups supplemented with selenium showed significant increase in the activity of catalase. Hepatopancreas is the organ, which is more active in both metabolism and detoxification.

This might be the reason for the observed difference in the response of catalase in muscle and hepatopancreas. SOD activity was also found increased in both muscle tissue and hepatopancreas on supplementing selenium (Fig 4.2.3.6.2).

4.2.4 *Trace element status*

Trace metal content of the control and selenium supplemented shrimps are given in table 4.2.4. Significant increase in the supplemented groups was observed in the case of selenium alone. The concentration of selenium was observed to be higher in the groups supplemented with 2.5 ppm and 5.0 ppm levels of dietary selenium. This indicates that selenium is effectively absorbed from the diet. A specialized excretory system might be absent in the shrimp due to which accumulation of the element within the tissues resulting in elevated concentration of this element. Similar relationship between the dietary selenium and serum selenium levels was reported in kittens supplemented with low selenium diet (Yu *et al.*, 2002).

4.2.5 *Electrophoretic pattern of tissue proteins*

The electrophoretic pattern of the proteins in the tissue homogenate is shown in figure 4.2.5. Selenium forms the co-factor for the enzyme GPx (Rotruck *et al.,* 1973). In addition there are certain structural proteins in which selenium is an integral part (Yeh *et al.*, 1997). Under these conditions, there is a possibility that dietary

Fig 4.2.3.1 Lipid peroxidation in the hepatopancreas and muscle tissue of control and selenium supplemented *P. monodon*

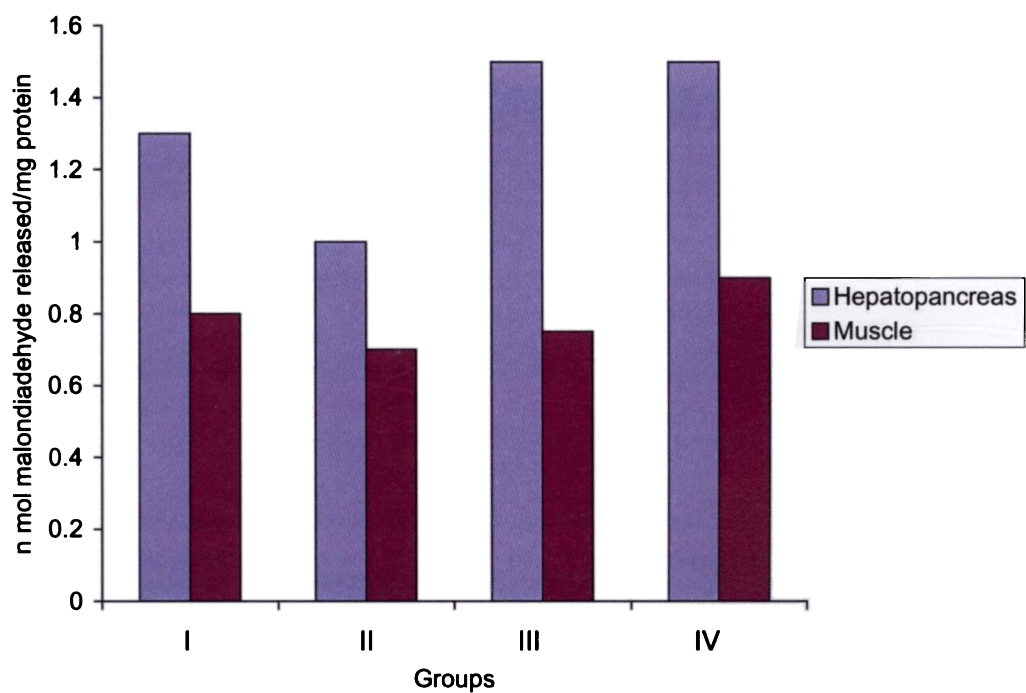


Fig 4.2.3.2.1 Level of reduced glutathione (GSH) in the hepatopancreas of normal and selenium supplemented *P. monodon*

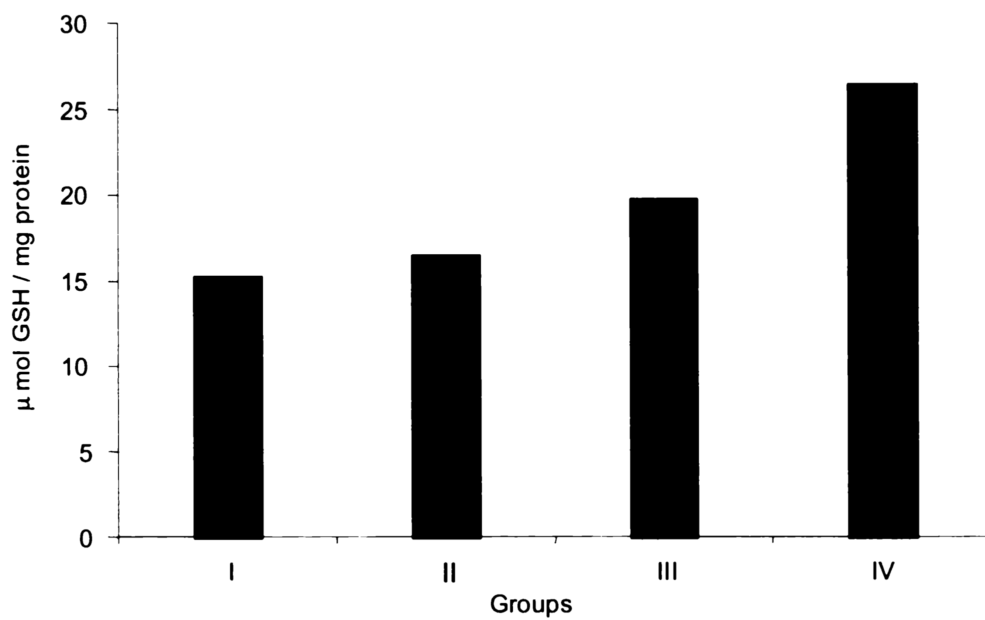


Fig 4.2.3.2.2 Level of reduced glutathione (GSH) in the muscle tissue of normal and selenium supplemented *P. monodon*

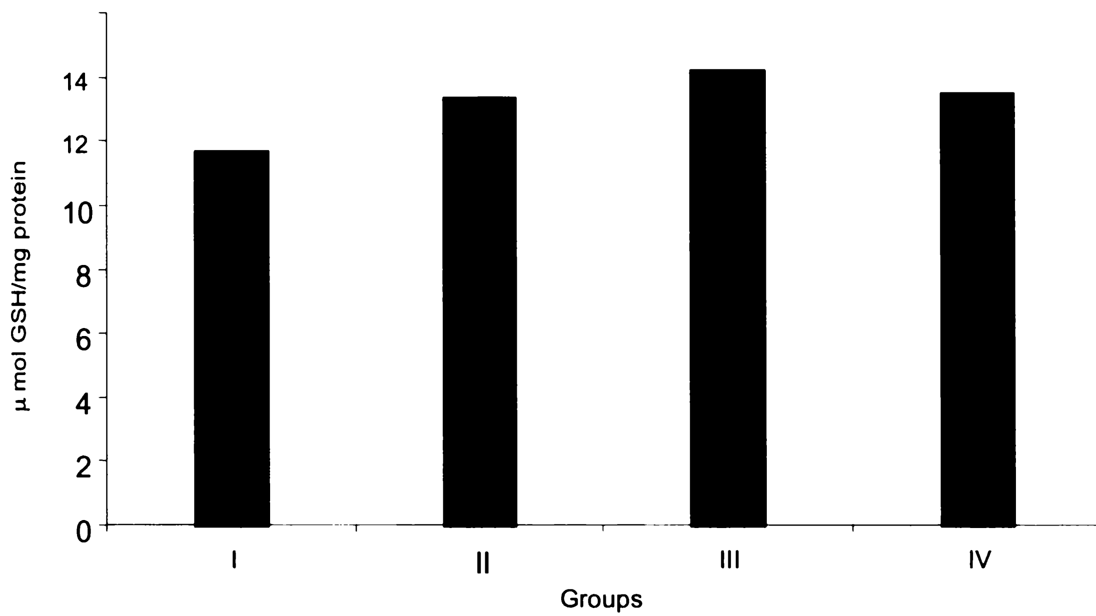


Table 4.2.3.3 TSH content of control and selenium supplemented *P. monodon*

TSH (ng/mg tissue)				
Tissue	Group I	Group II	Group III	Group IV
Hepatopancreas	261.6±3.17	479.2±5.82[a]	599.1±6.72[ab]	1633.0±5.82[abc]
Muscle tissue	173.8±4.38	243.5±3.29[a]	261.4±4.89[ab]	243.7±5.72[ac]

Values are expressed as Mean±SE; a = Significantly different ($p<0.05$) when compared to group I animals, b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals

Fig 4.2.3.4.1 Activity of selenium dependent GPx in the hepatopancreas of the control and selenium supplemented *P. monodon*

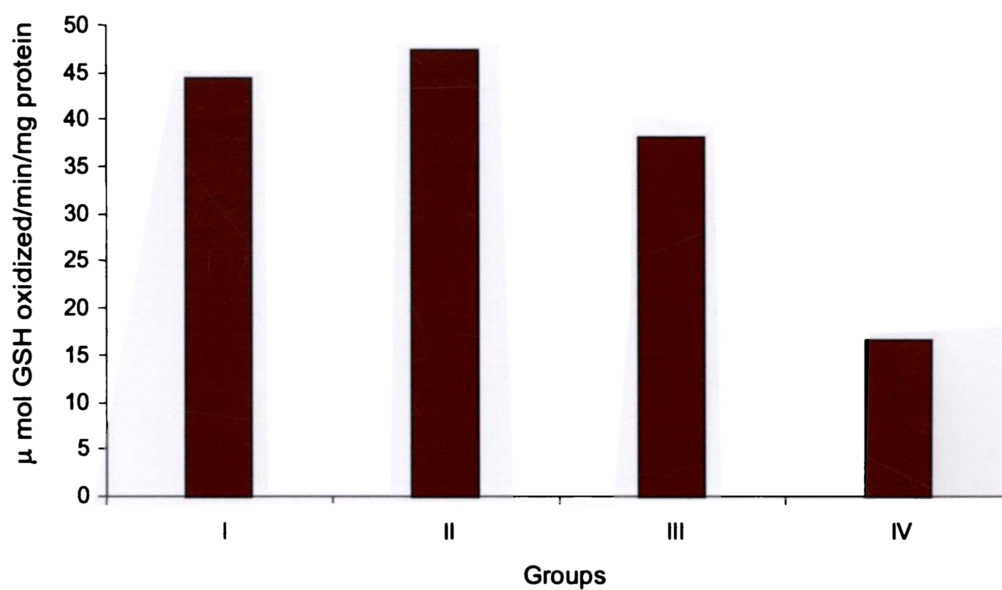


Fig 4.2.3.4.2 Selenium dependent GPx activity in the muscle tissue of control and selenium supplemented *P. monodon*

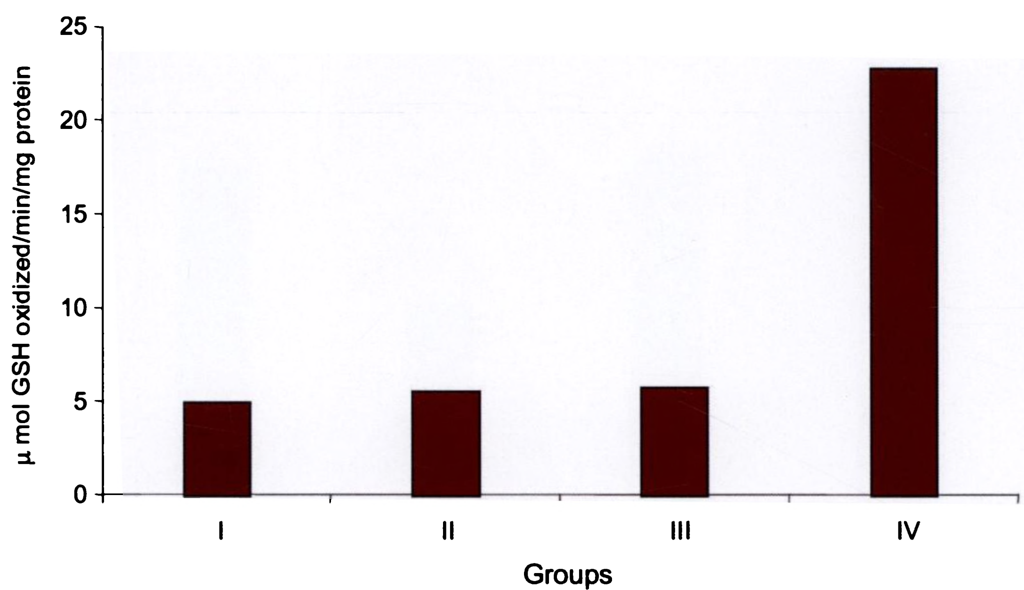


Fig 4.2.3.5.1 GST activity in the hepatopancreas of the control and selenium supplemented *P. monodon*

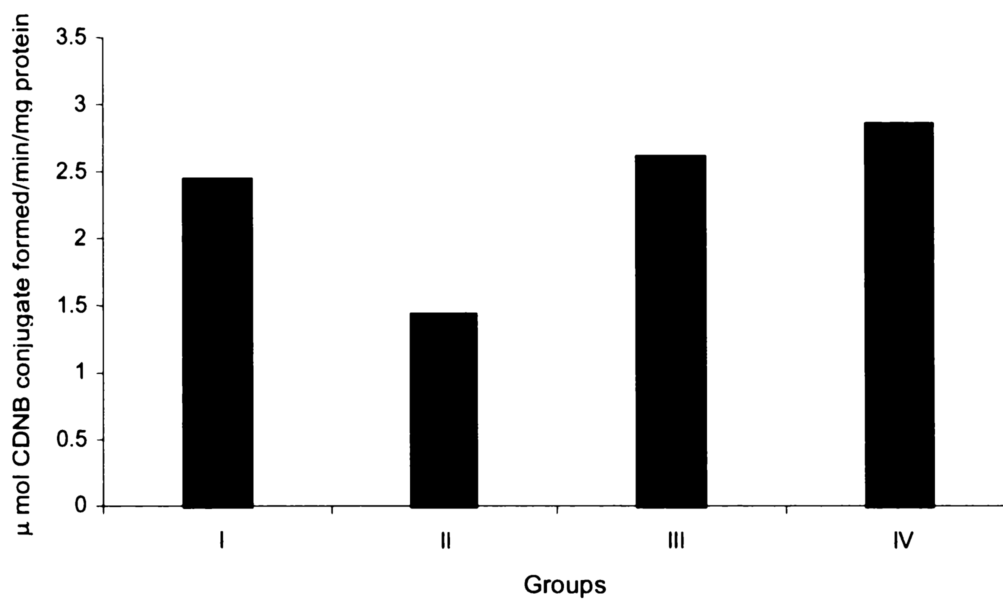


Fig 4.2.3.5.2 GST activity of the muscle tissue of control and selenium supplemented *P. monodon*

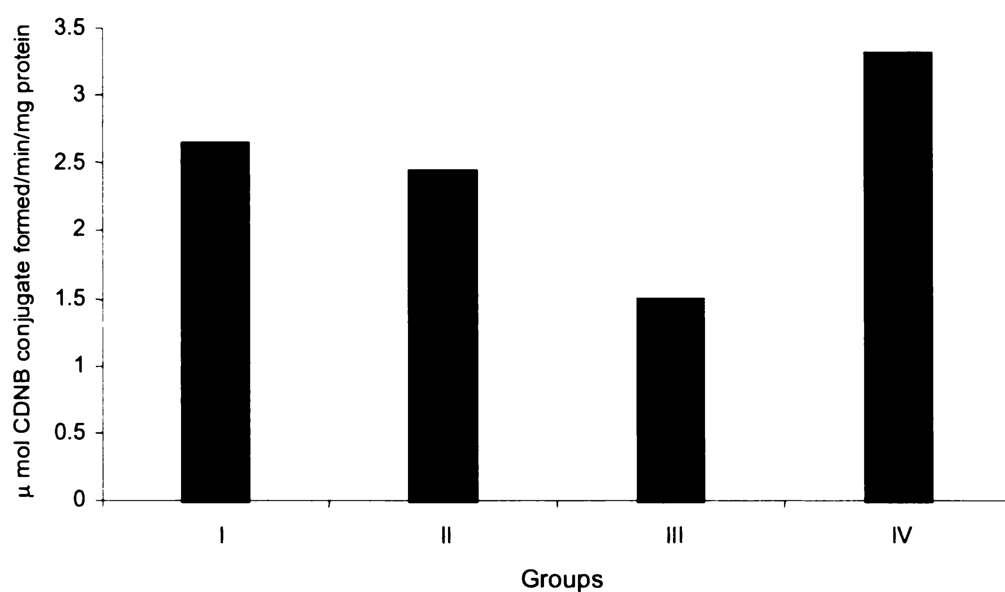


Fig 4.2.3.6.1 Catalase activity in the hepatopancreas and muscle tissue of control and selenium supplemented *P. monodon*

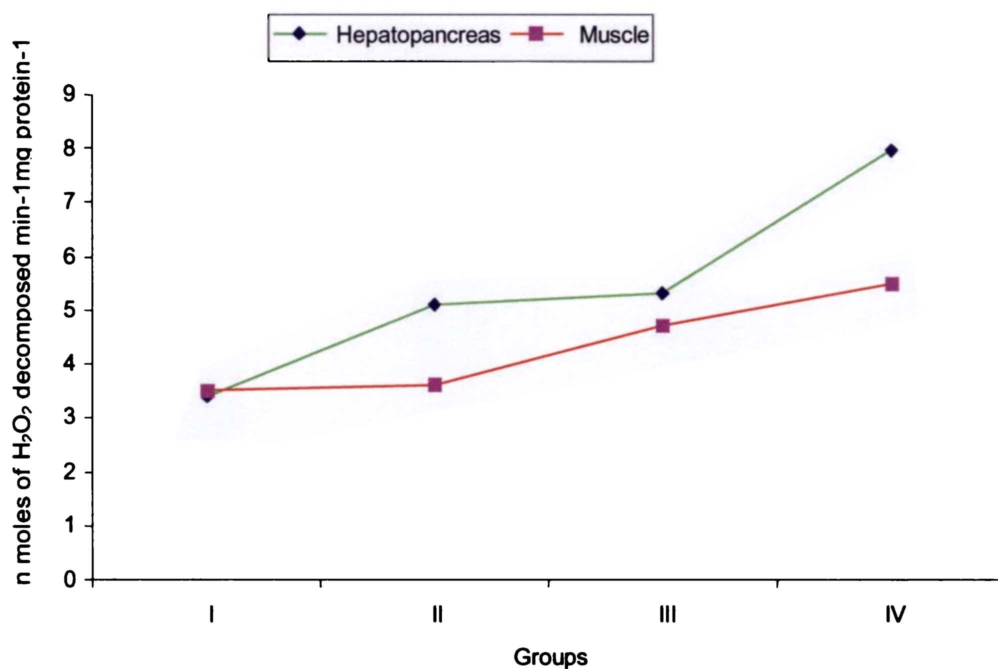
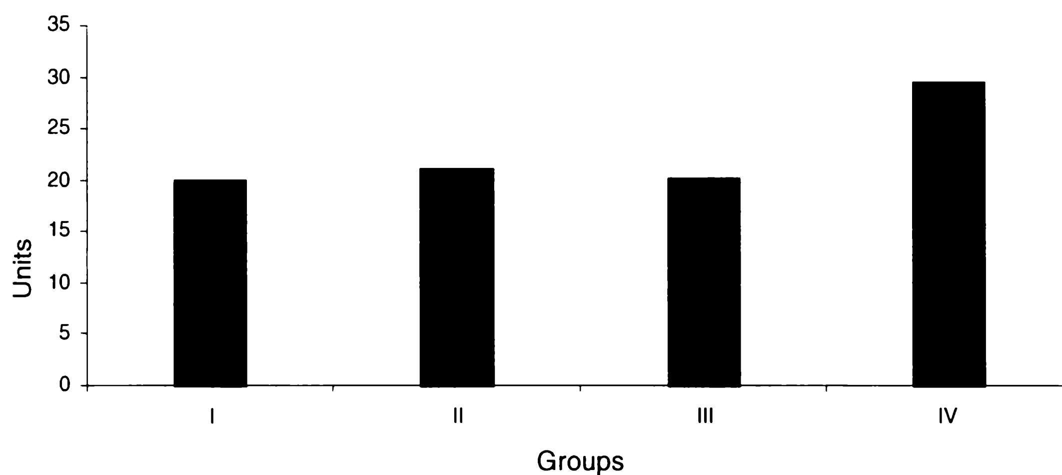
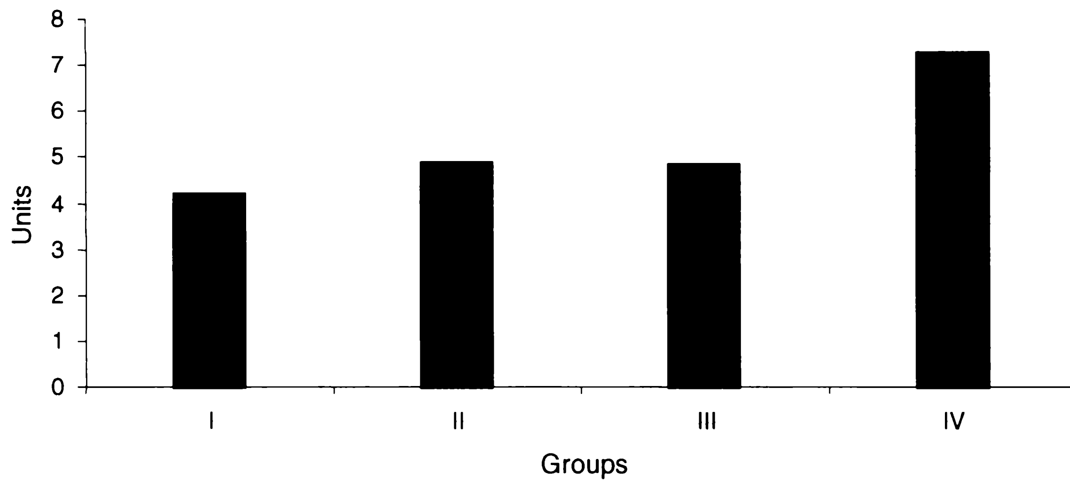


Fig 4.2.3.6.2 SOD activity in the hepatopancreas of the control and selenium supplemented *P. monodon*



One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

Fig 4.2.3.6.3 SOD activity in the muscle tissue of the control and selenium supplemented *P. monodon*



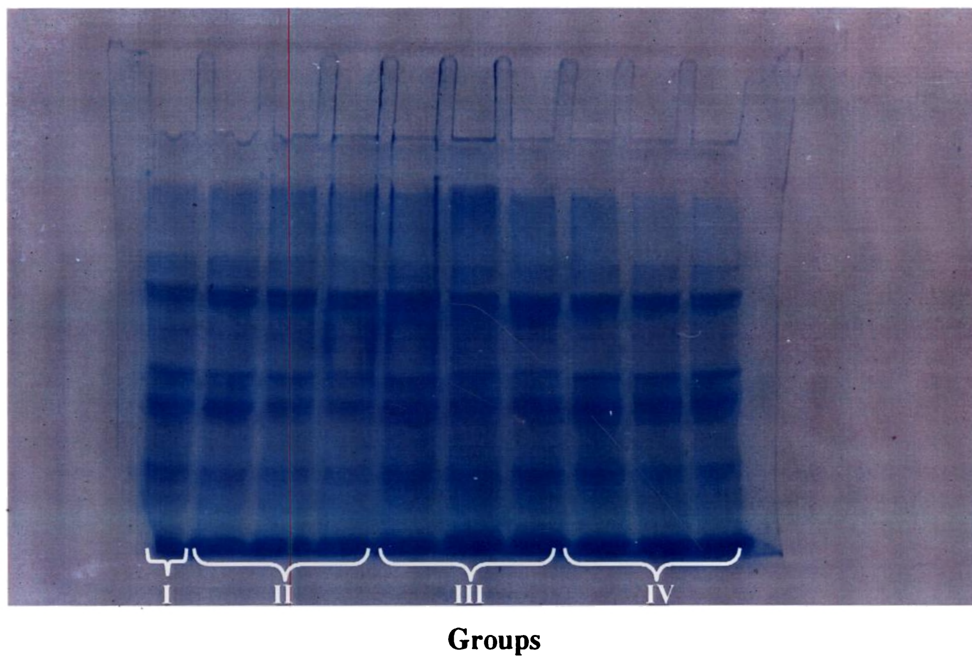
One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

Table 4.2.4 Trace element status of control and selenium supplemented experimental shrimps

Element	Group II (Control)	Group II	Group III	Group IV
Cu (μg%)	137.5±5.3	142.3±3.9	135.9±2.2	131.4±3.5
Co (μg%)	2.7±0.25	2.1±0.17	2.2±0.14	2.4±0.15
Fe (mg%)	0.38±0.02	0.45±0.02	0.49±0.03	0.45±0.02
Mg (mg%)	12.8±0.28	13.6±0.31	13.4+0.26	13.1±0.23
Mn (μg%)	136.3±4.6	137.0±3.2	131.5±3.1	140.8±3.1
Mo (μg%)	3.2±0.09	3.9±0.14	3.2±0.12	3.1±0.13
Se (μg/g)	0.26±0.01	0.28±0.01	0.32±0.01[a]	0.38±0.01[abc]
Zn (mg%)	23.08±0.70	21.6±0.95	24.0±1.0	22.2±1.0

Values are expressed as Mean ± SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals

Fig 4.2.5 Electrophoretic pattern of tissue protein of the control and experimental shrimps supplemented with selenium



Groups

selenium may affect the tissue levels of proteins. But in the present study, there is no change in the band pattern of the control shrimps and that of the experimental shrimps. So it could be assumed that selenium does not influence the protein synthesis in any significant manner.

4.3 EFFECT OF MOLYBDENUM ON GROWTH AND OTHER BIOCHEMICAL PARAMETERS

4.3.1 *Effect of Mo on growth of P. monodon*

Fig (4.3.1.1) gives the growth in control and Mo supplemented groups of *P. monodon*. Only group II animals, fed diet supplemented with 0.5 ppm levels of Mo showed a significant weight gain when compared to control animals. A further increase in Mo levels was found to be growth inhibitory as observed by Moffor and Rodway (1991) in ewe-lambs and by Frank *et al.* (2000) in other animals. Incorporation of Mo in the diet reduced the feed intake in all the supplemented groups. But at lower level of Mo supplementation (group II) the shrimps exhibited significantly increased SGR and a better FCR (Fig.4.3.1.2) and the feed conversion efficiency was also significantly increased in this group (fig.4.3.1.3), which ultimately resulted in the enhanced growth observed in this group. Reduced feed consumption together with reduced FCE and PER might be responsible for the growth retardation in groups III and IV animals. Fig.4.3.1.4 shows the mortality in control groups and groups supplemented with Mo. Group II and III animals showed a decrease in mortality where as elevated level of Mo enhanced the mortality. Molybdenum is considered to be a relatively non-toxic element for aquatic organisms (Hamilton and Buhl, 1990). Despite the nontoxic nature of molybdenum, (LC50 was greater than 2000mg/l) acute sub

lethal exposure to this metal had physiological consequences to those fish exposed even for only a brief period (Reid, 2002). The increased mortality observed at higher levels of molybdenum supplementation might be a reflection of these physiological consequences.

4.3.2 *Effect of molybdenum on the biochemistry of P. monodon*

4.3.2.1 *Proximate composition*

No significant change in any of the major muscle constituents was observed between control shrimps and shrimps supplemented with molybdenum (Table 4.3.2.1).

4.3.2.2 *Amino acid composition*

Amino acid composition of the control and experimental shrimps, supplemented with Mo is given in the table 4.3.2.2. The amino acids namely, glutamic acid, glycine and cysteine increased significantly in shrimp supplemented with molybdenum at 0.5 ppm and 2.5 ppm levels. There is no supportive evidence for the effect of molybdenum on these amino acids. It can be either at the level of

Fig.4.3.1.1 Effect of dietary molybdenum on growth of control and experimental shrimps

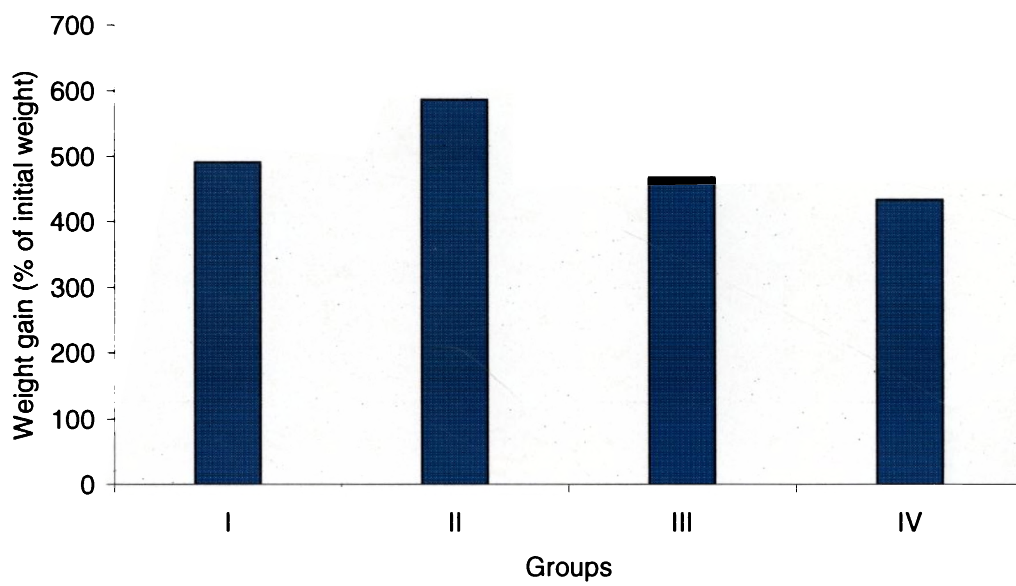


Fig.4.3.1.2 Effect of dietary molybdenum on FCR and SGR of control and experimental shrimps

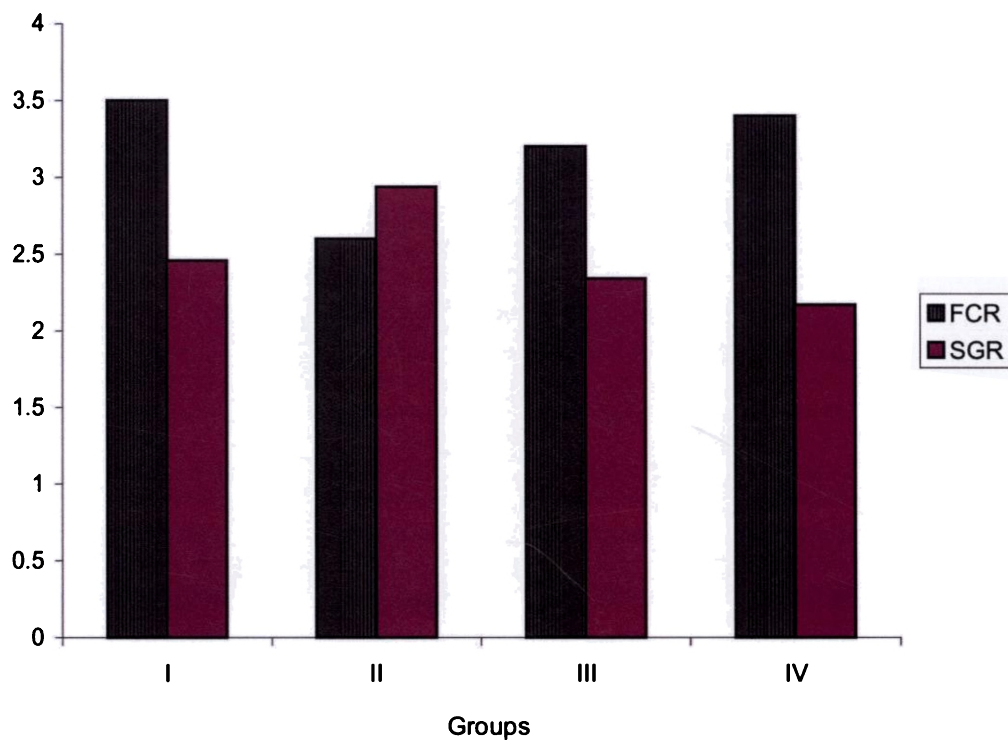


Fig.4.3.1.3 Effect of dietary molybdenum on FCE and PER of control and experimental shrimps

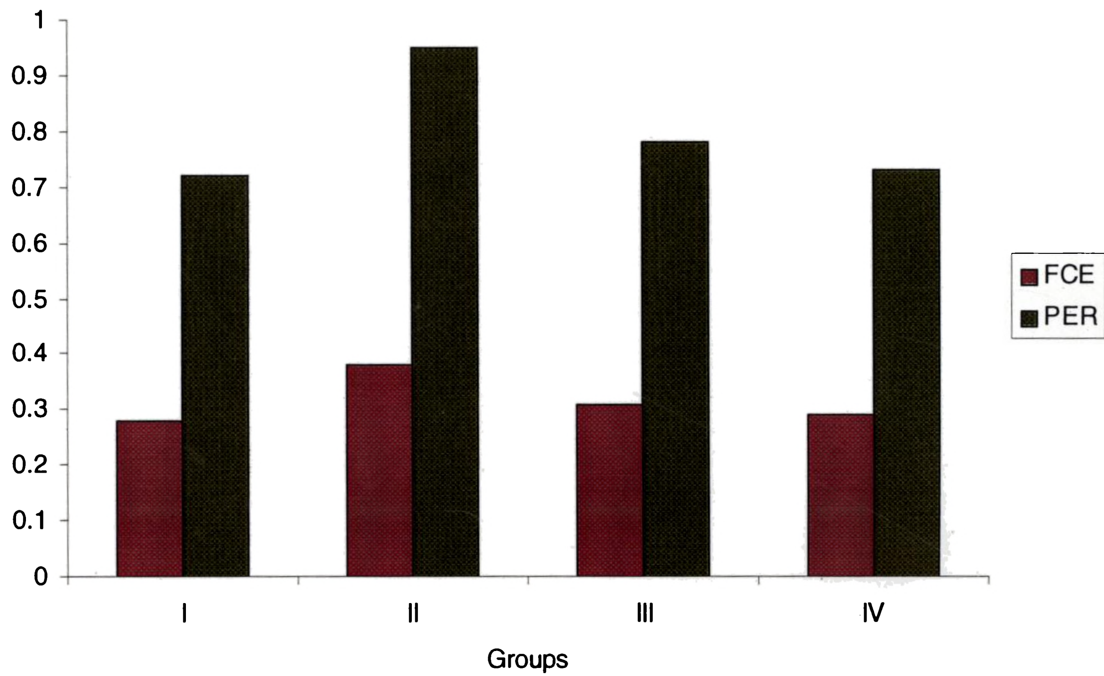


Fig.4.3.1.4 Effect of dietary molybdenum on mortality of control and experimental shrimps

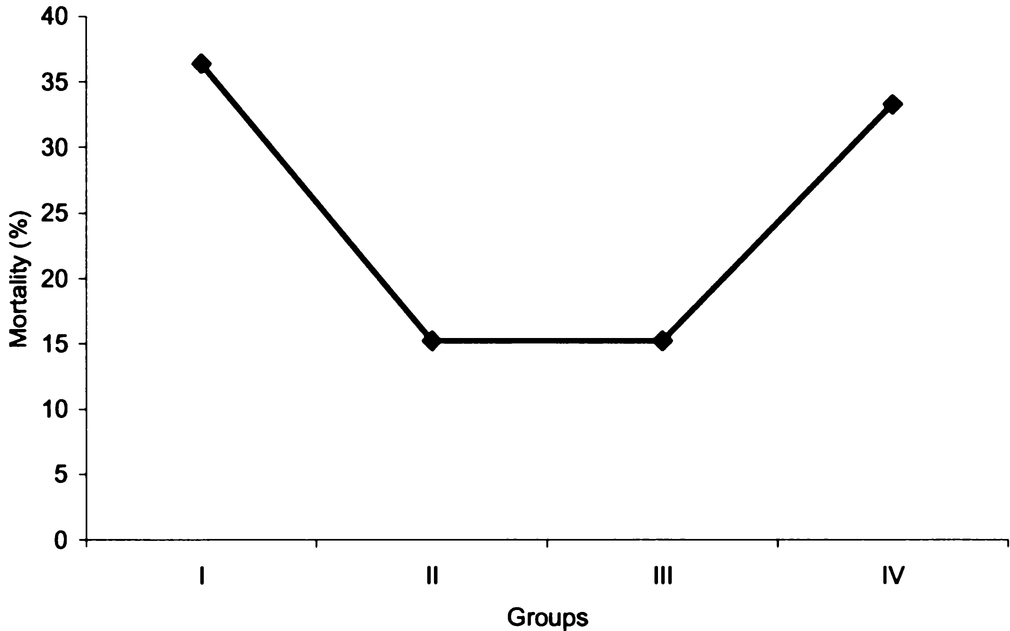


Table 4.3.2.1 Effect of dietary molybdenum on proximate composition of control and experimental shrimps

PARAMETERS (%)	GROUP I (CONTROL)	GROUP II (0.5ppm)	GROUP III (2.5ppm)	GROUP IV (5ppm)
Moisture	75.3±0.21	75.5±0.32	75.8±0.37	75.2±0.19
Protein	21.3±0.14	22.5±0.30	21.8±0.24	20.4±0.30
Crude fat	1.9±0.11	2.1±0.08	2.0±0.12	2.0±0.08
Ash	1.6±0.06	1.4±0.06	1.4±0.08	1.4±0.07

Values are expressed as Mean ± SE

Table 4.3.2.2 Effect of dietary molybdenum on amino acid composition of control and experimental shrimps

Amino acid (g/16g N2)	I	II	III	IV
Asp	10.74±0.44	10.78±0.26	10.84±0.48	10.61±0.48
Thr	3.72±0.08	3.59±0.24	3.54±0.12	3.59±0.24
Ser	3.73±0.18	3.70±0.18	3.56±0.76	3.70±0.26
Glu	15.68±0.47	18.65±0.29 [a]	19.41±0.31 [a]	17.84±0.35 [ac]
Pro	4.63±0.24	4.76±0.23	4.89±0.38	4.62±0.29
Gly	8.15±0.26	10.27±0.25 [a]	10.35±0.23 [a]	8.90±0.29 [bc]
Ala	8.10±0.34	8.29±0.23	8.71±0.44	8.60±0.26
Cys	0.41±0.02	0.65±0.03 [a]	0.78±0.03 [a b]	0.48±0.03 [c]
Val	5.26±0.34	4.9±0.34	4.8±0.15	4.8±0.23
Met	2.3±0.32	2.16±0.32	2.2±0.20	2.1±0.14
Ile	3.5±0.20	3.2±0.05	3.4±0.11	3.3±0.24
Leu	6.9±0.17	6.6±0.20	6.9±0.08	6.7±0.26
Tyr	1.5±0.15	1.5±0.12	1.7±0.21	1.5±0.26
Phe	3.7±0.16	3.3±0.14	3.8±0.17	3.4±0.18
His	2.0±0.08	2.0±0.14	2.1±0.17	2.0±0.13
Lys	2.9±0.14	2.3±0.14	2.5±0.20	2.4±0.10
Arg	7.8±0.23	7.8±0.26	7.9±0.31	7.9±0.32
Trp	1.34±0.03	1.07±0.08	1.03±0.08	1.20±0.11

Values are expressed as Mean±SE, a = Significantly different ($p<0.05$) when compared to control animals; b = Significantly different ($p<0.05$) when compared to group II animals; c = Significantly different ($p<0.05$) when compared to group III animals.

Table 4.3.2.3 Effect of dietary molybdenum on fatty acid composition of control and experimental shrimps

Fatty acids (%)	Group I	Group II	Group III	Group IV
C14:0	1.5±0.11	1.6±0.12	1.4±0.12	1.7±0.14
C16:0	24.3±0.29	24.5±0.23	24.5±0.17	24.6±0.63
C16:1	2.5±0.18	2.4±0.15	2.3±0.17	2.7±0.21
C17:0	1.8±0.14	1.9±0.12	1.8±0.17	1.7±0.14
C18:0	9.9±0.17	9.8±0.18	9.5±0.14	9.8±0.17
C18:1	14.2±0.17	14.2±0.23	14.5±0.20	14.7±0.24
C18:2	17.5±0.26	17.6±0.14	17.3±0.23	17.0±0.15
C18:3	0.8±0.08	0.9±0.08	0.8±0.11	0.7±0.08
C18:4	1.0±0.12	1.1±0.17	0.9±0.11	1.0±0.11
C20:2	0.7±0.08	0.9±0.12	0.9±0.15	1.0±0.18
C20:4	1.8±0.08	1.6±0.11	1.7±0.13	1.5±0.17
C20:5	9.3±0.31	9.2±0.21	9.1±0.14	8.6±0.18
C22:6	8.9±0.23	9.2±0.17	9.1±0.14	$8.5±0.20^{b}$

Values are expressed as Mean±SE, a = Significantly different ($p<0.05$) when compared to control animals b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals.

synthesis or at the level of absorption. These three amino acids are the amino acids present in the important cellular tripeptide, glutathione. In the present experiment these groups also exhibited an enhancement in the levels of GSH. This might be the reason for the observed increase in these amino acids.

4.3.2.3 *Fatty acid composition*

Table 4.3.2.2 shows the fatty acid profile of control and molybdenum supplemented *P. monodon*. Significant changes were not observed in any of the fatty acids other than the reduced level observed with the fatty acid C22:6, when compared to that of group supplemented with 0.5 ppm molybdenum. This fatty acid is one of the most important fatty acid required by *P. monodon*. It is the major component of phpospholipids, which play an important structural role in cell membranes. Along with this fatty acid, C20:5 also showed a moderate decrease.

4.3.3 *Effect of molybdenum on Tissue Defense System of P. monodon*

4.3.3.1 *Lipid peroxidation*

Lipid peroxidation in control shrimps and shrimps fed molybdenum supplemented diet are given in fig 4.3.3.1. Significant increase in lipid peroxidation was observed in both muscle tissue and

hepatopancreas at 2.5 ppm and 5 ppm level of molybdenum supplementation. In the case of hepatopancreas, 0.5 ppm level of molybdenum supplementation also showed significant change. Lipid peroxidation is regulated by the availability of substrate in the form of polyunsaturated fatty acids, the availability of inducers such as free radicals and excited state molecules to initiate propagation and the reaction. Increased lipid peroxidation with the supplementation of molybdenum has been reported earlier in experimental animals (Koizumi *et al.*, 1995; Cerone *et al.,* 2000). Molybdenum is a co-factor for enzymes such as XO, which will cause the release of super oxide radicals (Shaw, 1989). Mo supplementation might have enhanced the reaction leading to the increased peroxidation of lipid in the tissue, which is in accordance with earlier reported studies (Sugihara *et al.*, 2001; Darr and Fridovich, 1984).

4.3.3.2 *Reduced Glutathione content (GSH)*

Reduced glutathione content was found to increase significantly in both hepatopancreas and muscle tissue of all the groups supplemented with molybdenum (fig 4.3.3.2.1 and fig 4.3.3.2.2). The increased release of oxygen free radicals might have activated the GSH dependent anti oxidant defense system and simultaneously the increased production of GSH. But when compared to group II and group III, the group IV animals showed a significant reduction in their

levels of GSH content in the hepatopancreas. Similarly group IV animals showed a significantly lower GSH content than that of group III animals in the muscle tissue. This indicates the excess consumption of the reduced glutathione due to the increased lipid peroxidation observed in these groups. GSH is the most abundant non-protein thiol in the cells (Anderson and Meister, 1980). It is known to have several important physiological roles including amino acid transport, detoxification reaction and storage of transport form of cystein (Meister and Anderson, 1983). Maintenance of adequate levels of GSH is thought to be necessary to protect membrane proteins and lipids against oxidative damage resulting from the formation of H_2O_2, super oxide and other free radicals (Burk, 1989). Excessive lipid peroxidation can cause increased GSH consumption (Deneke and Fanburg, 1989). The higher activity of xanthine oxidase could also be a major factor for the observed decrease in GSH as reported by Schimple *et al.* (2000).

4.3.3.3 *Total sulfhydryl content (TSH)*

TSH level in the muscle tissue and hepatopancreas of the control and experimental shrimps treated with molybdenum are given in the table 4.3.3.3. Shrimp fed diet supplemented with 0.5 ppm and 2.5 ppm levels of molybdenum showed significant increase in the level of TSH. But in hepatopancreas, there was a significant reduction

in the level of TSH in the group supplemented with 5.0 ppm levels of molybdenum when compared to that of control group. In these groups of shrimps an enhancement in the level of lipid peroxidation was also observed. So it could be inferred that the SH groups are utilized to protect the cells from release of excess free radicals.

4.3.3.4 *Glutathione peroxidase (GPx)*

The activity of GPx of the control group and groups supplemented with Mo are given in Fig 4.3.3.4.1 and 4.3.3.4.2. No significant increase in the activity of GPx was observed in the case of muscle tissue of the molybdenum supplemented groups when compared to that of control animals. But in hepatopancreas, a significant increase was observed in group III animals. The metabolic function of GPx is part of the mechanism responsible for the detoxification of oxygen free radicals (Anandan *et al.*, 1999). The reduction of oxygen in the form of such active species H_2O_2, OH^- and organic peroxide is accomplished by the combined action of free radical scavengers such as Vitamin E, and Vitamin C in conjunction with reduced glutathione, catalyzed by GPx. These agents also alleviate the toxic effects of drugs and antibiotics as well as experimental chemical carcinogen (Spolar *et al.*, 1999). The increased lipid peroxidation might be responsible for the observed increase in activity of GPx. At an elevated concentration of Mo (5.0 ppm), the

activity of this enzyme was significantly (P<0.05) reduced in both muscle and hepatopancreas. Inhibitory effect of Mo on the activity of GPx has been reported in goats earlier (Frank *et al.*, 2000).

4.3.3.5 *Glutahione-S- transferase (GST)*

In the present study GST activity increased with the supplementation of Mo in both muscle and hepatopancreas (fig.4.3.3.5.1 and 4.3.3.5.2). GST aids in the protection of the cell by enhancing the excretion of toxic metabolites in animals. (Jackoby, 1977). Therefore the level of GST, have been suggested as important indicator of the susceptibility of organisms or tissues to the challenges posed by xenobiotics. Here significant increase (P<0.05) in activity was noticed at elevated concentrations of molybdenum supplementation (2.5 and 5 ppm levels) in hepatopancreas. But in muscle tissue the increase in activity was significant only at 5 ppm level (P<0.05). Even though low levels of Mo (0.5 ppm) enhanced the activity, the increase was not significant. Hepatopancreas showed a higher activity of GST when compared to muscle tissue. GST gets activated upon increased release of lipid peroxides. At low Mo concentration, the other enzymes such as SOD, CAT and GPx had higher activity. But at elevated levels of Mo both SOD and CAT showed a decline in activity. Moreover higher Mo concentrations had an inhibitory effect on the activity of GPx. These functions together might be responsible for the higher activity of GST in these groups.

Fig 4.3.3.1 Effect of dietary molybdenum on Lipid peroxidation of control and experimental shrimps

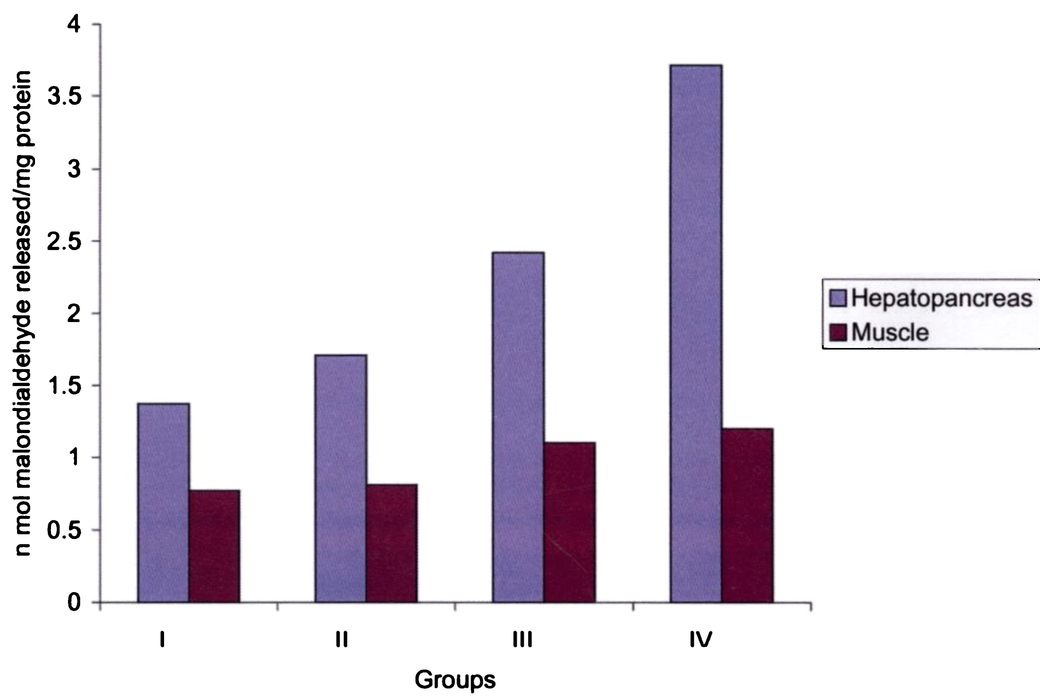


Fig 4.3.3.2.1 Effect of dietary molybdenum on GSH content in hepatopancreas of control and experimental shrimps

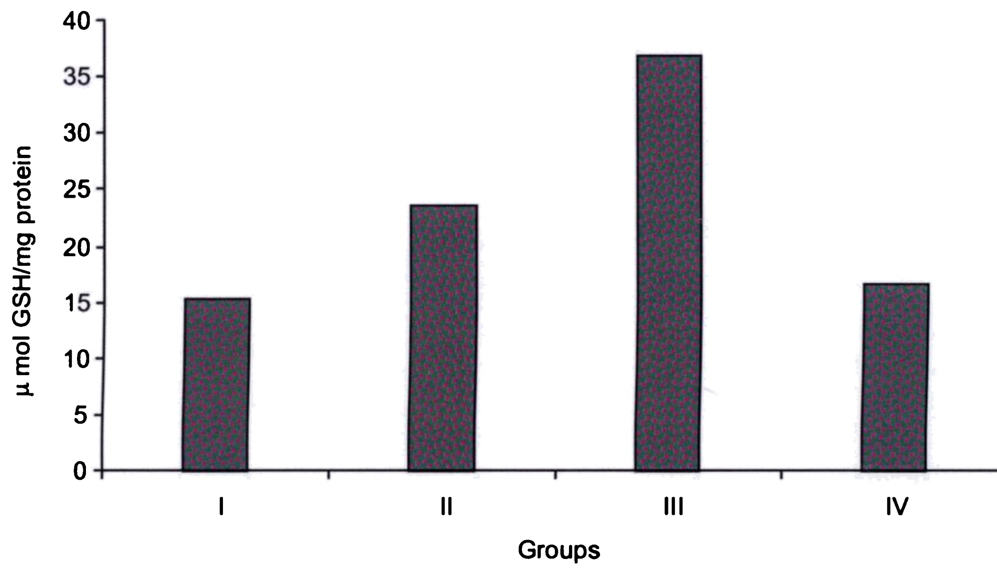


Fig 4.3.3.2.2 Effect of dietary molybdenum on GSH content of muscle tissue of control and experimental shrimps

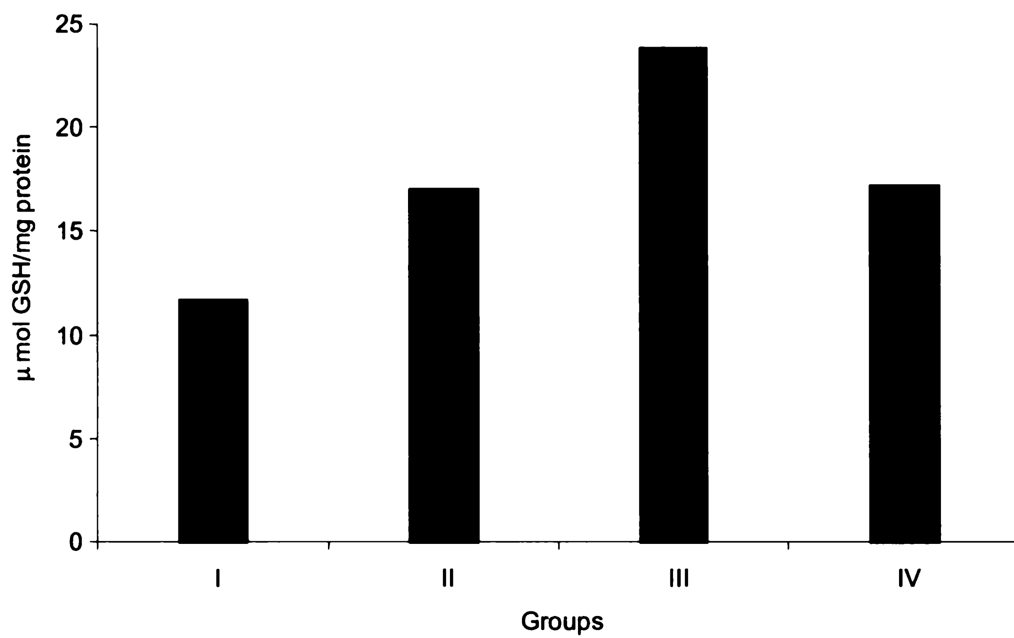


Table 4.3.3.3 Effect of dietary molybdenum on Total sulfhydryl content (TSH) of muscle tissue and hepatopancreas of control and experimental shrimps

TSH ng/mg tissue				
Tissue	Group I	Group II	Group III	Group IV
Hepatopancreas	241.3±4.0	454.9±4.7[a]	518.2±5.4[ab]	227.5±4.1[abc]
Muscle tissue	170.2±4.9	238.6±5.0[a]	321.4±4.4[ab]	195.1±5.3[ac]

Values are expressed as Mean±SE, a = Significantly different ($p<0.05$) when compared to control animals b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals.

Fig 4.3.3.4.1 Effect of dietary molybdenum on Glutathione peroxidase (GPx) activity of hepatopancreas of control and experimental shrimps

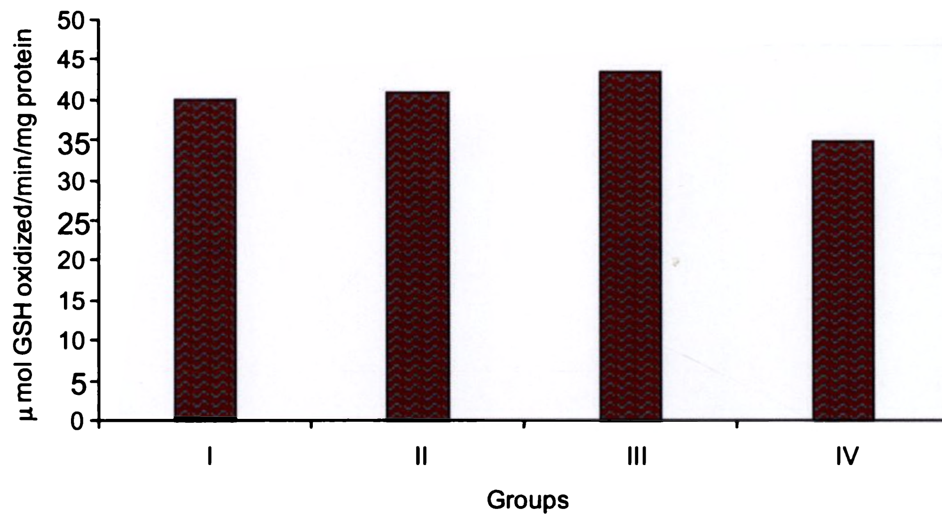


Fig 4.3.3.4.2 Effect of dietary molybdenum on Glutathione peroxidase (GPx) activity of muscle tissue of control and experimental shrimps

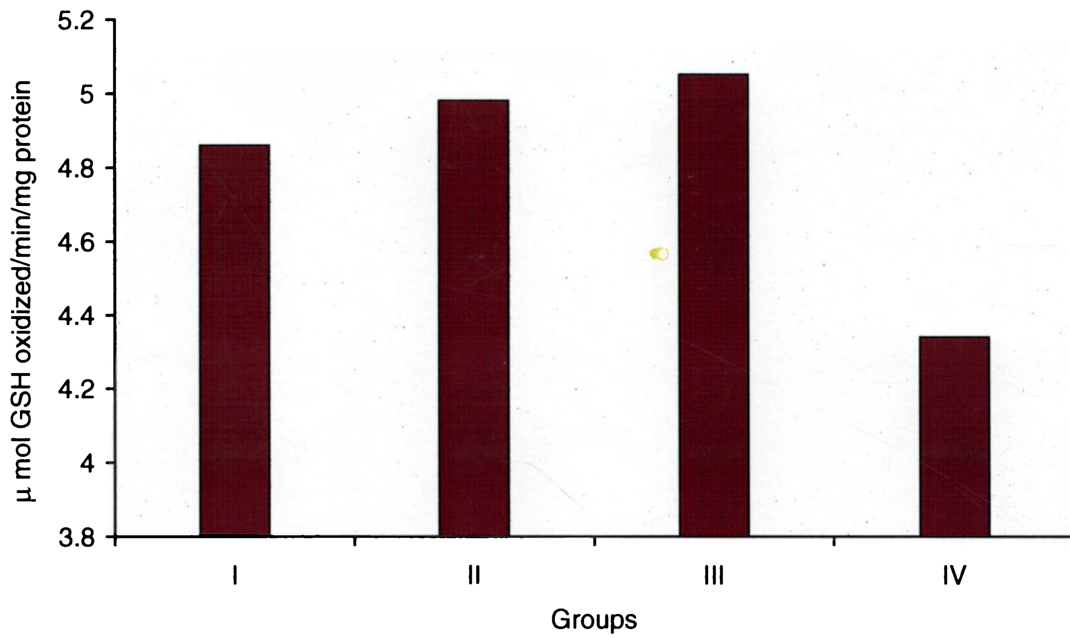


Fig 4.3.3.5.1 Effect of dietary molybdenum on Glutathione S-transferase (GST) activity of hepatopancreas of control and experimental shrimps

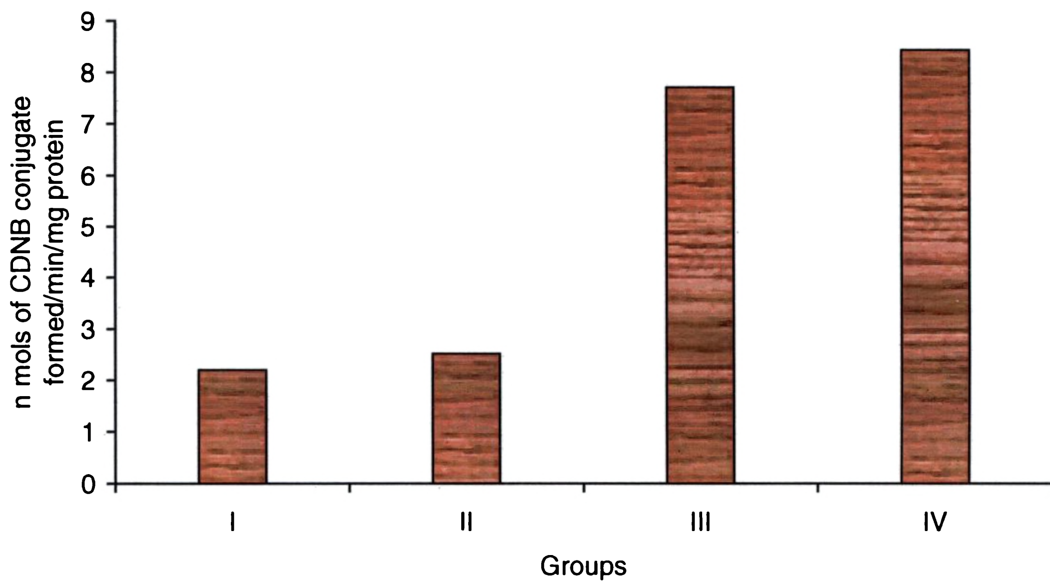
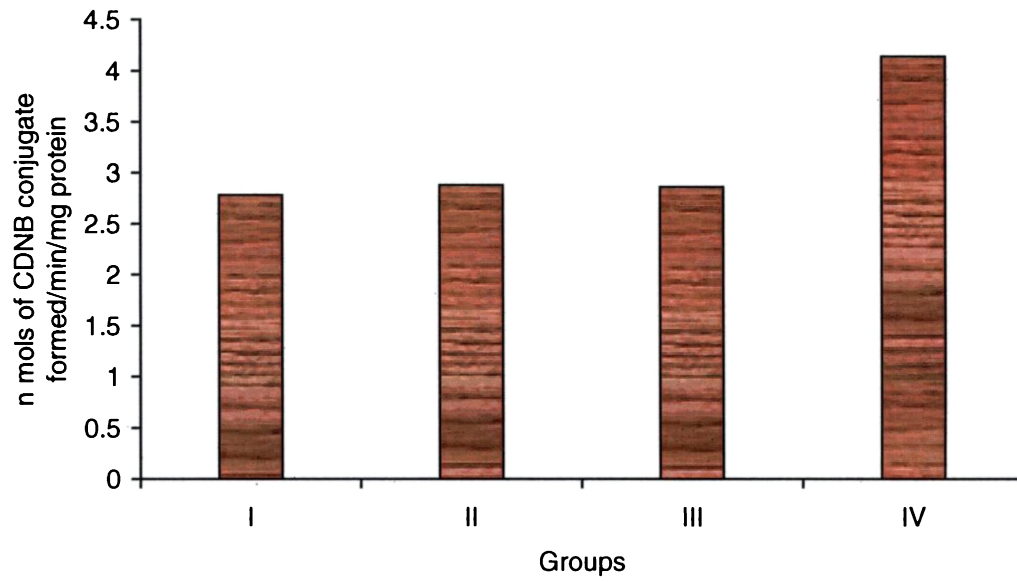
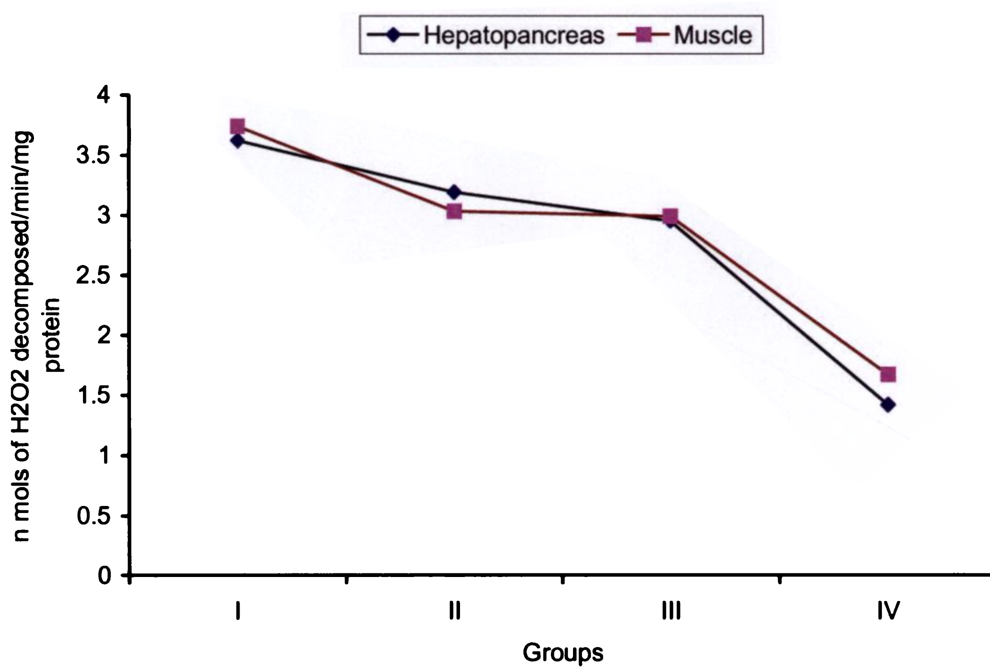


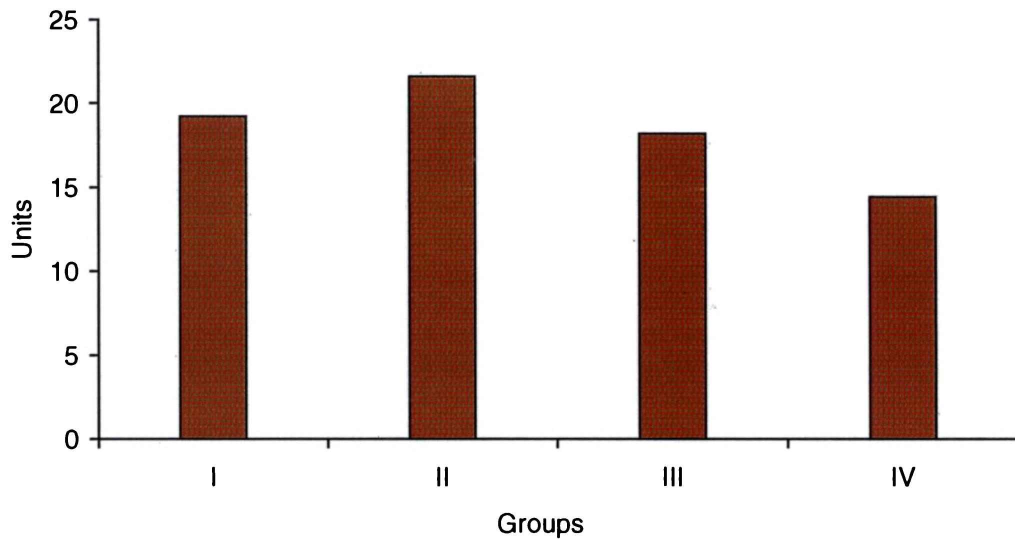
Fig 4.3.3.5.2 Effect of dietary molybdenum on Glutathione S-transferase (GST) activity of muscle tissue of control and experimental shrimps



4.3.3.6.3 Effect of dietary molybdenum on antiperoxidative enzyme (CAT) activity of hepatopancreas and muscle tissue of control and experimental shrimps

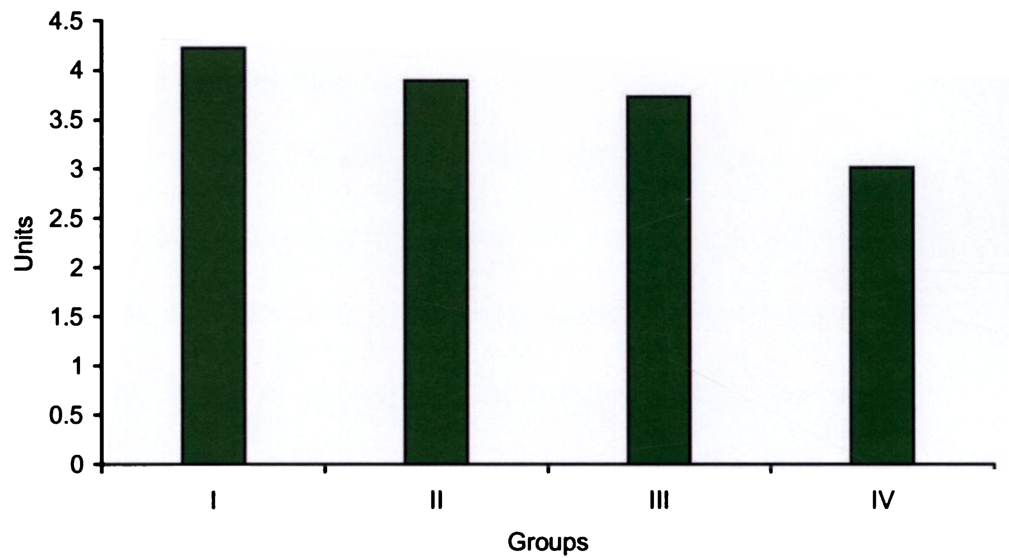


4.3.3.6.1 Effect of dietary molybdenum on antiperoxidative enzyme (SOD) activity of hepatopancreas of control and experimental shrimps



One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

4.3.3.6.2 Effect of dietary molybdenum on antiperoxidative enzyme (SOD) activity of muscle tissue of control and experimental shrimps



One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

4.3.3.6 ***Antiperoxidative enzymes (SOD & CAT)***

Present study shows the negative effect of Mo on the activity of the enzyme SOD (Fig. 4.3.3.6.1 and 4.3.3.6.2). This enzyme scavenges the super oxide radicals which appear to be an important agent of oxygen free radical toxicity and thus provides protection against free radical toxicity. It is the Cu/Zn form of this enzyme, which plays crucial role in the defense against free radicals. Mo is an antagonist to Cu. Excessive Mo intake causes secondary Cu deficiency and subsequently affects the iron Metabolism (Frank *et al.*, 2000). The elevated Mo level might have inhibited the absorption of Cu. Since SOD is a copper-containing enzyme, the deficiency of available copper ultimately might have resulted in decreased activity of this enzyme. This is in line with the previous reports (Xin *et al.*, 1991; Frank *et al.*, 2000).

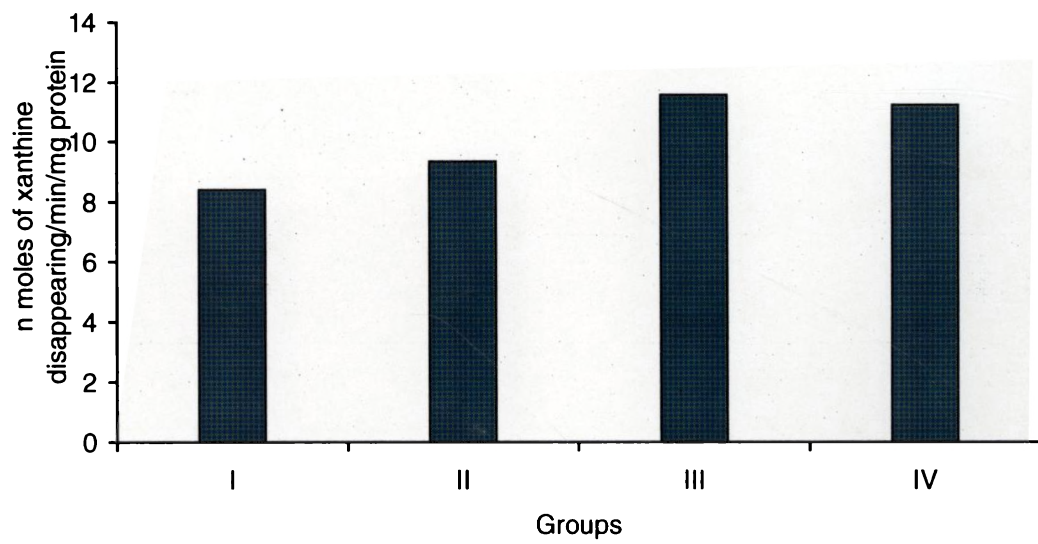
Catalase, another antiperoxidative enzyme is also a metallo enzyme in which iron forms the co-factor. Non-availability of iron affects the activity of this enzyme. Due to the inter relationship between iron and Cu, the deficiency of Cu often results in decreased activity of this enzyme as observed in molybdenum-supplemented groups (Fig 4.3.3.6.3).

4.3.3.7 *Molybdenum dependent oxidative enzymes (XO and SO)*

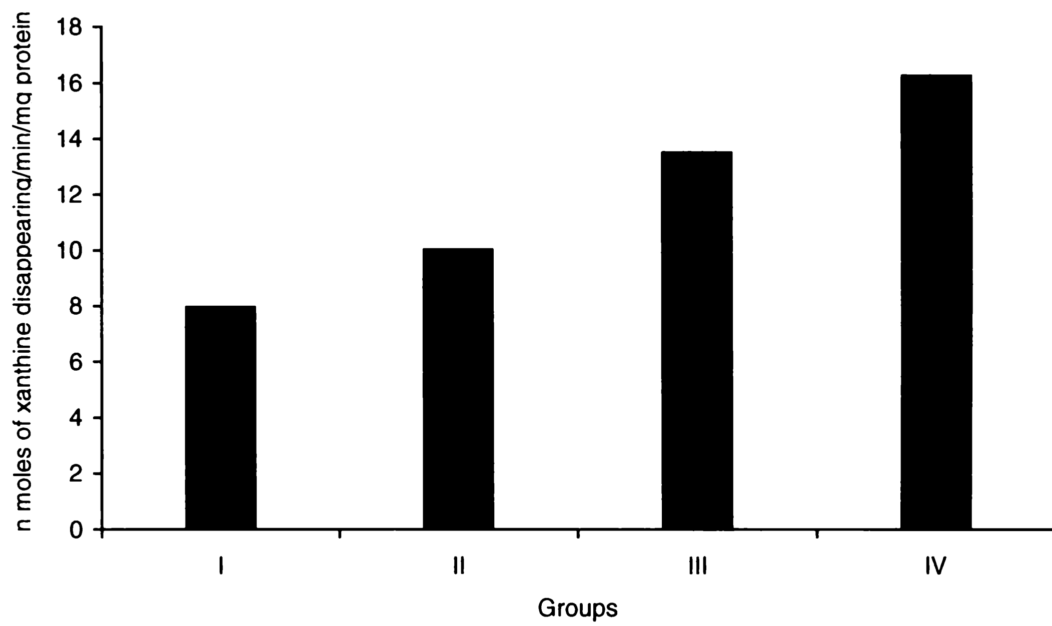
The activity of xanthine oxidase (XO) was significantly higher in muscle and hepatopancreas tissues of molybdenum-supplemented prawns compared to control animals (Fig 4.3.3.7.1). The results obtained in this study indicate that dietary molybdenum has a positive effect on the activity of the enzyme XO. The percentage of increase was found to be much higher in hepatopancreas than in muscle tissue. A similar increase in XO activity was earlier reported by Komada *et al.* (1990) in rats. Increased activity of this enzyme is harmful to the system as this results in increased production of super oxide radicals (Shaw, 1989; Borges *et al.*, 2002). Since superoxides are the precursors of reactive species such as hydroxyl radicals, peroxides and hydro peroxides, they are extremely toxic to the system.

In the present study, an increase in the activity of sulfite oxidase (SO) was observed in groups given diets supplemented with molybdenum (Fig 4.3.3.7.3 and 4.3.3.7.4). Increased SO activity by molybdenum supplementation was reported earlier in rats (Wang *et al.*, 1992). Compared to muscle tissue, increased activity of this enzyme was noticed in hepatopancreas. The activity of this enzyme proportionally increased in muscle tissue with increased supplementation of molybdenum. But hepatopancreas was found to exhibit a different pattern. The groups fed on diets with molybdenum

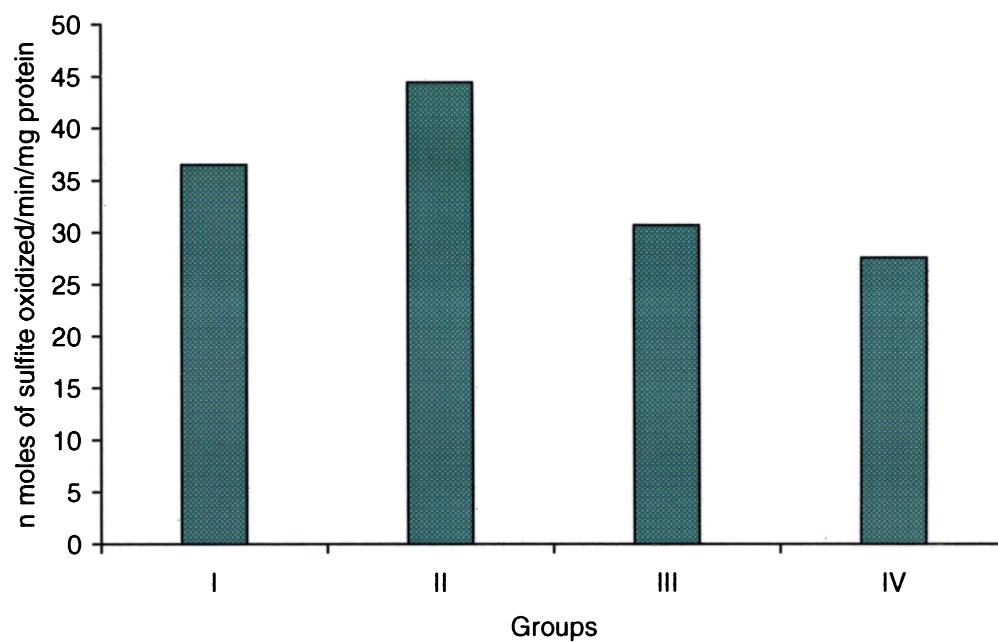
4.3.3.7.1 Xanthine oxidase activity of hepatopancreas of control and experimental shrimps



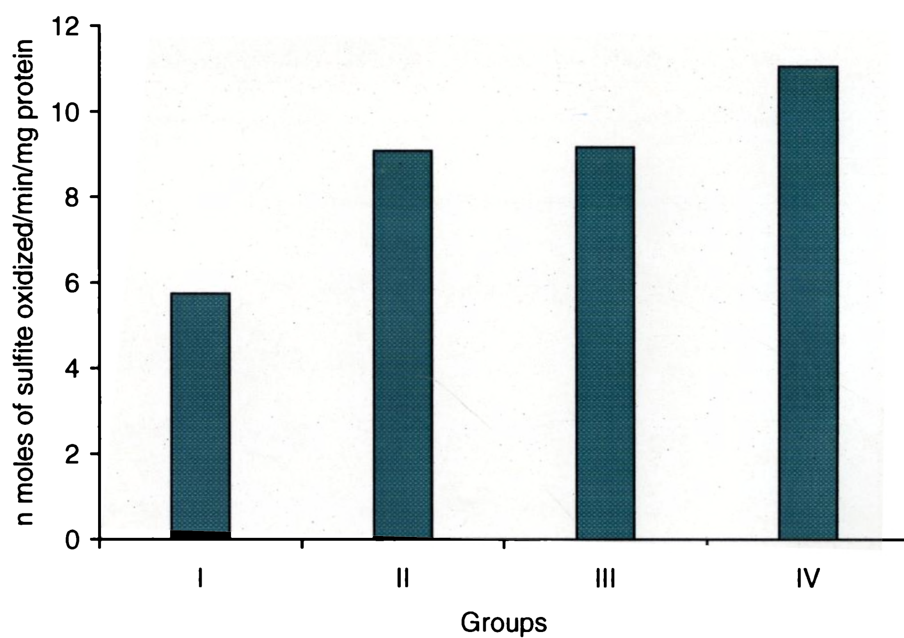
4.3.3.7.2 Xanthine oxidase activity of muscle tissue of control and experimental shrimps



4.3.3.7.3 Sulfite oxidase activity of hepatopancreas of control and experimental shrimps



4.3.3.7.4 Sulfite oxidase activity of muscle tissue of control and experimental shrimps



at 2.5 ppm and 5.0 ppm showed a significant decrease in the activity. SO is an important enzyme involved in the metabolism of sulfur containing amino acids and catalyzes the production of inorganic sulfates by sulfoxidation (Sardesai, 1993; Rajagopalan, 1988). A number of xenobiotics employ sulfation as the primary route of detoxification (Mc Fadden, 1996). Yang and Yang (1989) reported a similar decrease in the activity of SO at an elevated molybdenum concentration in female rats. Since liver is the major organ for detoxification, the higher activity of the enzyme in hepatopancreas might have resulted in increased concentration of sulfate. Binding of sulfate ions to the active site of the enzyme prevents intramolecular electron transport in SO (Pacheco *et al.*, 1999; Chaudhary *et al.*, 1996). This may be the reason for decreased activity of SO in hepatopancreas at elevated levels of molybdenum supplementation. The free radicals produced at higher concentration of molybdenum might also be possible factors to exert an inhibitory action on the activity of this enzyme in hepatopancreas.

4.3.4 *Trace element status*

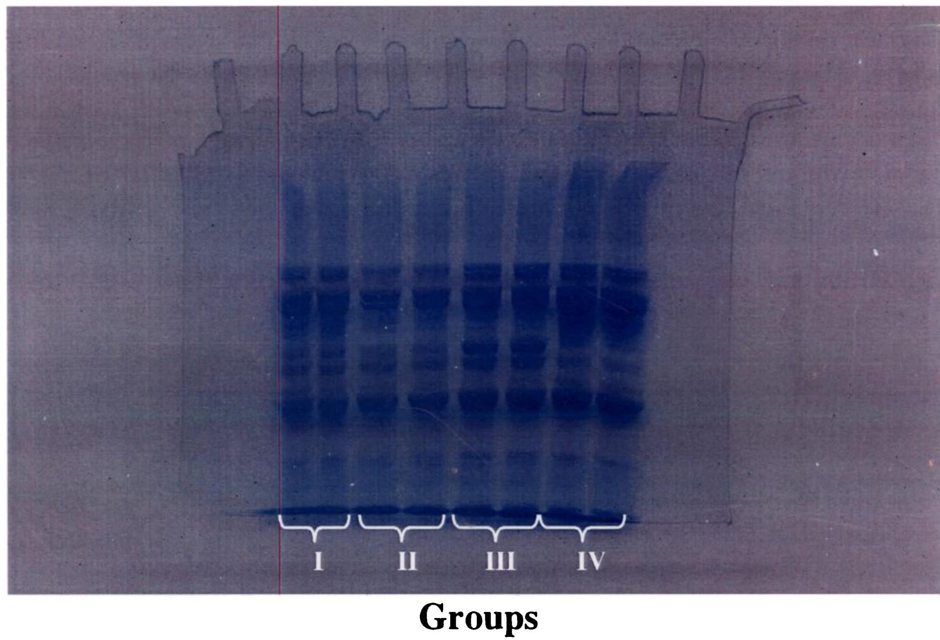
Dietary status of molybdenum is very important in maintaining normal levels of trace element in the tissues. Excessive molybdenum causes secondary copper deficiency and also a possible chromium deficiency (Frank *et al.*, 2000). In their study they could observe that the levels of Al, Ca, Fe, Co, Mo, Pb, Se and Mn were

4.3.4 Effect of dietary molybdenum on Trace element status of control and experimental shrimps

Element	Group I	Group II	Group III	Group IV
Cu (μg%)	135.0±2.8	127.0±3.3	92.6±4.1[ab]	35.4±2.6[abc]
Co (μg%)	2.7±0.11	2.3±0.16	2.9±0.20	2.7±0.17
Fe (mg%)	0.35±0.02	0.41±0.01	0.31±0.02[b]	0.39±0.02[c]
Mg (mg%)	12.4±0.38	13.5±0.55	13.3±0.46	14.1±0.11[a]
Mn (μg%)	103.0±2.0	128.0±1.5[a]	135.0±3.4[a]	130.5±1.1[a]
Mo (μg%)	3.06±0.17	3.6±0.20	8.79±0.14[ab]	13.4±0.53[abc]
Se (μg/g)	0.25±0.01	0.28±0.01	0.23±0.02	0.24±0.01
Zn (mg%)	25.9±0.79	22.0±1.08	25.8±0.93	23.4±0.95

Values are expressed as Mean±SE, a = Significantly different ($p<0.05$) when compared to control animals b = Significantly different ($p<0.05$) when compared to group II animals, c = Significantly different ($p<0.05$) when compared to group III animals.

Fig 4.3.5 Electrophoretic pattern of tissue protein of the control and experimental shrimps supplemented with molybdenum



Groups

increased considerably in the liver of tetrathiomolybdate supplemented male goats. Here in the present study also significant reduction in the level of copper was observed in groups treated with 2.5 ppm and 5.0 ppm levels of molybdenum (Table 43.4). This is a clear evidence for the antagonistic effect of molybdenum against copper. Iron content showed a fluctuating pattern. But when compared to the control group, the level did not show significant change. As reported by Frank *et al.* (2000), in the present study also, the magnesium content was found to increase at elevated level of molybdenum supplementation. Manganese levels were also significantly higher in molybdenum supplemented groups. These changes in the trace element levels are of considerable importance that it will adversely affect the activities of various enzymes as well as the absorption and metabolism of some other elements.

4.3.5 *Electrophoretic pattern of tissue proteins*

Electrophoretic pattern of tissue proteins of the control and experimental shrimps are given in the figure 4.3.5. As far as the literatures available, molybdenum is not a component of any of the structural proteins. But it forms co-factor for certain enzymes (Kisker *et al.,* 1997). As there is no change in the tissue protein pattern in the control and experimental animals, it is concluded that molybdenum did not have any effect on tissue proteins in the administered levels.

4.4 EFFECT OF COBALT ON GROWTH AND OTHER BIOCHEMICAL PARAMETERS

4.4.1 *Effect of Co on growth of P. monodon*

Cobalt deficiency was found to be a reason for reduced weight gain observed in sheep (kennedy *et al.*, 1991). At the same time excess cobalt resulted in growth depression in tomato plants (Chatterjee and Chatterjee, 2003). In fish it was found that both survival and behavior were changed by excessive dietary supply of cobalt (Janssen, 2000). The influence of Co on the growth of the shrimp is evident from the present study. The weight gain was found to be increased significantly in prawns fed feed supplemented with cobalt at 2.5ppm and 5.0ppm levels (Fig.4.4.1.1). Maximum growth was observed in group III. This group of shrimps showed better results with respect to FCR, SGR (Fig.4.4.1.2) and FCE and PER (Fig.4.4.1.3) also. Higher growth rate and PER was reported earlier in *Tilapia zillii* fingerlings fed diet supplemented with cobalt (Anadu *et al.*, 1990).

A further increase in the level of Co in the feed did not significantly affect the growth. The work conducted in rats by Ybarra *et al.* (1997) also showed the growth promoting effect of cobalt. Present study also revealed that in the supplemented levels, Co did not have any effect on the mortality of the shrimps (Fig.4.4.1.4). The

mortality did not show any regular pattern, indicating the influence of cobalt.

4.4.2 *Effect of Co on the biochemistry of P. monodon*

4.4.2.1 *Proximate composition*

Analysis of the proximate composition of the control group shrimps and shrimps supplemented with Co showed that, dietary supplementation of Co did not alter any of the parameters in proximate composition other than crude fat content (Table 4.4.2.1). Cobalt supplemented groups of shrimps showed a small increase in fat content when compared to that of control groups. It was reported earlier in rats that Co supplementation produced hyperlipidemic changes (Basu Mallik, 1989).

4.4.2.2 *Amino acid composition*

Amino acid composition of the control and cobalt supplemented groups of shrimps are given in table 4.4.2.2. Cysteine and proline showed a significant increase in all groups supplemented with cobalt. Glycine increased in 2.5 ppm and 5.0 ppm supplemented shrimps. Oven *et al.* (2002) reported a similar increase in the amino acid cysteine in plant cells exposed to cobalt ions. In addition, experiment conducted in rats showed a reduction in the activities of enzymes such as glutamate decarboxylase and aromatic amino acid decarboxylase (Emson and Joseph, 1975). The resulting alteration in the metabolism of amino acids may also change the tissue levels of amino acids.

Fig 4.4.1.1 Effect of dietary cobalt on the growth of control and experimental shrimps

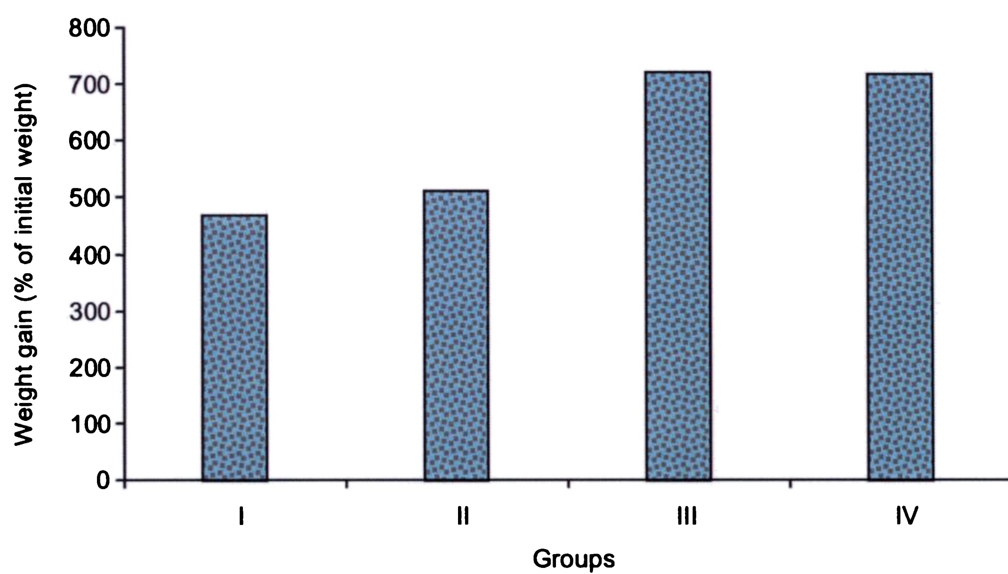


Fig.4.4.1.2 Effect of dietary cobalt on FCR and SGR of control and experimental shrimps

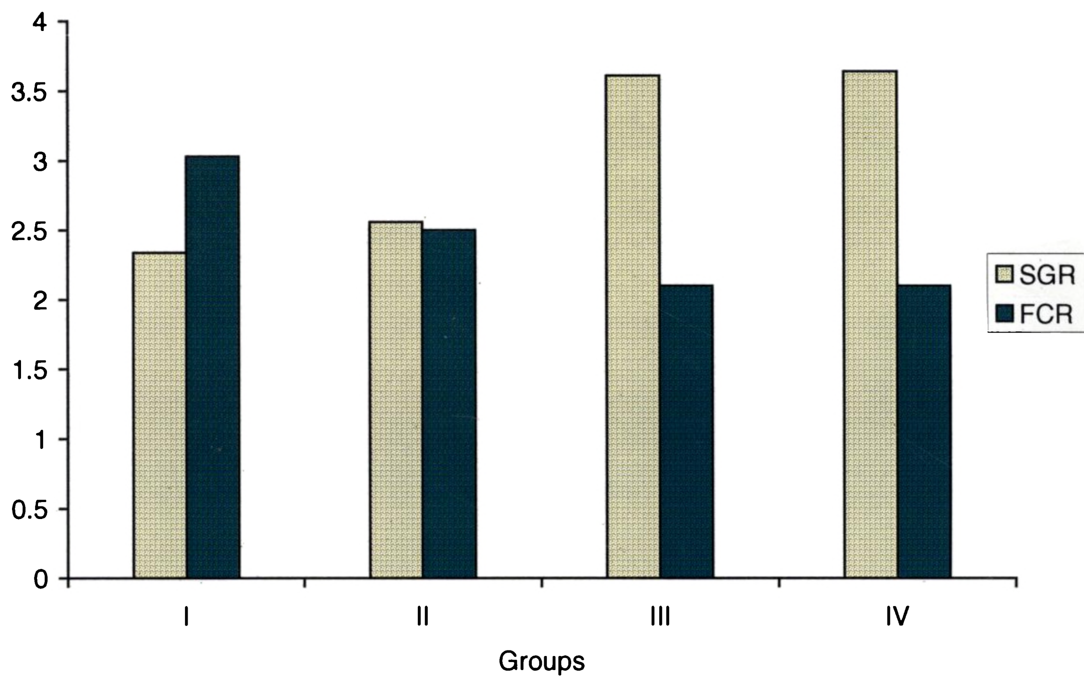


Fig.4.4.1.3 Effect of dietary cobalt on FCE and PER of control and experimental shrimps

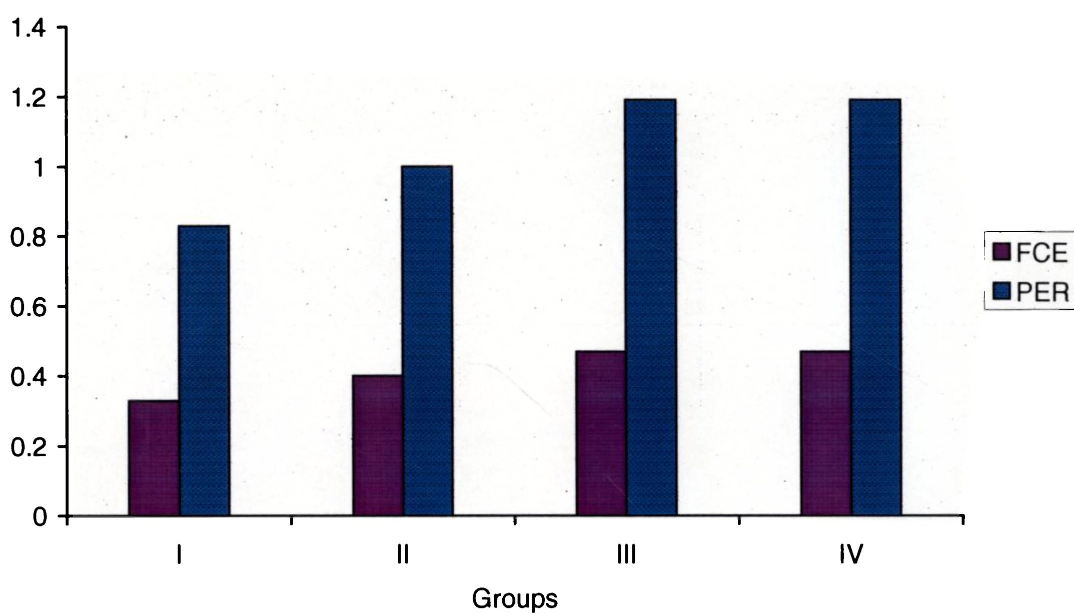


Fig.4.4.1.4 Effect of dietary cobalt on mortality of control and experimental shrimps

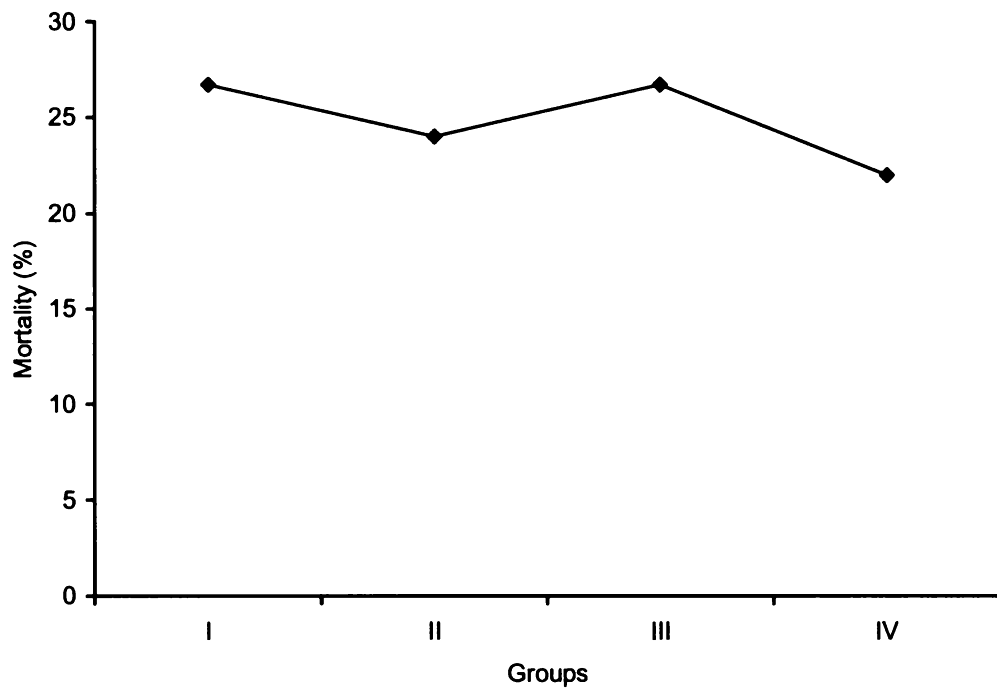


Table 4.4.2.1 Effect of dietary cobalt on proximate composition of control and experimental shrimps

	Group I (Control	Group II	Group III	Group IV
Moisture (%)	75.0±1.6	74.7±2.0	75.5±2.0	75.6±2.7
Total protein (%)	21.99±1.0	22.25±0.9	22.19±1.0	21.45±1.2
Crude fat (%)	1.8±0.1	2.1±0.2[a]	2.0±0.19[a]	1.9±0.19
Ash(%)	1.4±0.1	1.4±0.1	1.3±0.07	1.3±0.07

Values are expressed as Mean ± SD; a = Significantly different ($p<0.05$) when compared to control animals

Table 4.4.2.2 Effect of dietary cobalt on amino acid composition of control and experimental shrimps

Amino acid (g/16g N2)	I	II	III	IV
Asp	10.9±0.20	10.3±0.15[a]	10.4±0.11[a]	9.59±0.23[a]
Thr	3.4±0.05	3.45±0.12	3.33±0.17	3.23±0.14
Ser	3.7±0.12	3.65±0.04	3.72±0.08	3.72±0.03
Glu	10.3±0.08	10.3±0.12	10.3±0.12	10.4±0.05
Pro	3.40±0.11	3.75±0.12 a	4.16±0.06[a b]	4.05±0.03 a
Gly	11.1±0.14	12.0±0.12[a]	14.4±0.26[a b]	14.01±0.10[a b c]
Ala	7.02±0.03	7.05±0.14	7.38±0.03	11.3±0.08
Cys	0.04±0.00	0.09±0.00	0.41±0.00[a]	0.50±0.00[a]
Val	4.06±0.04	3.81±0.08	3.89±0.05	4.01±0.08
Met	1.94±0.03	1.94±0.03	2.09±0.03	1.61±0.03
Ile	2.50±0.05	2.54±0.08	2.80±0.11	2.53±0.03
Leu	5.99±0.14	6.08±0.13	6.15±0.03	5.94±0.03
Tyr	2.27±0.03	1.99±0.08	2.20±0.03	1.70±0.12
Phe	4.02±0.12	3.91±0.11	4.07±0.08	3.21±0.03
His	3.08±0.05	2.45±0.05	2.5±0.08	2.56±0.05
Lys	2.59±0.08	2.68±0.03	2.44±0.11	1.67±0.08
Trp	1.3±0.03	1.5±0.03	1.7±0.05	1.8±0.05

Values are expressed as Mean±SE, a= Significantly different ($p<0.05$) when compared to control animals b = Significantly different ($p<0.05$) when compared to group II animals, c= Significantly different ($p<0.05$) when compared to group III animals.

Table 4.4.2.3 Effect of dietary cobalt on fatty acid composition of control and experimental shrimps

FATTY ACIDS (%)	Group I	Group II	Group III	Group IV
C14:0	1.6±0.11	1.5±0.08	1.6±0.12	1.6±0.11
C16:0	24.5±0.60	24.3±0.49	24.6±1.0	24.2±0.65
C16:1	2.8±0.14	3.2±0.14	3.4±0.08	3.1±0.17
C17:0	1.7±0.05	1.8±0.09	1.6±0.07	1.7±0.09
C18:0	9.810.18	10.0±0.17	10.2±0.17	10.0±0.14
C18:1	14.0±0.14	14.9±0.12	15.1±0.20[a]	15.1±0.17[a]
C18:2	17.9±0.21	18.2±0.18	18.3±0.34	19.0±0.20[ab]
C18:3	0.9±0.08	0.9±0.13	0.8±0.06	0.8±0.04
C18:4	1.0±0.08	1.0±0.06	0.9±0.04	1.0±0.10
C20:2	0.9±0.09	1.0±0.09	1.1±0.10	1.0±0.09
C20:4	1.7±0.11	1.6±0.12	1.6±0.11	1.8±0.08
C20:5	9.2±0.20	9.8±0.20	9.9±0.23	8.9±0.46
C22:6	8.1±0.17	8.0±0.28	7.4±0.11[a]	6.4±0.17[abc]

Values are expressed as Mean±SE, a= Significantly different ($p<0.05$) when compared to control animals b= Significantly different ($p<0.05$) when compared to group II animals, c= Significantly different ($p<0.05$) when compared to group III animals.

4.4.2.3 *Fatty acid composition*

The fatty acid profile of control groups and cobalt supplemented groups are given in the table 4.4.2.3. There was a significant reduction in the levels of polyunsaturated fatty acid, DHA, at elevated levels of cobalt supplementation. The changes in the lipid metabolism of cattle in response to the prolonged, moderate cobalt deficiency were demonstrated by Stangl *et al.* (1999). In their study they could observe decrease in DHA and arachidonic acid followed by an increase in C18:2 fatty acids, in cobalt deficient animals. This effect can be either by the influence of cobalt on synthesis of fatty acids as explained by Stangl *et al.* (1999) or it could be assumed that the metal might have exerted a nearly toxic effect on these higher PUFA, which are easily susceptible to oxidative damage. These might be broken down to lower fatty acids. An increase in the levels of fatty acids C18:1 and C18:2 was also observed in cobalt supplemented shrimps when compared with that of control group. Since the experiment in shrimp with cobalt is a comparatively virgin area, there are no sufficient literature evidences to support the present view.

4.4.3 *Tissue defense system*

Cobalt exerts significant effect on the tissue anti-oxidant defense system. The reaction of cobalt with water, especially in the presence of biological chelators is capable of generating a spectrum of

reactive oxygen species that play a key role in the Co induced cell injury (Leonard *et al.,* 1998). Much progress has been made in understanding the roles of classic antioxidant enzymes such as SOD, CAT, GPx etc, in mediating resistance against oxidative damage. Thus studying the anti oxidant status of the shrimp is important in assessing the toxicological effect of dietary Co.

4.4.3.1 *Lipid peroxidation*

Cobalt was defined as an oxidative stress-inducing factor since Co (II) was shown to react with hydrogen peroxide to produce hydroxyl radicals. The activation of free radical formation is usually judged by the dynamics of lipid peroxidation. Lipid peroxidation in hepatopancreas and muscle tissue of control and Co supplemented *P. monodon* is given in Fig.4.4.3.1. In hepatopancreas, lipid peroxidation was significantly lower in all the groups supplemented with cobalt. Lipid peroxidation and subsequent tissue injury is the major symptoms of Cobalt toxicity (Christova *et al.*, 2003; Christova *et al.*, 2001). In the present study, results showed that Co is not toxic to the shrimp in the administered levels, as lipid peroxidation was not induced even in the highest level of Co supplementation. On the other hand Co is having a role in bringing down the lipid peroxidation when compared with the normal level. Lipid accumulation and increased lipid peroxidation were reported in sheep, as a consequence of cobalt deficiency (Kennedy *et al.,* 1994).

4.4.3.2 *Reduced glutathione content (GSH)*

Changes in the GSH level under the present experimental condition are given in Fig 4.4.3.2.1 and Fig 4.4.3.2.2. Hepatopancreas showed a significant increase in the GSH level in all groups supplemented with Co. But muscle tissue showed a significant increase in 2.5 ppm and 5 ppm supplemented groups. According to Christova *et al.* (2001) chronic administration of $CoCl_2$ increases the GSH levels.

4.4.3.3 *Total Sulfhydryl content (TSH)*

Total sulfhydryl content of the control and cobalt supplemented groups of *P. monodon* are depicted in table (4.4.3.3). TSH level increased significantly in all the groups supplemented with cobalt. It is already observed that cobalt supplementation reduced the level of total lipid peroxidation. The increased synthesis of sulfhydryl groups might be considered as one of the reasons for this reduction in lipid peroxidation.

4.4.3.4 *Glutathione peroxidase (GPx)*

Extremely low activity of GPx in guinea pig liver was found greatly enhanced after acute cobalt loading (Christova *et al.*, 2002). But in the above cited study, the increase was reported due to the enhanced lipidperoxidation caused by excessive cobalt loading.

However in the present study it was observed that cobalt itself causes the activation of GPx as observed in the elevated levels of activity of GPx in muscle tissue and hepatopancreas of the cobalt supplemented shrimps (Fig 4.4.3.4.1 and 4.4.3.4.2). This was not in response to increased oxidative stress.

4.4.3.5 *Glutathione S-transferase (GST)*

GST is a member of a complex super gene encoded family of detoxification enzyme, found in a wide variety of animal tissues. GST activity increased significantly in muscle tissues of Co supplemented group of shrimps when compared to control group (Fig.4.4.3.5.1 and 4.4.3.5.2). Glutathione antioxidant system plays a fundamental role in cellular defense against reactive free radicals and other antioxidant species. In the present study GST activity was found to be significantly higher in shrimps supplemented with Co. This shows that cobalt may play a positive role in strengthening the antioxidant system.

4.4.3.6 *Anti peroxidative enzymes (SOD and CAT)*

In the hepatopancreas, group III showed significant increase in SOD activity when compared with that of control, where as group IV showed a significant reduction when compared with group II and group III. In the case of muscle tissue, group II and group III showed

significant increase in the activity. But the activity was found to be lower in group III than that of group II. Group IV showed significantly low activity than that of II and III group (Fig.4.4.3.6.1). It has been reported that cobalt binds stoichiometrically at the zinc site in the SOD protein and the Cu-Co-SOD derivative has low enzymatic activity (Banci *et al.*, 1999; Lyons *et al.*, 2000). This is the possible explanation for the reduced activity of SOD in these groups. But CAT activity was found significantly increased in both muscle tissue and hepatopancreas of the shrimps supplemented with Co (Fig.4.4.3.6.2). This is in line with the observation of Christova *et al.* (2003) in guinea pigs.

The results obtained in the present study indicate that cobalt in general promotes antioxidant system.

4.4.3.7 *Vitamin B_{12}*

Cobalt plays an important role in maintaining animal performance due to its role in vitamin B_{12} synthesis. Vitamin B_{12} has been found in all crustaceans and its function appears to be fundamental to many of the important metabolic processes. It involves the biosynthesis of the labile methyl groups in connection with choline metabolism and the reduction of thiol compounds especially GSH and system affecting the metabolism of carbohydrate and fat.

Fig.4.4.3.1 Lipid peroxidation in the hepatopaancreas and muscle tissue of the control shrimps and shrimps supplemented with cobalt

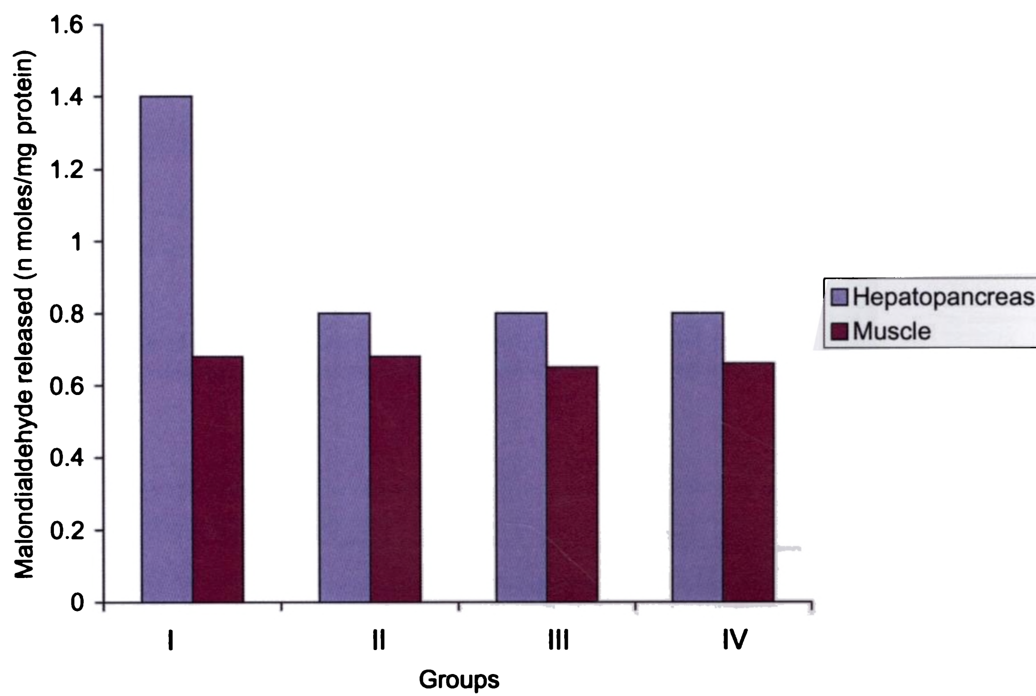


Fig 4.4.3.2.1 Effect of dietary cobalt on the GSH content of the hepatopancreas of control and experimental shrimps

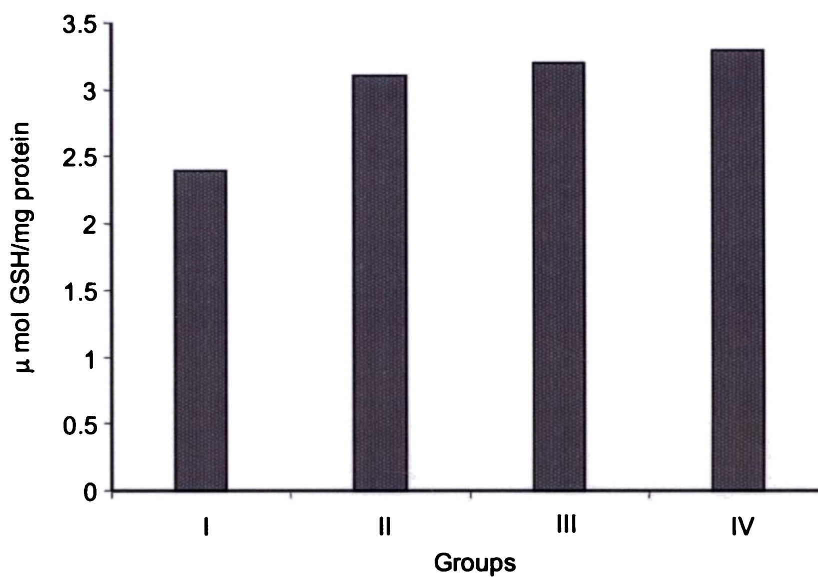


Fig 4.4.3.2.2 Effect of dietary cobalt on the GSH content of the tissue of control and experimental shrimps

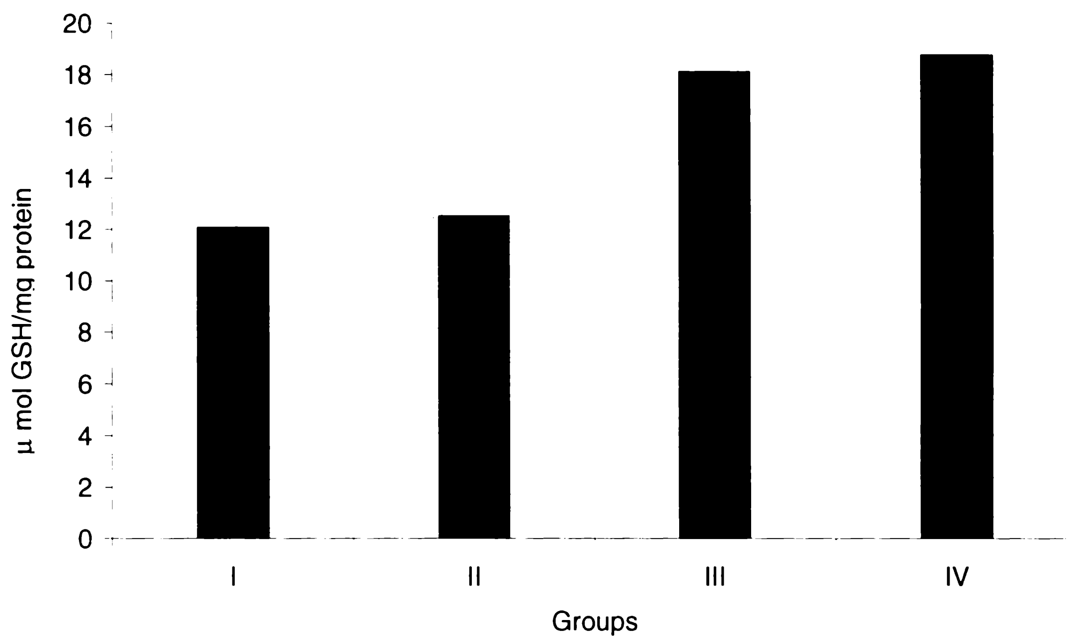


Table 4.4.3.3 Effect of dietary cobalt on the TSH content of the hepatopancreas and muscle tissue of control and experimental shrimps

Tissue	TSH (ng/mg tissue)			
	Group I Control	Group II	Group III	Group IV
Hepatopancreas	233.81±14[a]	302.54+8.0[a]	324.61±10.6[ab]	330.01±7.8[ab]
Muscle tissue	166.5±9.5[a]	240.39+12.9[a]	270.82±13.3[ab]	274.69±9.6[ab]

Values are expressed as Mean ± SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals.

Fig 4.4.3.4.1 Effect of dietary cobalt on the GPx activity of the hepatopancreas of control and experimental shrimps

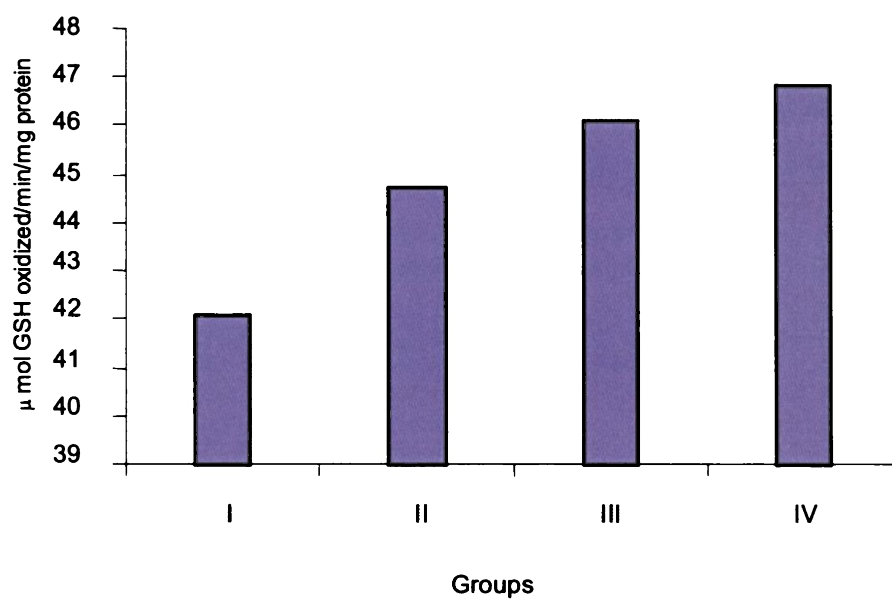


Fig 4.4.3.4.2 Effect of dietary cobalt on the GPx activity of the muscle tissue of control and experimental shrimps

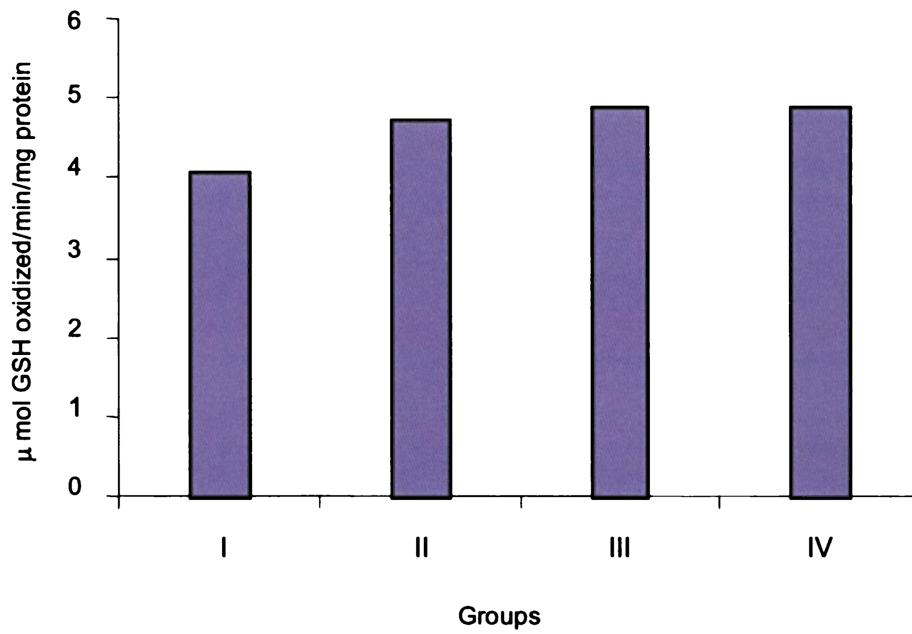


Fig 4.4.3.5.1 Effect of dietary cobalt on the GST activity of the hepatopancreas of control and experimental shrimps

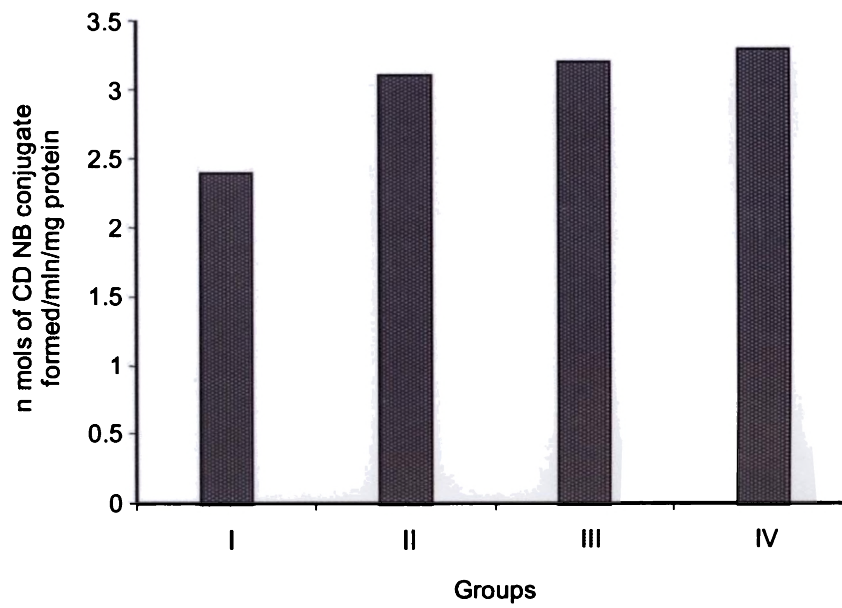


Fig 4.4.3.5.2 Effect of dietary cobalt on the GST activity of the muscle tissue of control and experimental shrimps

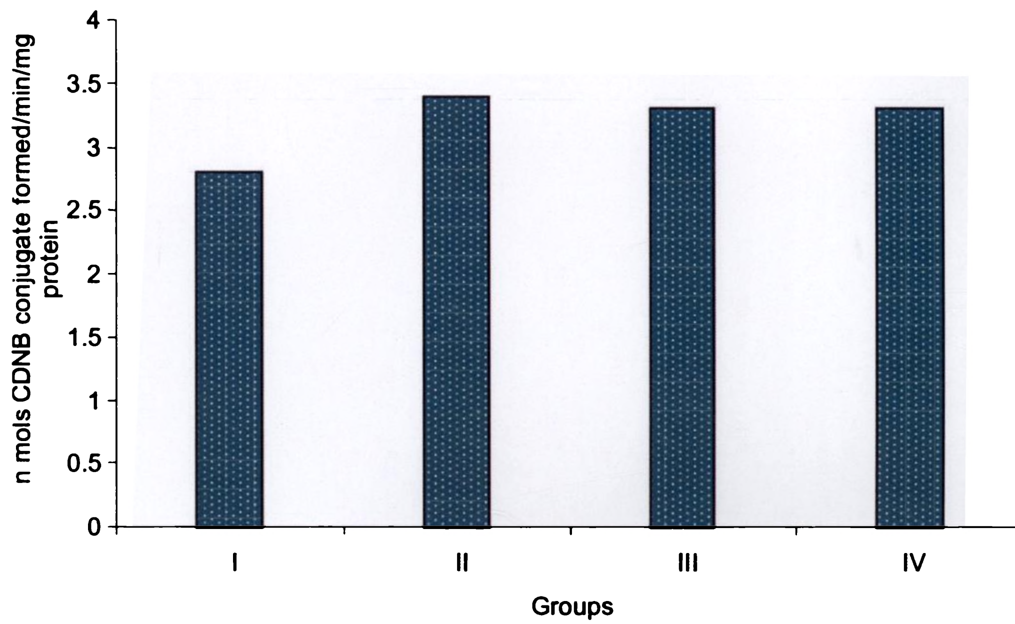
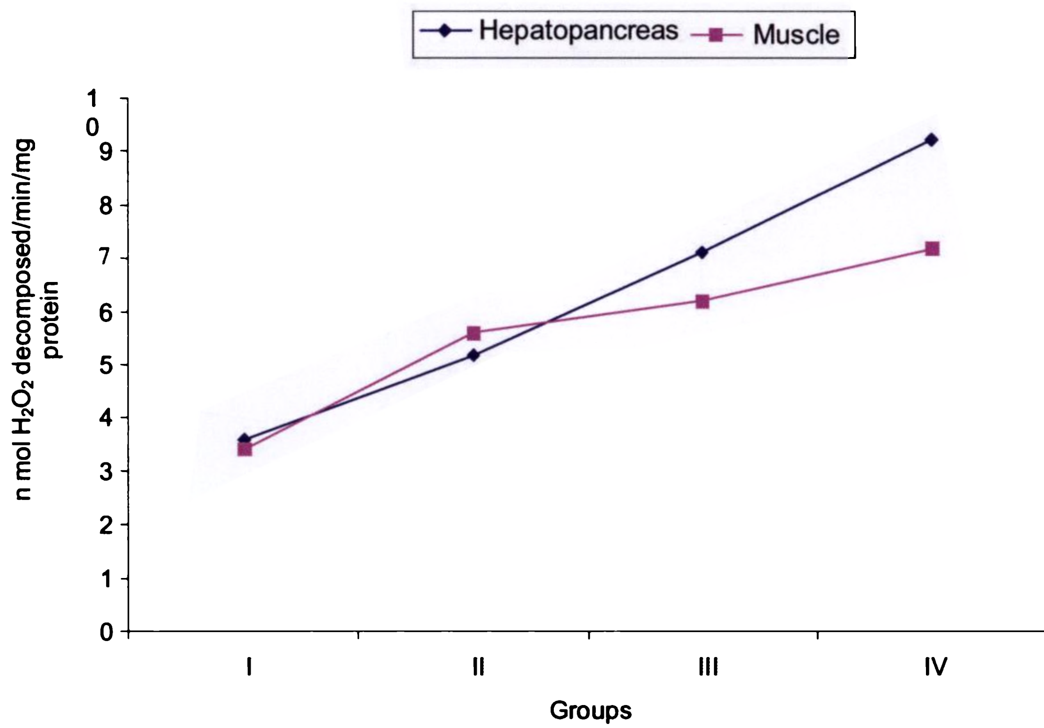
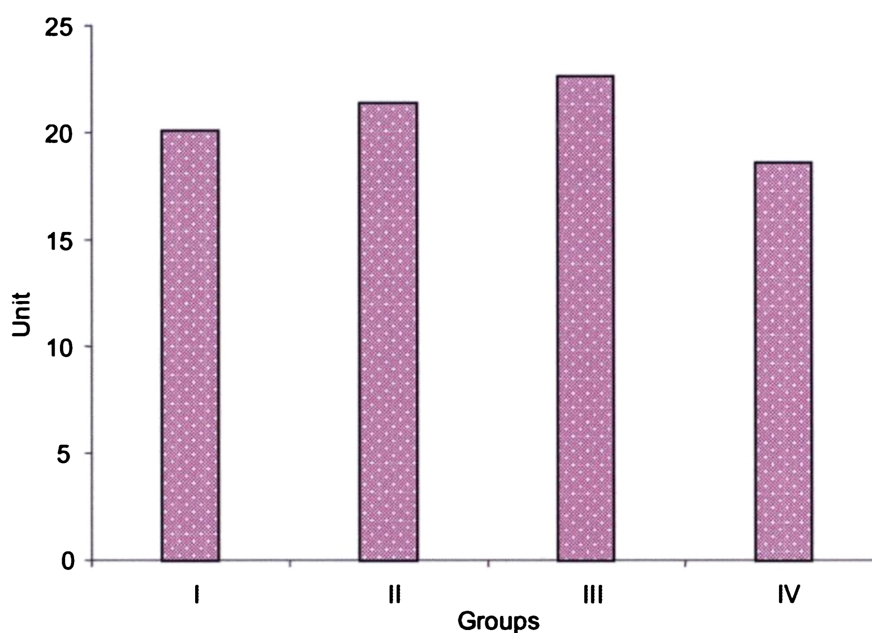


Fig 4.4.3.6.1 Effect of dietary cobalt on antiperoxidative enzyme (CAT) activity of hepatopancreas and muscle tissue of control and experimental shrimps

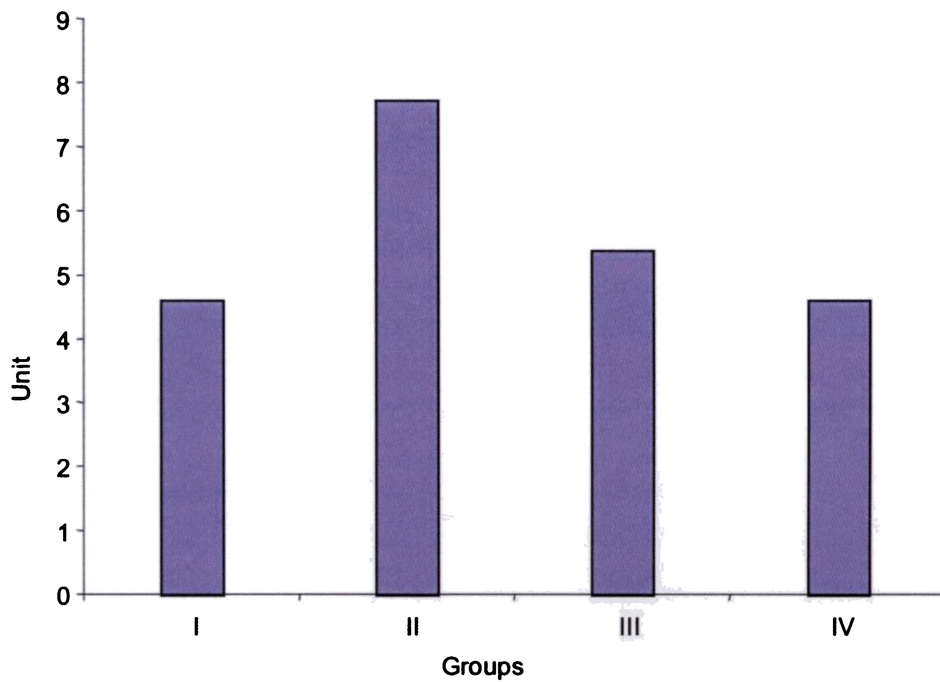


4.4.3.6.2 Effect of dietary cobalt on antiperoxidative enzyme (SOD) activity of hepatopancreas of control and experimental shrimps



One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

4.4.3.6.3 Effect of dietary cobalt on antiperoxidative enzyme (SOD) activity of muscle tissue of control and experimental shrimps



One unit of SOD activity is the amount of protein required to give 50% inhibition of epinephrine auto-oxidation

In the present study detectable levels of Vitamin B_{12} was not found in muscle tissue of the shrimps (Table 4.4.3.7). Vitamin B_{12} is the only vitamin known to contain a metal. This is Cobalt attached to a complex molecule in which the nucleotide part may be varied. The vitamin is certainly present in phytoplankton and associated bacteria (Cavari and Grossowier, 1977) from where it could come to obtained by herbivores. In ruminants dietary supplementation of cobalt had been found to be effective in elevating tissue levels of the vitamin (Kadim *et al.*, 2004). This was explained as due to the increased availability of cobalt for incorporation in the vitamin by the activity of the microbial flora inhabiting the rumen of the animals (Kadim *et al.*, 2004). But according to the present study, in shrimp, dietary cobalt did not have any effect on the tissue level of vitamin B_{12}. Therefore it may be assumed that shrimps are incapable of synthesizing this vitamin and the requirement must be met through diet.

4.4.3.8 *Phospholipids and Cholesterol*

The levels of phospholipids and cholestrol in control group and groups supplemented with Co are given in Table 4.4.3.7. Significant increase was observed in the level of cholesterol in Co supplemented groups, indicating the lipogenic action of Co. Phospholipid levels also increased in Co supplemented groups. Kennedy *et al.* (1992) observed that in the absence of cobalt, alteration occurs in the activity of

methionine synthase and methylation ratio. This will result in impaired phospholipid metabolism. But slight reduction in the levels of both phospholipids and cholesterol could be observed in the group supplemented with elevated level of cobalt (5.0 ppm). There are also reports giving the opposite effect of cobalt on lipid (Dzhuraev and Nasriddinova, 1992). Here Co-35 is recommended as an effective means against atherosclerosis. This is because of its ability to reduce the levels of cholesterol, phopholipids and lipo-protein in the tissues.

4.4.4 *Trace element status*

Cobalt content of the tissue increased in animals fed diet supplemented with cobalt (Table 4.4.4). Accumulations of cobalt in the tissues indicate the absence of an efficient excretory system for this element in shrimps. Accumulation of cobalt in the gills, viscera and kidneys were reported in rainbow trout exposed to cobalt in water (Baudin *et al.*, 1997). Tissue levels of manganese and magnesium were also found to be significantly increased in cobalt supplemented groups of shrimps. Since the variable factor here is the dietary cobalt, it could be assumed that the cobalt affected the metabolism of the above metals. This view is further supported by the increased manganese accumulation in kidney, bile and tibia of chicken by excess dietary cobalt (Brown and Southern, 1995), and increased magnesium in the heart and skeletal muscle in calves in response to high levels of cobalt (Van Ryssen *et al.*, 1987).

Fig 4.4.3.7 Effect of dietary cobalt on the cholesterol, phospholipid and vitamin B_{12} content of the control and experimental shrimps

	Group I	Group II	Group III	Group IV
Cholesterol (nmol/mg protein)	3.58±0.11	6.31±0.06^{a}	6.08±0.07^{a}	5.94±0.16^{ab}
Phospholipids (nmol/mg protein)	2.36+0.09	3.45+0.06^{3}	3.25±0.06^{a}	3.18±0.07^{ab}
Vitamin B12 (ng/g tissue)	10.01±0.33	10.50±0.38	10.52±0.30	10.40±0.24

Values are expressed as Mean±SE; a= Significantly different ($p<0.05$) when compared to control animals, b= Significantly different ($p<0.05$) when compared to group II animals.

Fig 4.4.4 Effect of dietary cobalt on the trace metal content of the control and experimental shrimps

Element	Group I	Group II	Group III	Group IV
Cu (μg%)	140.0±2.2	133.2±2.2	133.6±3.5	132.1±4.1
Co (μg%)	1.7±0.14	2.3±0.17	2.5±0. 14^{a}	3.2±0.08^{abc}
Fe (mg%)	0.34±0.02	0.34±0.01	0.32±0.01	0.40±0.01
Mg (mg%)	10.2±0.20	11.4±0.18^{a}	13.3±0.15^{ab}	12.7±0.20*
Mn (μg%)	126.0±2.4	133.2±2.1	136.8±3.9"	143.4±2.6^{ab}
Mo (μg%)	3.5±0.17	3.3±0.17	3.1±0.11	2.6±0.17^{ab}
Se (μg/g)	0.26±0.01	0.23±0.01	0.20±0.01	0.24±0.00
Zn (mg%)	25.1±0.71	25.7±0.80	25.4±1.2	22.1±0.58

Values are expressed as Mean±SE; a = Significantly different ($p<0.05$) when compared to control animals, b = Significantly different ($p<0.05$) when compared to group II animals.

4.4.5 *Electrophoretic pattern of tissue proteins*

According to Kennedy *et al* (1992), vitamin B_{12} plays an important role in the regulation of muscle protein metabolism. So cobalt deficiency may affect the normal protein metabolism. In the experiment conducted by Kadim *et al* (2004) in goats, a considerable degree of alteration of the muscle structural elements and their related proteins were observed which was measured in terms of shear force in cobalt deficient goats. But in the present study, notable change in the tissue proteins, as observed in the electrophoretic pattern of the tissue proteins (Fig 4.4.5) were not observed. This indicates that tissue protein in shrimp is not directly affected by dietary cobalt at the administered levels. The ability of cobalt to alter the tissue protein may be through vitamin B_{12}, as observed by Kadim *et al* (2004). In this study it was found that cobalt did not influence the vitamin B_{12} levels in prawn.

Summary

5. SUMMARY

From the present study, it is clear that all the three metals, selenium, molybdenum and cobalt have significant effect on the antioxidant status of the shrimps. Selenium and molybdenum were observed to induce peroxidative damage at elevated levels. But at the same level, cobalt did not show such an effect.

Selenium was found to be growth promoting at lower levels of dietary supplementation. Even though low levels of dietary selenium had a protective effect against the lipid peroxidation, the present study indicates that high levels of dietary selenium could promote lipid peroxidation. The selenium-dependent antioxidant enzyme, GPx behaved differently in muscle and hepatopancreas. A high concentration of selenium was required for the active expression of the enzyme in the muscle, where as in hepatopancreas maximum activity was observed at lower selenium concentration. Selenium supplementation had a positive effect on GSH concentration. The other antioxidant enzymes such as GST, SOD and CAT showed enhanced activity at higher concentration of selenium.

Molybdenum supplementation significantly reduced the free radical scavenger enzymes SOD and CAT. This resulted in enhanced lipid peroxidation in tissues. The activity of antioxidant enzyme GPx and the concentration of the substrate for the enzyme, GSH also were

lower at elevated levels of molybdenum supplementation. In addition to this amino acids and fatty acids were also altered in molybdenum supplemented groups. In trace amounts, dietary molybdenum exerts a beneficial effect on the growth and also in the activities of the enzymes XO and SO. At the same time it also indicates a possibility of oxidative damage as a result of the peroxidation caused by the activities of the enzymes SO and XO at elevated concentrations of molybdenum is also indicated. The absorption of various trace elements was also altered by molybdenum supplementation.

Among the three metals studied, cobalt was the least toxic one at the administered levels. But this metal has a significant effect on the lipid content, amino acid composition, cholesterol levels and phospholipid levels. Increased growth was also observed as a result of cobalt supplementation in shrimps. The antioxidant system of the animal was activated by dietary cobalt. Tissue levels of the trace metals were also found to be altered in cobalt supplemented groups of shrimps.

These studies, thus shows that influence of dietary trace metals calls for more detailed studies in farmed shrimp. They may hold the key to growth and even disease resistance in shrimp. But this still remains as a virgin field which demands more attention, especially in view of the increasing importance of shrimp farming.

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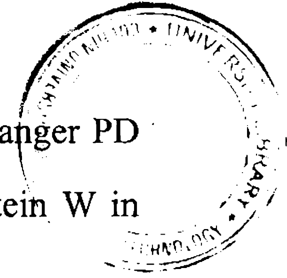

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