BIOCHEMICAL EFFECTS OF CADMIUM, SALINITY AND TEMPERATURE ON THE CATABOLISM OF PROTEINS AND PURINES IN *OREOCHROMIS MOSSAMBICUS* (PETERS)

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Under the Faculty of Marine Sciences

By

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Certificate

This is to certify that the thesis entitled ***"Biochemical Effects of Cadmium, Salinity and Temperature on the Catabolism of Proteins and Purines in Oreochromis mossambicus (Peters)"*** *is an authentic record of the research work carried out by* ***Mrs. Jisha Jose*** *under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry of Cochin University of Science and Technology, and no part thereof has been presented before for the award of any other degree, diploma or associateship in any university.*

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This is to certify that ***Mrs. Jisha Jose*** *has incorporated all relevant corrections and modifications suggested by the audience during the synopsis presentation and recommended by the doctoral committee in her thesis entitled* ***"Biochemical Effects of Cadmium, Salinity and Temperature on the Catabolism of Proteins and Purines in Oreochromis mossambicus (Peters)"***

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January 2014

Dr. Babu Philip

… To God Almighty

Declaration

I hereby declare that the thesis entitled ***"Biochemical Effects of Cadmium, Salinity and Temperature on the Catabolism of Proteins and Purines in Oreochromis mossambicus (Peters)"*** *is a genuine record of research work done by me under the supervision and guidance of* ***Prof. Dr. Babu Philip****, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology for the award of the degree of* ***Doctor of Philosophy*** *in Biochemistry. The work presented in this thesis has not been submitted for any other degree or diploma earlier.*

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Jisha Jose

%	-	Percentage
°C	-	Degree Celsius
µg	-	Microgram
µg/l	-	Microgram per litre
µl	-	Micro litre
µM	-	Micromolar
µmol	-	Micromole
2, 4 DNPH	-	2,4 dinitro phenyl hydrazine
ADP	-	Adenosine di phosphate
ALT	-	Alanine aminotransferase
AMP	-	Adenosine monophosphate
ANOVA	-	Analysis of Variance
APHA	-	American Public Health Association
AQ	-	Ammonia quotient
AST	-	Aspartate aminotransferase
ATP	-	Adenosine tri phosphate
B.C.	-	Before Christ
Ca	-	Calcium
Ca^{++}	-	Calcium ion
Cd	-	Cadmium
Cd^{++}	-	Cadmium ion
$CdCl_2$	-	Cadmium chloride
Cl^-	-	Chloride ion
Co^{++}	-	Cobalt ion

CO_2	-	Carbon dioxide
CTAB	-	Cetyl tri methyl ammonium bromide
CTMax	-	Critical thermal maximum
Cu	-	Copper
Cu^{++}	-	Copper ion
df	-	Degrees of freedom
Dist.H_2O	-	Distilled water
dl	-	Decilitre
DO	-	Dissolved oxygen
EDTA	-	Ethylene diamine tetra acetic acid
EPA	-	Environmental Protection Agency
EU	-	European union
F	-	Variance ratio
FAA	-	Free amino acid
FAO	-	Food and Agricultural Organization
Fe^{++}	-	Ferrous ion
g	-	Gram
g/l	-	Gram per litre
GDH	-	Glutamate dehydrogenase
GTP	-	Guanosine triphosphate
h	-	Hour
H_2	-	Hydrogen
H_20_2	-	Hydrogen peroxide
$HCIO_4$	-	Perchloric acid
HCl	-	Hydrochloric acid
HSP	-	Heat shock protein
IARC	-	International Agency for Research on Cancer

IMP	-	Inosine mono phosphate
K^+	-	Potassium ion
l	-	Litre
LC_{50}	-	Lethal concentration causing 50% mortality
M	-	Molar
mg	-	Milligram
mg/dl	-	Milligram per decilitre
mg/g	-	Milligram per gram
mg/l	-	Milligram per litre
Mg^{++}	-	Magnesium ion
min	-	Minute
ml	-	Milli litre
mM	-	Millimolar
mm/l	-	Millimole/litre
Mn^{++}	-	Manganese ion
$MnCl_2$	-	Manganese chloride
$MnSO_4.4H_2O$	-	Manganous sulphate
mRNA	-	Messenger Ribo nucleic acid
N	-	Normal
Na^+	-	Sodium ion
Na^+- K^+ ATPase	-	Sodium potassium adenosine triphosphatase
NaCl	-	Sodium chloride
NAD^+	-	Nicotinamide adenine dinuclcotide (oxidised)
NADH	-	Reduced nicotinamide adenine dinucleotide
NaOH	-	Sodium hydroxide
NH_3	-	Ammonia
NH_4^+	-	Ammonium ion

nm	-	Nano metre
NQ	-	Nitrogen quotient
O.D	-	Optical density
$O_2^{\cdot -}$	-	Super oxide radical
Pb	-	Lead
ppm	-	Parts per million
ppt	-	Parts per thousand
RBC	-	Red blood corpuscles
ROS	-	Reactive oxygen species
rpm	-	Revolutions per minute
S.D	-	Standard deviation
$SnCl_2.2H_2O$	-	Stannous chloride
SPSS	-	Statistical Package for Social Sciences
TCA	-	Tri chloro acetic acid
UILT	-	Upper incipient lethal temperture
USEPA	-	United States Environmental Protection Agency
WHO	-	World Health Organization
Wt	-	Weight
XD	-	Xanthine dehydrogenase
XO	-	Xanthine oxidase
XOR	-	Xanthine oxido reductase
Zn	-	Zinc
Zn^{++}	-	Zinc ion

Contents

Chapter 1
General Introduction

Living organisms maintain a stable internal environment called homeostasis. The means of maintaining homeostasis is vital to the life of all organisms. Stress can be considered as a state of threatened homeostasis. Hans Selye defined stress as ‘‘the nonspecific response of the body to any demand made upon it’’ (Selye, 1973). The response to stress is considered as an adaptive mechanism that allows the organism to cope with stressors in order to maintain its normal or homeostatic state. These "stressors," whether natural or human-induced, disrupt cellular and molecular activity. Cells and organisms respond to stressors with mechanisms that restore normal function and repair of stress-induced damage. Excessive stress can overwhelm the stress response pathways and lead to cell injury, disease or death.

Physiological responses to environmental stressors have been grouped as primary, secondary and tertiary responses. Primary responses, which involve the initial neuroendocrine responses, include the release of catecholamines from chromaffin tissue (Randall and Perry, 1992; Reid *et al.*, 1998a), and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis culminating in the release of corticosteroid hormones into circulation (Donaldson, 1981; WendelaarBonga, 1997; Mommsen *et al.*, 1999). Secondary responses include biochemical and physiological changes. These responses relate to physiological adjustments such as in metabolism, respiration, acid-base status, hydromineral balance, immune function and cellular responses

(Pickering, 1981; Iwama *et al.*, 1997, 1998; Mommsen *et al.*, 1999). Tertiary responses refer to aspects of whole-animal performance such as changes in growth, overall resistance to disease, metabolic scope for activity, behaviour and ultimately survival (Wedemeyer and McLeay, 1981; Wedemeyer *et al.*, 1990). However, the stress, depending on its magnitude and duration, may affect the organism at all levels of organization, from molecular and biochemical to population and community (Adams, 1990).

The environment may display daily and seasonal fluctuations. The fluctuation in the environment is a source of stress to the animal. All cells and organisms are exposed to changes in their environment. Most organisms must sense environmental changes and respond accordingly to optimize metabolism and growth. The stress on an aquatic ecosystem can be the result of physical, chemical or biological alterations of the environment. These environmental stressors cause harmful impact on the organisms. Exposure to environmental stressors can result in biochemical, physiological and histological (tissue) alteration in living organism. As compared to terrestrial inhabitants, fish and other aquatic organisms are subject to a wide variety of stressors because their homeostatic mechanisms are highly dependent on prevailing condition in their surroundings. The organisms are typically subjected to variations in physicochemical parameters (varying hydraulic, temperature and salinity regimes), changes in food and habitat availability, exposure to contaminants and increase in nutrient inputs.

In the present investigation, three important stressors: cadmium ion (Cd^{++}), salinity and temperature were selected to study their effects on protein and purine catabolism of *O. mossambicus*.

Cadmium ion (Cd^{++})

Fish have been exposed to a vast array of chemical and particulate contaminants, of both natural and man-made origin. Examples include pharmaceuticals, agricultural chemicals, manufacturing by products, animal and human waste materials, mining effluents, and substances released as a consequence of natural disasters such as fires. At sufficient concentration, almost any contaminant is capable of inducing a stress response. The reckless disposal of chemicals and heavy metal wastes from agriculture, industries and mining activity is known to have an adverse effect on aquatic life and water quality. Heavy metals in the environment have long biological half-lives and are therefore a major threat to aquatic organisms, especially fishes (Waldichuk, 1979). At high concentrations heavy metals will kill aquatic organisms; in subacute concentrations heavy metals are gradually accumulated in various aquatic organisms as they reach higher tropic levels of the food chain (Zitko, 1979). Health hazards created by heavy metals have become a great concern only when they affected humans via the food chain as in Minamata disease in Japan (Kurland *et al*., 1960). The seas, rivers and lakes are the eventual sinks for many of the harmful or waste substances disposed by man. Aquatic life, including food fish, is capable of absorbing and concentrating these pollutants. In the meantime, the physicochemical properties of water are extensively modified. As a result, fish are exposed to frequent stresses. Cadmium (Cd) is a biologically nonessential metal that can be toxic to aquatic animals. Cadmium is a trace element which is a common constituent of industrial effluents. It is a non-nutrient metal and toxic to fish even at low concentrations. Cadmium ions accumulate in sensitive organs like gills, liver, and kidney of fish in an unregulated manner (Brown *et al*., 1986; McGeer *et al*., 2000b). Thus; the toxic effects of cadmium are related to changes in natural physiological and biochemical processes in organisms.

Salinity Fluctuations

Terrestrial and aquatic organisms have to control and maintain the osmotic pressure of their cells by regulating fluxes of ions and water through the cell membrane, often with some metabolic cost. The ability of an aquatic organism to tolerate wide variation of salinity without compromising life processes is called euryhalinity. There are a number of euryhaline fish species that tolerate great fluctuations in water salinity (Stickney, 1986; Plaut, 1999; Fiol and Kultz, 2007), including acute changes on a daily basis (Swanson, 1998; Scott *et al*., 2004). Teleosts, inhabiting environments with various salinities, have complicated and sophisticated mechanisms of osmoregulation to maintain the internal osmotic and ionic homeostasis, which allows normal functioning of cellular and physiological processes and survival (Evans *et al*., 2005; Hwang and Lee, 2007). Marine teleost fishes tend to lose water through osmosis and to gain ions (essentially Na^+ and Cl^-) through diffusion (ingestion of seawater, excretion of small volume of urine and active excretion of salt through gills), whereas the reverse mechanism occurs in freshwater fishes (excretion of relatively dilute urine, active uptake of salt across the gills and possibly some ingestion of salt in the food) (Alderdice, 1988). Salinity adaptation by euryhaline teleosts is a complex process involving a suite of physiological and behavioural responses to environments with differing osmoregulatory requirements. The mechanics of osmoregulation (i.e. total solute and water regulation) are reasonably well understood (Evans, 1984, 1993), and most researchers agree that salinities that differ from the internal osmotic concentration of the fish must impose energetic regulatory costs for active ion transport. There is limited information on protein and purine catabolism of euryhaline fish during salinity adaptation.

Temperature Variation

Fish are subject to stress from rapid temperature fluctuations (beyond the high or low range of tolerance). Ectothermic fish, which vary their body temperature according to the environmental temperature, are widespread in a variety of environments. Temperature controls and limits all physiological and behavioural parameters of ectotherms (Fry, 1947). In fact, water temperature has been described as the 'abiotic master factor' for fishes (Brett, 1971). Optimal temperature range, as well as upper and lower lethal temperature, vary widely between and among species and are dependent on genetics, developmental stage and thermal histories (Beitinger *et al*., 2000; Somero, 2005). Within a range of non-lethal temperatures, fishes are generally able to cope with gradual temperature changes that are common in natural systems. However, rapid increases or decreases in ambient temperature may result in sub lethal physiological and behavioural responses.

The environmental stressors induce severe physiological and biochemical alterations leading to impairment of major metabolic pathways. During the stress condition, the organism is generally in a catabolic state. The term catabolic state refers to a specific condition in which catabolic processes are dominant. In response to stress, the body secretes epinephrine, norepinephrine, cortisol and other hormones. The glucocorticoids (such as cortisol) have a catabolic action at high levels. That is, they suppress the synthesis of protein, and triglycerides and mobilize them from stored forms. Instead, these are broken down into amino acids and fatty acids respectively. This process is necessary to counteract stress. However, if the process is prolonged, the resulting catabolism is very damaging to the body and causes excessive tissue breakdown. In addition, a prolonged stress

response suppresses the immune system, the digestive organs, growth hormones and other important vital functions.

The catabolic pathways of proteins and purines are important biochemical processes. The catabolism of proteins and purines results in the production of energy and waste nitrogen. Proteins and nucleotides are the most important nitrogen compounds in living organisms. Proteins make up the structural tissue for muscles and tendons, transport oxygen (eg. Hemoglobin), catalyzes all biochemical reactions as enzymes, and regulates reactions as hormones. Proteins in excess are used to supply energy or build reserves of glucose, glycogen or lipids. Purine and pyrimidine nucleotides are precursors of nucleic acids, as well as metabolites participating in bio-energetic processes and in the synthesis of macromolecules, including polysaccharides, phospholipids and glycolipids (Ross, 1981). Nitrogen metabolism in animals has to deal with excess nitrogen and excrete it in a nontoxic form. Animals not only ingest N-containing organic molecules as building blocks for cellular substances but also for the generation of metabolic energy via carbon oxidation. It is mostly during carbon oxidation that nitrogen is released as waste. The catabolism of proteins and purines results in the production of excretory nitrogenous compounds. Animals excrete three main nitrogenous products: ammonia, urea and uric acid as well as some minor nitrogen excretory products, including trimethylamine oxide, guanine, creatine, creatinine and amino acids. Ammonia is toxic to the animals when it is accumulated in body tissues. A major factor in determining the mode of nitrogen excretion is the availability of water in the environment. Generally, aquatic animals excrete mostly ammonia, whereas terrestrial animals excrete either urea or uric acid.

Ammonia is mostly formed from the catabolism of proteins usually in the liver. Most L- amino acids are first transaminated to form glutamate, catalyzed by a group of transaminase enzymes. Glutamate is then deaminated to form NH_4^+ and α-ketoglutarate, catalyzed by glutamate dehydrogenase (GDH). Transdeamination is the term given to this two-step process.

In addition to amino acid catabolism, another pathway known as purine nucleotide cycle also produces ammonia. The purine nucleotide cycle directly liberates ammonia as NH_3, a potentially acid base disruptive route. The pathway is active in fish muscle, especially post exercise, when it is used to scavenge AMP produced by hydrolysis of ATP during muscle contraction. The NH_3 produced would then consume protons to form NH_4^+ at a time when tissue is lactacidotic, thereby aiding in correction of depressed muscle pH back towards resting values.

Purine metabolism is an essential biochemical pathway that is conserved across a wide-range of phyla and is considered a likely candidate for the most ancient metabolic pathway on the planet (Caetano- Anolles *et al.*, 2007). The end products of purine catabolism, however, vary among vertebrates and have been the subject of comparative biochemical studies since the early 20th century (Hunter *et al.*, 1914). Animals degrade purines only partially and excrete purine nitrogen. In primates (including humans), birds and many reptiles, urate is the end product of purine degradation, whereas most mammals generate allantoin for excretion. Most teleost fishes and amphibians excrete urea as the end product of purine degradation. The terminal portion of purine catabolism begins with the degradation of hypoxanthine to uric acid by xanthine dehydrogenase. The next step in the pathway is the degradation of uric acid to allantoin by uricase. Allantoinase then degrades allantoin to allantoate, which is the final

product of the pathway in some teleost fish. In other teleost fish and amphibians, allantoicase catalyzes the hydrolysis of allantoate to ureidoglycolate and urea, which is followed by degradation of ureidoglycolate to glyoxylate and urea by ureidoglycolate lyase (Hayashi *et al*., 2000). Finally, urease activity, whose presence has been detected in the gut of some fish but is encoded within bacteria living in the host and not the vertebrate genome, can generate the most terminal products of the pathway, ammonia and carbon dioxide (Urich, 1994).

Majority of teleost fish excrete nitrogen waste primarily as ammonia (55-80%). A small but significant component is also excreted as urea (5-40%). Urea excretion in teleost fish is a secondary but significant component of total nitrogen excretion. In freshwater and marine teleosts, ammonia excretion constitutes 60%-95% of nitrogen wastes, with most of the remainder excreted as urea (Campbell and Anderson, 1991; Wood, 1993; Wright, 1993). In the majority of teleosts, urea is produced from the catabolism of purines (uricolysis) and from dietary arginine (catalyzed by arginase) (Forster and Goldstein, 1969; Mommsen and Walsh, 1992; Wright, 1993). The three uricolytic enzymes, uricase, allantoinase and allantoicase as well as arginase are present in the liver of many species (Wright and Land, 1998).

Environmental stress, including temperature and salinity stress, affects metabolism and nitrogen excretion in fish, possibly as part of the adaptive response which allows survival under adverse conditions (Wright *et al*., 1995; Altinok and Grizzle, 2004; Wood *et al*., 1994; Polez *et al*., 2003). In the present investigation a baseline attempt to investigate the effects of three different stressors viz, cadmium ion (Cd^{++}), salinity and temperature on fresh water adapted euryhaline teleost *Oreochromis mossambicus* (Peters) has been carried out.

Oreochromis mossambicus (Peters) is found in abundance in the rivers and back waters of Kerala. It is one of the most commonly cultivated fish species owing to their taste and fast growing characteristics and seems to be the fourth most commonly cultured food fish (FAO, 1995). The local availability of the fish throughout the year, low cost, reasonable size, its restricted niche, omnivorous feeding habit etc make it an ideal candidate for laboratory studies.

Systemic position of the experimental animal, *Oreochromis mossambicus* (Common name –Tilapia) employed in this study is as follows (Fig 1.1)

Kingdom	:	Animalia
Phylum	:	Chordata
Class	:	Teleostomi
Order	:	perciformes
Family	:	Cichlidae
Genus	:	*Oreochromis*
Species	:	*mossambicus*

Fig 1.1 *Oreochromis mossambicus* (Peters)

Organization of the Thesis

The thesis is divided in to 9 chapters with the following objectives:

- To study the changes in the protein catabolism on exposure to cadmium ion (Cd^{++}) by investigating selected metabolic parameters and enzymes involved in protein catabolism
- To assess and evaluate the effect of Cd^{++} on enzymes and metabolic parameters associated with purine catabolism.
- To study the effect of acute salinity change on protein catabolism
- To assess the changes in metabolic parameters and enzymes involved in purine catabolism on exposure to acute salinity change.
- To determine the effect of acute exposure to different temperatures on protein catabolism.
- To examine the changes in purine catabolism on exposure to different temperatures.

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Chapter 2
Review of Literature

Stress is defined as a condition in which the dynamic equilibrium of organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Chrousos and Gold, 1992). The stressors generally produce effects that threaten or disturb the homeostatic equilibrium and they elicit a coordinated set of behavioural and physiological responses thought to be compensatory and adaptive, enabling the animal to overcome the threat (Wendelaarbonga, 1997). If an animal is experiencing intense chronic stress, the stress response may lose its adaptive value and become dysfunctional, which may result in inhibition of growth, reproductive failure and reduced resistance to pathogens (Wendelaarbonga, 1997).

All biological molecules and all biochemical reactions are directly susceptible to perturbation by multiple environmental parameters including temperature, pressure, pH, ionic strength, water availability, radiation, and attack by free radicals. Excretion of nitrogen is a liable character and the pattern may change with the life cycle, availability of water, nutrition and water environmental factors (Prosser, 1973). Nitrogenous excretory products

are derived from the catabolism of proteins, by way of amino acids which may be transaminated and deaminated or by the degradation of nucleic acids (Bayne *et al*., 1985).

The major end product of nitrogen metabolism in animals is ammonia, which is highly toxic and must be detoxified or excreted. Mammalian and other terrestrial and amphibian vertebrate species and the lungfishes are ureotelic, that is, they maintain blood levels of ammonia below 0.03 mM by converting ammonia to urea via the classical urea cycle in the liver (Anderson, 2001). Birds and terrestrial reptiles are uricotelic, converting ammonia into uric acid. Marine elasmobranchs (sharks, skates and rays) are ureosmotic and have an active urea cycle, synthesizing and retaining urea at high concentrations (0.3–0.6 M) primarily for the purpose of osmoregulation (Perlman and Goldstein, 1988; Anderson, 1991, 1995a; Ballantyne, 1997).

The vast majority of teleost fish are ammonotelic, that is, ammonia generated in the liver and other tissues is simply excreted directly across the gills where it is diluted by the surrounding aqueous environment (Anderson, 2001). Ammonia is mostly formed from the catabolism of proteins usually in the liver. Most L- amino acids are first transaminated to form glutamate, catalysed by a group of transaminase enzymes. Glutamate is then deaminated to form $NH4^+$ and α-ketoglutarate, catalyzed by glutamate dehydrogenase (GDH). Transdeamination is the term given to this two-step process (Braunstein, 1985; Torchinsky,1987).

The purine nucleotide cycle directly liberates ammonia as NH_3, a potentially acid base disruptive route. The pathway appears to be active in fish muscle, especially post exercise, when it is used to scavenge AMP produced by hydrolysis of ATP during muscle contraction (Wood, 1988; Dobson, and Hochachka, 1987; Mommsen and Hochachka, 1988).

Although primarily and generally ammonotelic, most teleost fish do excrete a significant proportion of their total excreted nitrogen as urea (5–20%) (Campbell and Anderson, 1991; Wood, 1993; Wright, 1993). In the majority of teleosts, urea is produced from the catabolism of purines (uricolysis) and from dietary arginine (catalyzed by arginase) (Forster and Goldstein, 1969; Mommsen and Walsh, 1992; Wright, 1993; Korsgaard *et al.,* 1995; Anderson, 1995a). The three uricolytic enzymes, uricase, allantoinase and allantoicase as well as arginase are present in the liver of many species (Brown *et al.,* 1966; Wright, 1993; Wright *et al.,* 1993; McGeer *et al.,* 1994). In teleosts, alterations of environment (water pH, salinity, or heavy metal pollution) cause physiological responses, such as secretion of hormones (growth hormone, prolactin and cortisol); fluctuations of plasma ion, osmolality, and glucose; and changes in water balance and oxygen consumption rate (Potts, *et al*., 1987; McCormick *et al*., 1989*b*; McCormick, 1996; Lin *et al*., 2000).

2.1 Biochemical studies on cadmium

Cadmium is a naturally occurring ubiquitous element, but it is also rare and is not found in a pure state in nature (Mc Geer *et al;* 2012). Cadmium is considered as a potential human carcinogen (group 2B) by the US Environmental Protection Agency (EPA) and a human carcinogen (group 1) by the International Agency for Research on Cancer of the World Health Organization (WHO). Beyersmann *et al*. (2008) reported that exposure to cadmium is associated with increased risk of lung and kidney cancer in humans. Kang *et al*. (2013) reported that environmental cadmium exposures were associated with an elevation in serum liver enzyme levels in Korean adults.

Sources to the environment include the weathering of rock (particularly phosphate rock), volcanic activity, windblown dust and aerosols from sea spray;

as well as anthropogenic sources related to the mining and smelting of Zn, Pb, and Cu ores, use of phosphate fertilizers, burning of fossil fuel, peat, and wood, and the manufacture of cement (Mc Geer *et al;* 2012). Uses and applications of cadmium have varied considerably over time and currently include batteries, pigments, stabilizers, coatings, and as a minor constituent in some alloys (Mc Geer *et al;* 2012). Battery production accounts for 83% of cadmium use (Mc Geer *et al;* 2012).

Cadmium (Cd) is a biologically nonessential metal (Baker *et al*., 2002) that can be toxic to aquatic animals (Almeida *et al*., 2001). The toxic effects of cadmium have been reviewed extensively, including bioaccumulation (Usha Rani, 2000), mild anemia, osteoporosis, and emphysema (Peraja *et al*., 1998).

One important acute effect of Cd^{++} is disruption of ion homeostasis, particularly calcium regulation. Matsuo *et al*. (2005) studied the effect of cadmium on Amazonian teleost tambaqui (*Colossoma macropomum*) and found that cadmium disrupted calcium balance. Waterborne cadmium exposure of rainbow trout at 3 mg/l resulted in significant reductions in whole-body sodium over the first 4 days of exposure (Hollis *et al*., 1999; McGeer *et al*., 2000a). Fu *et al*. (1990) found that cadmium exposure of tilapia (*Oreochromis mossambicus*) resulted in reductions in plasma sodium and calcium. This loss of sodium is likely related to inhibition of uptake, as branchial Na^{+}-K^{+}-ATPase activity can be inhibited by cadmium exposure (Atli and Canli, 2007).

Basha and Usha Rani (2003) studied the induction of antioxidant enzymes in liver and kidney of freshwater teleost *Oreochromis mossambicus* (tilapia) during prolonged exposure to heavy metal cadmium ion (Cd^{++}).

The effect of exposure to cadmium upon water electrolyte status in the goldfish *Carassius auratus* has been examined by McCarty and Houston (1976).

Pascoe and Mattey (1977) studied the toxicity of cadmium to the three-spined stickleback *Gasteresteus aculeatus*. Shaffi (1978) examined the effect of cadmium intoxication on tissue glycogen content in three freshwater teleosts. Banerjee *et al*. (1978) have shown impairment of the carbohydrate metabolism in *Clarias batrachus* and *Oreochromis mossambicus* exposed to cadmium. These authors also noticed stimulated activity of acid phosphatase in *Clarias batrachus*. The toxic effects of cadmium on the digestive system of *Heteropneustes fossilis* have been examined by Sastry and Gupta (1979). Roberts *et al*. (1979) studied the effect of cadmium on enzyme activities and accumulation of the metal in tissues and organs of fishes. The harmful effect of cadmium is attributed to its binding to the sulfhydryl enzymes, especially dehydrogenases (Beliles, 1975). Sastry and Subhadra (1985) investigated the in vivo effects of cadmium on some enzyme activities of fresh water cat fish *Heteropneustes fossilis*. Cadmium exposed fish may show skeletal deformities, alterations in several enzymatic systems, including those involved in neurotransmission, trans epithelial transport and intermediate metabolism, alteration of mixed function oxidase activities, abnormal swimming, changes in individual and social behaviour and metabolic disorders, among others (Scott and Sloman, 2004; Wright and Welbourn, 1994).

Ferrari *et al*. (2009) found that exposure of *Cyprinus carpio* to sub lethal cadmium concentrations resulted in gill epithelium damage, which may lead to alterations in ion and gas exchange and energy balance. Goering *et al*. (1995) observed that at the cellular level, heavy metals can cause a number of adverse effects, such as alterations in the communication between cells and in the interaction with intracellular signal transduction proteins, which may in turn lead to alterations in cell growth and differentiation. Eissa *et al*. (2006, 2010) found that sub lethal concentration of cadmium also causes important changes in the swimming activity of *C. carpio* in captivity. In acute water

pollution incidents, the physiological disturbances of fish are well known, e.g., respiratory distress, loss of locomotor ability, and behaviour alterations.

Freshwater fish exposed to waterborne cadmium at total concentrations well below 100 µg/l exhibit substantial pathophysiology (Wood, 2001). Some of the physiological effects of chronic exposure to waterborne cadmium at sub lethal levels are manifested in the form of disturbances in respiration (Majewski and Giles, 1981; Shaffi *et al*., 2001), disruption in whole-body or plasma ion regulation (Haux and Larsson, 1984; Giles, 1984; Pratap *et al*., 1989; McGeer *et al*., 2000a; Baldisserotto *et al*., 2004b), changes in hematology (Haux and Larsson, 1984; Gill and Epple, 1993; Zikic *et al*., 2001) and other blood parameters, such as cortisol and glucose, that reveal the stress response in fish (Fu *et al*., 1990; Pratap and WendelaarBonga, 1990; Gill *et al*., 1993; Brodeur *et al*., 1998; Lacroix and Hontela, 2004).

AST and ALT are the most important enzymes acting as transaminases involved in amino acid metabolism and they are known to be sensitive to metal exposures (Almeida *et al*. 2001; Levesque *et al*. 2002; Gravato *et al*. 2006). Tormanen (2006) observed the inhibition of rat liver and kidney arginase by cadmium ion. De Smet and Blust (2001) reported that Metal-induced gill lesions such as thickening and lifting of respiratory epithelium result in an increase of diffusion distance between the water and blood which makes oxygen absorption difficult

De Smet and Blust (2001) indicated that elevated activities of alanine amino transferase (ALT) and aspartate amino transferase (AST) in liver and kidney of *Cyprinus carpio* following cadmium exposures were due to increased protein breakdown to deal with the energy requirement. A decrease in the protein content was found in the hepatopancreas of edible crab *Scylla*

serrata exposed to cadmium and the gills, liver, kidney, muscle and intestine of the common carp exposed to mercury (Suresh *et al*., 1991; Reddy and Bhagyalakashmi, 1994).

2.2 Biochemical effects of salinity

Salinity adaptation by euryhaline teleosts is a complex process involving a suite of physiological and behavioural responses to environments with differing osmoregulatory requirements. The mechanism of osmoregulation is reasonably well understood (Evans, 1984, 1993). The energetic cost of ionic and osmotic regulations seems to play a significant role in growth rates (Boeuf and Payan, 2001). Some studies support the idea of growth enhancement arising from reduced metabolic cost for osmoregulation (Woo and Kelly, 1995). De Silva and Perera (1976) suggested higher energy/protein requirement in high salinities, an effect that possibly reflects an elevated metabolic cost of osmoregulation in such salinities. Although most teleost fish are ammonotelic (Wood, 1993), nitrogen metabolism and excretion are environmentally influenced (Hollingworth, 2002). Very few studies have addressed the influence of water salinity on nitrogen excretion (Wright *et al*., 1995).

Oxygen consumption has been used as an indirect indicator of metabolism in fish (Cech, 1990) and its measurement at different salinities has been employed in an attempt of assessing the energetic cost of osmoregulation in several species (Da Silva Rocha *et al.,* 2005). Farmer and Beamish (1969) observed low oxygen consumption rates at the isosmotic salinity in the Nile tilapia *Oreochromis niloticus.* Rao (1968) also found low oxygen consumption rates at the isosmotic salinity in the Rainbow trout *Oncorhynchus mykiss* (Walbaum). Woo and Kelly (1995) observed similar results in Sea bream *Sparus sarba*. Morgan and Iwama (1991) found low oxygen consumption rates

in fresh water, and the consumption increased with the increase in salinity with juvenile Rainbow trout and Steelhead trout *O. mykiss.*

There are a number of euryhaline fish species that tolerate great fluctuations in water salinity (Stickney, 1986; Plaut, 1999; Fiol and Kultz, 2007) including acute changes on a daily basis (Swanson, 1998; Scott *et al*., 2004). The information on the effects of salinity on fish nitrogen excretion is scanty and somewhat contradictory. Following salinity increment (usually chronic), no change has been observed in *Salmo trutta* (Dosdat *et al*., 1997) and *Allenbatrachus grunniens* (Walsh *et al*., 2004). An increase in ammonia excretion with a decrease in urea excretion has been observed in *Cyprinus carpio* (De Boeck *et al*., 2000) and *Rivulus marmoratus* (Frick and Wright, 2002) while a decrease or constant ammonia excretion with an increase in urea excretion has been observed in the Hybrid sturgeon (Gershanovich and Pototskij, 1995) and *Opsanus beta* (Walsh *et al*., 2004). Gracia-Lopez *et al*. (2006) have reported that high salinity reduces ammonia excretion in *Centropomus undecimalis.* Zheng *et al*. (2008) reported that ammonia excretion is affected by both salinity and temperature in *Miichthys miiuy*. Changes in urea excretion in response to variable salinity have also been reported in catfish and goldfish (Altinok and Grizzle, 2004).

Martinez-Alvarez (2002) investigated the Physiological changes of Sturgeon *Acipenser naccarii*, when subjected to growing environmental salinity up to 35% and observed a number of physiological responses such as disturbance in body fluid (detected by increased plasma osmolality, altered number of red blood cells and decreased levels of muscle hydration), activation of osmoregulatory mechanisms (increased cortisol levels) augmented antioxidant enzyme activities in the blood and alteration of energetic metabolites (changes in protein concentration in the plasma and liver), indicating that the

acclimation of Sturgeons to increased salinities involves osmotic stress counteracted by osmoregulation.

The adaptation of Tilapia to sea water is characterized by the readjustment of several physiological and biochemical processes: the drinking rate, the sodium exchange and the net outward transport of NaCl all increase considerably (Potts *et al*., 1967). Foskett *et al*. (1981) found that the chloride cells, which are responsible for the actual NaCl transport, proliferate during salinity acclimation. The investigations by Bashamohideen and Parvatheswararao (1972) on effects of osmotic stress in the blood glucose, liver glycogen and muscle glycogen levels of fresh-water euryhaline teleost *Tilapia mossambica* have confirmed the changes in carbohydrate metabolism during salinity stress.

Frick and Wright (2002) have found that non-essential amino acids, such as proline and taurine, are responsible for the increase of free amino acids at high salinities in the tissues of the mangrove Killifish, *Rivulus marmoratus.* Increase in amino acids was reported in other teleosts acclimated to sea water (Huggins and Colley, 1971; Lasserre and Gilles, 1971; Colley *et al*., 1974; Ahokas and Sorg, 1976). Aas-Hansen *et al*., 2005 reported that increased liver ALT and AST activities during downstream migration of Arctic char prior to seawater exposure. Studies on Climbing perch (*Anabas testudineus*) showed significant increases of both aspartate and alanine in muscles after six days of acclimation to sea water of 30 ppt (parts per thousand) salinity (Chang *et al*., 2007a). Increase in the activity of ALT and AST was also observed during the sea water acclimation of arctic char (Bystriansky *et al.,* 2007).

Jurss *et al*. (1986) showed elevated glutamate dehydrogenase (GDH) in liver of rainbow trout exposed to increasing salinity. Jurss *et al*. (1985) speculated that metabolic adaptation to elevated salinity may involve regulation

by metabolite activation (e.g., activation of GDH by leucine). Thus, as amino acid levels (including leucine) rise in tissues as part of the osmotic adaptation, the activity of GDH would be enhanced. Similarly Kultz and Jurss (1993) reported increased GDH activity in the gill and kidney tissues. Frick and Wright (2002) found an increase in ammonia excretion in *Rivulus marmoratus* exposed to 15% and 30% sea water compared to those exposed to fresh water. Raffin (1986) reported an increased activity of AMP deaminase, when trout were transferred to sea water or during reverse transfer to fresh water. Cheng *et al*. (2004) observed activities of xanthine dehydrogenase and xanthine oxidase in hepatopancreas increased directly with salinity level in Kuruma shrimp *Marsupenaeus japonicus*.

McCormick (1995) suggests that changes in plasma concentrations of several hormones, including cortisol, growth hormone and prolactin, have been associated with the process of ion regulation and consequently with sea water acclimation in fish. In a euryhaline freshwater teleost, the *Oreochromis mossambicus*, cortisol and growth hormone levels were elevated 1st and 4th days after transfer to sea water but not when these animals were transferred to brackish water or fresh water (Morgan *et al*., 1997).

2.3 Biochemical effects of temperature

Temperature and salinity have long been recognized as two of the most important abiotic factors affecting biological metabolisms in aquatic organisms (Moser and Hettler, 1989; Via *et al*., 1998). Temperature directly affects the rate of all biological processes, such as food intake, metabolism and nutritional efficiency (Brett, 1979; Burel *et al*., 1996). Protein synthesis increases with temperature (Fauconneau and Arnal, 1985; Loughna and Goldspink, 1985; Watt *et al.,*1988). McCarthy and Houlihan (1997) indicated that white muscle and

whole-body protein synthesis rates actually rise in an exponential fashion as temperature increases, and this conclusion has now been reinforced by an experimental study on a single species, the marine wolf fish, fed to satiation at four different acclimation temperatures (McCarthy *et al.,* 1999).

Acclimation of *Labeo rohita* to 31°C, 33°C and 36°C compared with ambient temperatures (26°C) for 30 days was studied by Das *et al*. (2006). The results indicated that higher acclimation temperatures enhance metabolism in *L. rohita* and it maintains homeostasis between 26°C -36°C *via* an acclimation episode. Such adaptation appears to be facilitated by resorting to gluconeogenic and glycogenolytic pathways for energy mobilization and induction of heat shock proteins (HSPs).

Influence of different temperatures on the growth performance, survival rate and some physiological parameters of Nile tilapia (*Oreochromis niloticus*) were studied by El-Sherif and El-Feky (2009). The fishes were exposed for 15°C, 20°C, 25°C and 30°C for 60 days. Results showed that growth performance for tilapia was decreased at 15°C and 20°C. Survival rate was high at temperature 20°C, 25°C and 30°C. Decreasing temperature resulted in decreasing hematocrit and hemoglobin parameters. In Atlantic wolf fish (*Anarhichas lupus*), when the temperature increased to the upper thermal limit protein degradation increased while retention efficiency and growth decreased (McCarthy *et al*., 1999).

Optimum temperatures can be estimated indirectly based on the relationship between oxygen consumption and acclimation temperature (Kita *et al*., 1996). The increase in the respiration rate of juvenile Miiuy croaker, *Miichthys miiuy* (Basilewsky) with increasing temperature was observed by Zheng *et al.* (2008). It has been reported that temperature influences the osmoregulatory ability of fishes, where a reduction in temperature below an

optimal value appears to induce greater osmoregulatory disturbances than a similar elevation in temperature (Al Amoudi *et al*., 1996; Handeland *et al*., 2000; Staurnes *et al*., 2001; Imsland *et al*., 2003). Krishnamoorthy *et al.* (2008) observed a significant increase in the oxygen consumption of fish fingerlings of *Alepes djidaba* exposed to high temperature. Decreased oxygen consumption was observed in Nile tilapia exposed to low temperature (Alsop *et al.,* 1999).

Diverse works evaluate the effect of the critical thermal maximum (CTMax) and upper incipient lethal temperature (UILT), as stress indicators (Cherry *et al*., 1977; Paladino *et al*., 1980; Tsuchida, 1995; Luttersmidt and Hutchison, 1997). Zaragoza *et al*. (2008) studied the effect of the thermal stress on hematological parameters of *O. mossambicus* and observed altered modified coagulation time, sedimentation rate, mean corpuscular volume, haematocrit, number of erythrocytes and leukocytes, percentage of lymphocytes and granulocytes, number of thrombocytes, osmotic pressure and glucose concentration. Thermal stress led to a greater increase in glycemia, cortisol and peroxidase activity (Roche and Boge, 1996). Houston and DeWilde (1968) observed that the red blood cells count, the packed cell volume and the content of hemoglobin vary directly with temperature in *Cyprinus carpio*.

A change of temperature can influence the catalytic properties of enzymes (Klyachko and Ozernyuk, 1998). The adaptability of fishes and their ability to exhibit normal activity at extremes of temperature suggest that cellular processes may be maintained at appropriate levels following a period of thermal acclimation or adaptation (Gerlach *et al*., 1990). Manifestations of stress due to elevated temperatures include increased cardiovascular output, increased metabolic rate, and triggering of the synthesis of specific HSPs (Morimoto *et al*., 1990; Currie and Tufts, 1997; Iwama *et al*., 1998).

Davis (2004) studied the effects of low-water confinement stressor at temperatures ranging from 5°C to 30°C in Sunshine bass. An initial increase in hematocrit was noted, followed by a delayed decrease in hematocrit and chloride and an increase in plasma glucose and cortisol. In general, fish stressed at temperatures below 20°C had lower and more delayed changes in plasma glucose and cortisol than fish tested at 20°C, 25°C and 30°C.

In *Sarotherodon mossambicus*, a temperature rise triggers more phosphorylase activity, enhances hepatic glycogenesis and increases the glucose concentration in blood (Radhakrishnaiah and Parvatheswararao, 1984). Rao and Ramachandra (1961) reported that the osmotic pressure and the content of chloride and free amino acids of the blood undergo systematic change during acclimation to high temperature in the freshwater field crab, *Paratelphusa* sp and the freshwater mussel, *Lamellidens marginali.* Jagtap and Mali (2011) observed that at higher temperature of exposure, *Channa punctatus* showed decreased protein content in the muscle tissue.

In fish, acclimation to colder temperatures has been shown to greatly affect physiological and biochemical homeostasis. Cellular alterations such as increased activities of key oxidative enzymes (Sidell, 1980; Johnston and Dunn, 1987), increased density of mitochondria and lipid droplets (which increases oxygen storage and diffusivity) have been observed with lower acclimation temperature (Egginton and Sidell, 1989). Also, changes at the organ level occur, such as decreased cardiac output (Farrell, 1997), decreased blood flow to all organs except red muscle (Taylor *et al*., 1993, 1996; Wilson and Egginton, 1994) and increased amounts of red muscle (Sidell, 1980). Whole-animal effects such as altered behaviour (Crawshaw and O'Connor, 1997) and decreased swimming performance (Beamish, 1978; Johnston and Ball, 1997) at lower temperature have also been observed. Oxygen consumption is a widely studied indicator of

metabolic rate and temperature has a profound influence on metabolic processes in poikilothermic animals such as fish (Brett and Groves, 1979).

Jurss (1979) reported that the two main transaminases, ALT and AST, are responsive to temperature change in some teleost fishes. Jurss (1981) found that ALT does not increase in trout muscle acclimated to low temperature, but is elevated in the cold-adapted muscle of the pond loach, *Misgurnus fossilis* (Mester *et al.,* 1973). Total liver AST increases with cold acclimation (Jurss, 1981), although this is primarily due to an increase in liver size and hepatosomatic index. Vellas *et al*. (1982) studied the effect of increasing temperature on the activity of hepatic arginase in Rainbow trout (*Salmo gairdneri*). They observed an increase in the activity of arginase during first few days of high temperature acclimation, but after 17 days of acclimation to high temperature, arginase displayed complete thermal compensation. Jurss *et al*. (1987) reported that arginase activity was reduced at a low water temperature in the liver of Rainbow trout (*Salmo gairdneri*).

In many fish species, enhanced lipogenesis is observed at colder temperatures. Shikata *et al*. (1995) reported that fatty acid synthesis from amino acids is elevated with cold acclimation in carp liver. Another strategy for temperature acclimation is to produce different enzyme isoforms that function better at the new temperature. Some evidence for thermal isoforms of ALT was provided in a study of the pond loach (Mester *et al.,* 1973). There is some evidence that thermal isoforms of the aminoacyl-tRNA synthetases exist in eurythermal fish (Haschemeyer, 1985), presumably for the purpose of maintaining rates of protein synthesis at different temperatures.

The dietary protein requirement of fish increases at higher temperatures and it has been suggested that this is due to increased oxidation of amino acids

(DeLong *et al.,* 1958). Cold acclimation has little effect on the utilization of protein as an energy source in resting juvenile Rainbow trout, but reduces the utilization of protein as an energy source during exercise (Kieffer *et al.,* 1998). The studies described above indicate that temperature may differentially affect the metabolism of specific amino acids as well as the overall importance of amino acids as energy sources.

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Chapter 3

Effect of Cadmium Ion (Cd^{++}) on the Protein Catabolism of *Oreochromis mossambicus* (Peters)

3.1 Introduction

Since the beginning of industrial revolution, considerable progress of human civilization has occurred. The industrial development however, has been accompanied by an increasing harmful impact on the environment in terms of its pollution and degradation. Industrialization carries with it the seeds of environmental damage, assisted by both needs and greed of man. Most of the industries not only deplete the natural resources but also add stress to the environment by accumulating waste materials. The pollution is a by-product of industrialization and urbanization. Global warming and acid precipitation are the well-known effects of pollution. Nowadays the pollution caused by endosulfan in Kasargod district in Kerala has received international attention. This may be the most familiar example of dangerous chemical pollution to us. Industrialization is necessary for the progress of the society but the environmental pollution due to toxic chemical products, emissions and waste generated from these industries cause hazardous effect on all living

organisms. The pollution has the potential to cause irreversible reactions in the environment and hence is posing a major threat to our very existence.

A number of studies have shown that air and water pollution are taking a heavy toll of human life, particularly, in the developing countries through ill-health and premature mortality. Pollution control, thus, assumes greater significance in the context of ensuring sustainable development through planned industrialization.

Water pollution, like other environmental concerns, has been the focus of widespread public interest for about three decades and in the recent years this interest seems to be increasing. Many estuarine and coastal aquatic environments have been sinks for industrial and agricultural effluents. Heavy metals are ranked as highly toxic substances and are among the major contaminants of the marine environment (Dailianis and Kaloyianni, 2004). The two most important factors that contribute to the deleterious effects of heavy metals as pollutants are their indestructible nature through bioremediation unlike organic pollutants and their tendency to accumulate in environment especially in the bottom sediments of aquatic habitats in association with organic and inorganic matter. In many parts of the world, rivers have become contaminated with heavy metals such as zinc (Zn), lead (Pb) and copper (Cu) as a result of mining and associated activities. The tragic incidence of Itai-Itai disease in Japan during World War II has been attributed to an excessive dietary intake of cadmium through rice grown in contaminated water. On the other hand, Minamata disease was the case of methyl-mercury poisoning, which principally attacks the central nervous system, through the consumption of contaminated fish.

Contamination of aquatic environments by heavy metals, whether as a consequence of acute or chronic events, constitutes an additional source of stress

for aquatic organisms (Kori-Siakpere and Ubogu, 2008; Kargin, 2010). The impact of contaminants on aquatic ecosystems can be assessed by the measurement of biochemical parameters in fish that respond specifically to the degree and type of contamination (Petrivalsky *et al*., 1997). Therefore, the enzymatic and non-enzymatic parameters gain importance as sensitive tools to estimate the effects of metal exposures before the occurrence of hazardous effects in organisms.

Heavy metal exposure evokes severe alteration in the physiological and biochemical parameters of the animal. To counteract the stress caused by the metal, energy reserves, which might otherwise be utilized for growth, and reproduction will have to be diverted towards enhanced synthesis of detoxifying ligands (metal binding proteins, granules), or expended in order to maintain an elevated efflux of metal. Consequently, various enzymes related to energy metabolism alter their activity pattern depending on the nature of stress. Excess energy is required to carry out defensive behavioural responses that help animals to adapt and survive.

Out of the several heavy metals in the industrial waste streams, cadmium is often used in environmental studies because it is a non-essential metal (Baker *et al*., 2002), and a non-degradable cumulative pollutant. It is highly toxic, widely distributed in the environment and can adversely affect organisms at relatively low concentrations (Almeida *et al*., 2001). The toxicological effects of cadmium on humans and other higher organisms are well documented (Axelson and Piscator, 1966; Kopp *et al*., 1982). Chronic exposure to this metal results in progressive accumulation, mainly in liver and kidney and can lead to renal tubular dysfunction (Sakurai, 1978).

Cadmium concentrations can be traced in soil, water and food. Tobacco smoke is one of the most common sources of cadmium (Moore, 2004; Soengas *et al*., 1996). Cadmium is widely used in steel industry alloys, batteries and in pigments used in paints, inks, plastic and enamels (Timbrell, 2000). Because of its long biological half-life of 15 to 30 years, cadmium excretion is nearly impossible and it will, therefore, accumulate in blood, kidneys, liver and reproductive organs making it a very toxic metal. Cadmium and its ionic forms have become a serious problem in human health (Baker *et al*., 2003; Henson and Chedrese, 2004) and have also been found to inhibit drug metabolism in rats. Because, it is not an essential trace element, once it is incorporated by the organism it does not have a metabolic pathway and net accumulation occurs. An important fact is that cadmium may interact with other metals such as iron, calcium, copper and zinc (Khan *et al*., 1991) and influence the enzyme activities of metabolic pathways.

Proteins are the most abundant organic molecules of living system and form the basis of structure and function of life. Proteins have many different physiological functions. They are associated with enzymes, transport, and regulation of metabolism, defence, structural elements, and storage and hence represent an important biochemical constituent. Teleost fishes use protein as the main source of energy for their metabolic processes (Van Waarde, 1983). Proteins are polymers of amino acids. In energetic terms, a major function of amino acids is that they serve as catabolic substrates to provide ATP for biomechanical, synthetic, and transport processes. Amino acids provide 14 –85% of the energy requirements of teleost fish (Van Waarde, 1983). This is a substantially higher rate of catabolism than in mammals (20%) (Fauconneau and Arnal, 1985).

Due to the fact that proteins are major molecules in the metabolism of teleost fishes and heavy metals may be involved in the normal working of these molecules, it is important to study the changes in protein metabolism after metal

exposure in detail. Changes that may occur are the increased synthesis or breakdown of proteins and the inhibition or activation of certain enzymes. These can be observed as alterations in, for example, the total protein content, free amino acid (FAA) concentration and the activity of transaminases such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Hilmy *et al*., 1985; Baksi *et al*., 1988; Suresh *et al*., 1991; Reddy and Bhagyalakashmi, 1994; Canli, 1996). Protein metabolism was studied in gills, liver, kidney and muscle, which play important roles in the uptake and accumulation of the toxic metal (Kuroshima, 1992; Kraal *et al*., 1995; De Conto *et al*., 1997). AST and ALT are the most important enzymes acting as transaminases involved in amino acid metabolism and they are known to be sensitive to metal exposures and estimation of total protein levels could be beneficial in estimating the toxicity of metals (Almeida *et al*., 2001; Levesque *et al*., 2002; Gravato *et al*., 2006; Oner *et al*., 2009). As most of the nitrogenous end products of freshwater fish originate from protein catabolism, with ammonia as the principal end product, the contribution of protein catabolism to the total energy production of the fish can be assessed by determination of the ammonia quotient.

The objective of the present study was to determine the levels of total protein, free amino acid and plasma ammonia and to investigate the response of enzymes like AST, ALT, GDH and arginase in tissues of *Oreochromis mossambicus* (Peters) exposed to sub lethal concentrations of $CdCl_2$ for 7 days. In order to obtain a more general idea of the changes caused by Cd^{++} on protein catabolism, ammonia quotient, rate of ammonia excretion and rate of oxygen consumption were also analyzed.

3.2 Materials and Methods

3.2.1 Toxic substance used for the study

Cadmium chloride (Dried Anhydrous pure) purchased from Sisco Research Laboratories (SRL), India was used.

Chemical and physical properties of cadmium chloride

Systematic name- Cadmium chloride anhydrous

Molecular formula-$CdCl_2$

Molecular weight-183.32

Melting point -568 °C

Boiling point- 960 °C

Density- 4.047

Appearance- White crystalline powder

3.2.2 Experimental animal

The fresh water adapted euryhaline teleost cichlid fish *Oreochromis mossambicus* (Peters, 1852) commonly known as Tilapia was selected as the animal model for the study owing to its wide availability, ease of rearing, maintenance, sustainability in laboratory conditions and economic viability. The fish shows well adaptive nature with the changing environment.

3.2.3 Experimental design

3.2.3.1 Collection and maintenance of test fish

Oreochromis mossambicus of (15±3 g) were collected from the culture ponds of fisheries station of Kerala University of Fisheries and Ocean studies,

Puduvyppu, Cochin, Kerala. The fishes were acclimatized to laboratory conditions in large tanks, where a continuous and gentle flow of dechlorinated tap water was maintained. The physicochemical parameters of water were estimated daily according to the procedure of APHA (1998). The tap water had dissolved oxygen content of 7.8 ppm, pH 7.0 ± 0.32, temperature 26 ± 3^0C, salinity 0 ppt and hardness below detectable amounts. The fishes were fed on a commercial diet *ad libitum* and were acclimated in tanks for a month before the experiment. The laboratory acclimatized fishes were sorted in to batches of six each and were kept in 60 l tubs for the experiment. Water in the tubs was changed daily. During the experimental period the fishes were fed on the same commercial diet so as to avoid the effects of starvation on normal physiological processes. Suitable controls were maintained to nullify any other effect that likely to affect the fish.

3.2.3.2 Determination of median lethal concentration (LC_{50}) of cadmium chloride in *Oreochromis mossambicus* (Peters)

The median lethal concentration (LC_{50}) is the concentration of a toxin in water which kills 50% of a test batch of fish within a continuous period of exposure. The fishes were exposed to the test substance added to water at a range of concentrations. Most acute studies have been conducted for 96 hour as recommended by APHA (1971) and Portmann (1972). LC_{50} test was carried out using semi static method. For the experiment; several batches of the acclimatized fishes (6 nos. in each batch) were transferred to large experimental tubs, each containing 60 litres of dechlorinated tap water. Different cadmium chloride concentrations ranging from 3 mg/l (no mortality) to 10 mg/l (100 % mortality) were chosen for the final 96 hour test to determine the 50% lethal concentration (LC_{50}). Each experiment was repeated three times at the selected toxin

concentration. Fishes transferred to tanks containing no toxicant served as control. The contents of the control tanks (water) and the experimental tanks (water and $CdCl_2$) were renewed daily to remove the debris, taking care to give minimum disturbance to the fish. The fishes were not fed during the entire exposure period. The duration of the experiment was 96 hours. Fishes were checked for mortality at every 24 hours interval. Even though lethality is considered to be a crude measurement of toxic response, many workers have proved its importance and utility in environmental management. Hence lethal toxicity tests remain unsurpassed as a screening technique, a comparative tool and a classic approach to the study of stress response due to pollution. The LC_{50} levels were calculated using Probit analysis (Finney, 1971). The lethal toxicity experiments were repeated wherever necessary.

3.2.3.3 Bioassay method

The acclimatized fishes were sorted in to batches of six each for sub lethal toxicity studies. The bioassays were conducted in 60 l tubs containing dechlorinated tap water. For each experiment fish weighing 15±3g were used. From the LC_{50} value**,** it was calculated that 0.92 mg/l was the sub lethal concentration of cadmium chloride (which corresponds to 0.564 mg/l Cd^{++}) for *O.mossambicus*. To know the effect of higher concentrations of cadmium chloride, apart from 0.92 mg/l, two more concentrations: 1.84mg/l and 3.06 mg/l (1/5 and 1/3 of LC_{50} value) of cadmium chloride (which corresponds to 1.128 mg/l Cd^{++} and 1.876 mg/l Cd^{++} respectively) were also selected for the present study.

3.2.3.4 Experimental design for the study of the effects of cadmium chloride exposure

For conducting biochemical studies *O.mossambicus* (15±3g) were taken in three separate tubs which contained desired concentration of cadmium chloride (0.92 mg/l, 1.84 mg/l, 3.06 mg/l respectively) or (0.564 mg/l, 1.128 mg/l and 1.876 mg/l Cd^{++} respectively) along with tap water. Six replicates were kept for each experiment. The fishes were exposed for 7 days. During the experimental period of 7 days the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. The water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). Any other factor likely to influence toxicity was nullified by maintaining a suitable control.

3.2.3.5 Preparation of tissue samples for the biochemical study

After the 7 day experimental period the fishes were sacrificed by pithing (by damaging the brain and severing the spinal cord between the head and trunk region using a sharp needle) and the tissues viz. gills, liver, kidney and muscle were removed from the body, wiped thoroughly using blotting paper to remove blood and other body fluids. Then they were washed in ice cold 0.33 M sucrose solution and again blotted dry and the desired amount of the tissues were weighed and used.

3.2.3.6 Preparation of serum and plasma samples for the study

Blood was drawn from the common cardinal vein in 1 ml syringe. For serum preparation, the blood was allowed to clot at room temperature for 15 to 30 minutes. When the blood was clotted completely, it was rimmed with a

needle and then centrifuged for 5-10 minutes at 2,500 rpm. The supernatant fluid was then separated and it was then stored at 20°C until assayed.

Plasma is the supernatant fluid obtained when anti-coagulated blood has been centrifuged. The blood was mixed with an appropriate amount of anticoagulant like ethylene diamine tetra acetic acid (EDTA). This preparation should be mixed immediately and thoroughly to avoid clotting. The solution was then centrifuged for 5-10 minutes at 2000 - 2500 rpm. The supernatant fluid was then separated just like serum and assayed.

3.2.4 Methods used for the biochemical analysis

3.2.4.1 Estimation of protein

Protein was estimated by the method of Lowry *et al*. (1951).

Reagents

1. 2% Sodium carbonate in 0.1 N NaOH (Reagent A)
2. 0.5% Copper sulphate in 1% potassium sodium tartrate (Reagent B)
3. Alkaline copper reagent: 50 ml of A and 1.0 ml of B were mixed prior to use (Reagent C)
4. Folin-Ciocalteau reagent: 1 part of reagent was mixed with 2 parts of water (1:2 ratio)
5. Stock standard: 50 mg of bovine serum albumin was weighed. It was then made up to 50 ml in a standard flask with distilled water.
6. Working standard: 10 ml of the stock was diluted to 50 ml with distilled water. 1.0 ml of this solution contains 200 µg of protein.

Procedure

After isolating the tissues, 5% homogenates of gills, liver, muscle and 1% homogenate of kidney were prepared in 0.33 M cold sucrose solution. The homogenates were centrifuged at 1000 g for 15 minutes. To the supernatant equal volume of 10% tri chloro acetic acid was added to precipitate the proteins. The contents were allowed to stand for 30 min at room temperature and centrifuged at 1000g for 15 minutes. The sediment of protein was dissolved in 1ml of 0.1N NaOH. After suitable dilution a known volume of the solution was used as sample. A set of working standard solution ranging from 0.2 to 1.0 ml were pipetted out in to the test tubes. The volumes in all the tubes were made up to 1.0 ml with distilled water. 5.0 ml of alkaline copper reagent was added to all of the test tubes. The contents in the tubes were mixed well and were allowed to stand for 10 minutes. Then 0.5 ml of Folin-Ciocalteau reagent was added to each tube and the tubes were mixed well and incubated at room temperature for 30 minutes. A reagent blank was also prepared in the similar manner. After 30 minutes, the blue colour developed was read at 660 nm in spectrophotometer against the reagent blank. Tissue samples were also treated in a similar manner. The values were expressed as mg/g wet weight of tissue.

3.2.4.2 Estimation of free amino acids

Total free amino acids (also known as ninhydrin positive substances) were estimated by the method of Moore and stein (1948).

Reagents

1. 10% Tri chloro acetic acid (TCA)
2. Ninhydrin reagent- Dissolved 0.8 g stannous chloride ($SnCl_2.2H_2O$) in 500 ml of 0.2 M citrate buffer pH 5.0. Added this solution to 20 g of ninhydrin in 500 ml of methyl cellosolve (2 methoxy ethanol).

3. 0.2 M citrate buffer pH 5.0
4. Diluent solvent- Mixed equal volume of water and *n*-propanol
5. Stock standard Tyrosine: Dissolved 50 mg of tyrosine in 50 ml of distilled water
6. Working standard Tyrosine: 10 ml of stock standard was made up to 100ml.

Procedure

5% homogenates of gills, liver, muscle and 1% homogenate of kidney tissue were prepared in 10% TCA. The tissues were then centrifuged at 1000g for 15 min. The supernatant was taken for the assay. To 0.5 ml of the supernatant, 1.0 ml of ninhydrin reagent was added. The tubes were shaken well and covered with marble. The tubes were kept in a boiling water bath for 15 minutes. It was then cooled immediately. After suitable cooling 5 ml of diluent solvent was added and mixed well. The absorbance was read at 570 nm in a spectrophotometer using a blank. The blank consists of 0.5 ml of 10% TCA and 1 ml of ninhydrin reagent and received the same treatment as that of the sample. The amino acid content was expressed as mg of tyrosine equivalent/g wet weight of tissue.

3.2.4.3 Assay of alanine aminotransferase (ALT) (EC 2.6.1.2)

Alanine aminotransferase was assayed by the method of Mohun and Cook, 1957.

Reagents

1. Buffered substrate (0.1 M phosphate buffer, pH 7.4; 0.2 M DL- alanine; 2mM 2- oxoglutarate).
2. 2, 4 – Dinitro phenyl hydrazine (2, 4-DNPH)

3. 0.4 N NaOH
4. Standard pyruvate: Dissolve 11.01 mg of sodium pyruvate in 10 ml of distilled water. Diluted this further to 100 ml with distilled water and prepare fresh each time.
5. 0.33M Sucrose

Procedure

5% homogenates of gills, liver, muscle and 1% homogenates of kidney tissue were prepared in 0.33 M sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatant obtained was used as the enzyme source. 1 ml each of buffered substrate was pipetted out into two test tubes labelled 'test' and 'control'. Added 0.2 ml of the enzyme source into the test tube labelled 'test' and incubated the tubes at 37 ^{0} C for 60 minutes. After incubation, 0.2 ml enzyme was added to the control tube. 1ml of 2, 4 – DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 540 nm in a spectrophotometer against a blank. The blank preparation was the same as that of the test, except that the corresponding volume of distilled water substitutes the supernatant. The ALT activities were expressed as µ moles of pyruvate liberated / h / mg protein.

3.2.4.4 Assay of aspartate aminotransferase (AST) (EC 2.6.1.1)

Aspartate aminotransferase was assayed by the method of Mohun and Cook, 1957.

Reagents

1. Buffered substrate (0.1 M phosphate buffer, pH 7.4; 1.0 M aspartic acid; 2 mM 2-oxoglutarate).
2. 2, 4 – Dinitro phenyl hydrazine (2, 4 – DNPH)
3. 0.4 N NaOH
4. Standard pyruvate: Dissolve 11.01 mg of sodium pyruvate in 10 ml of distilled water. Diluted this further to 100 ml with distilled water and prepared fresh each time.
5. 0.33 M Sucrose.

Procedure

5% homogenates of gills, liver, muscle and 1% homogenate of kidney tissue were prepared in 0.33 M sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatant obtained was used as the enzyme source. 1 ml each of buffered substrate was pipetted out into two test tubes labelled 'test' and 'control'. Added 0.2 ml of the enzyme source into the test tube labelled 'test' and incubated the tubes at 37 ^{0} C for 60 minutes. After incubation, 0.2 ml enzyme was added to the control tube. 1 ml of 2, 4 – DNPH reagent was added to each tube and kept at room temperature for 20 minutes. The reaction was stopped by the addition of 10 ml of 0.4 N NaOH, vortexed and kept at room temperature for 5 minutes. The absorbance was measured at 540 nm in a spectrophotometer against a blank. The blank preparation was the same as that of the test, except the corresponding volume of distilled water substitutes the supernatant. The AST activity was expressed as μ moles of pyruvate liberated / h / mg protein.

3.2.4.5 Assay of glutamate dehydrogenase (GDH) (EC 1.4.1.2)

Glutamate dehydrogenase was assayed by the method of Plummer, 1987.

Reagents

1. Sodium phosphate buffer (0.1 M, pH 7.4).
2. 2-oxoglutarate (0.15 M), prepared in phosphate buffer and pH adjusted to 7.4.
3. Ammonium acetate (0.75 M), prepared in phosphate buffer and pH adjusted to 7.4.
4. EDTA (30 mM), prepared in phosphate buffer and pH adjusted to 7.4.
5. NADH (2.5 mg/ml in phosphate buffer, prepared fresh).
6. Triton X-100.

Procedure

5% homogenates of gills, liver, muscle and 1% homogenate of kidney tissue were prepared in 0.33 M sucrose solution and centrifuged at 1000g for 15 minutes. The supernatant obtained was used as the enzyme source The reaction mixture consisted of 2.1 ml phosphate buffer, 0.2 ml enzyme source, 0.1 ml NADH, 0.2ml ammonium acetate, 0.2 ml EDTA and 0.1 ml triton X-100. The above mixture was equilibrated at room temperature for 10 minutes. The reaction was started by adding 0.1 ml of 2-oxoglutarate and the rate of change of extinction at 340 nm with time were noted. Molar extinction coefficient of NADH is 6.3×10^3 litres mol^{-1} cm^{-1}. The enzyme activity was calculated as micromoles of NADH oxidized / minute / mg protein.

3.2.4.6 Assay of arginase (EC 3.5.3.1)

Principle

Arginase activity in tissues was estimated by the method of Brown and Cohen (1959).

Reagents

1. L-Arginine (25 mM)
2. CTAB (cetyl tri methyl ammonium bromide) (0.1%)
3. $MnCl_2$ (0.25 mM)
4. Sodium glycinate buffer (25 mM) at pH 9.5
5. $HClO_4$ (perchloric acid) (0.5 M)
6. TCA, 10%
7. Stock diacetyl monoxime (25 g/l)
8. Stock thiosemicarbazide, (2.5 g/l)
9. Acid ferric chloride solution: added 1.0 ml sulphuric acid to 100 ml of ferric chloride solution.
10. Acid reagent: added 10 ml of ortho phosphoric acid, 80 ml of sulphuric acid and 10 ml acid ferric chloride solution to one litre of water. The contents were then mixed well.
11. Colour reagent: To 300 ml of acid reagent, 200 ml of water, 10 ml stock diacetyl monoxime and 2.5 ml of thiosemicarbazide solution was added
12. Stock urea standard: 5 to 50 mmol/l of pure urea was taken which contained concentration ranging from 30- 300 mg/l

Procedure

5% homogenates of gills, liver, and muscle and 1% homogenate of kidney were prepared in 0.1% CTAB solution and centrifuged at 4000g for 10 minutes at 5 °C. The supernatant obtained (S_1) was stored in ice. The pellet R_1 is re-homogenised with a volume of CTAB that was employed in original homogenization. It was again centrifuged at 4000g for 10 minutes at 5 °C. The supernatant obtained (S_2) was stored in ice. Equal volume of S_1 and S_2 was the enzyme source. The reaction mixture consisted of 0.1 ml sample, 20µl $MnCl_2$, 200 ml sodium glycinate buffer pH 9.5 and 100 µl distilled water. The reaction mixture was incubated at 37 °C for 10 minutes. The reaction was started by adding 20 µl L-arginine. The above mixture was incubated at 37 °C for 30 minutes. Reaction was stopped by the addition of perchloric acid (5ml for 2.0 ml reaction mixture). Precipitated protein was removed by centrifugation. The clear supernatant was assayed for urea by diacetyl monoxime method. In a test tube 0.2 ml of the supernatant was taken and 3.0 ml of colour reagent was added. The tubes were heated in a boiling water bath for 20 minutes and were cooled to room temperature and the colour developed was then read at 520 nm within 15 minutes against a blank containing 0.2 ml of water and 3 ml colour reagent. A set of standard urea solutions was also treated similarly. The enzyme activity was calculated as micromoles of urea produced/ minute /g tissue.

3.2.4.7 Estimation of plasma ammonia

Principle

Ammonia in the serum sample was estimated using the method of Boltz and Howel (1978).

Reagents

1. Reagent A: Dissolved 10 g of phenol with 50 mg of sodium nitro prusside in 500 ml of distilled water (This solution is stable for one month if kept in stoppered amber coloured bottle in refrigerator).
2. Reagent B: Dissolved 5 g of sodium hydroxide in 10 ml of sodium hypochlorite and dilute to 500 ml with distilled water.
3. Standard ammonia solution: Dissolved 0.03819 g of anhydrous ammonium chloride in 100 ml of distilled water.
4. Working standard: Diluted 5 ml of Stock solution to 100 ml with distilled water
5. Deproteinizing agent: 80% ethanol

Procedure

To 0.2 ml of plasma, 2 ml deproteinizing agent was added. It was then centrifuged at 5000 rpm for 5 minutes. Supernatant was taken for the assay. To 1 ml of the supernatant, 2.5 ml of reagent A was added. All the tubes were stoppered. The tubes were kept at room temperature for 5 min. To each tube 2.5 ml of reagent B was added. The tubes were again kept for 5 min at room temperature. After 5 min the tubes were incubated at 37 °C for 20 minutes. The optical density was read after 30 minutes at 625 nm against a blank containing 1 ml of 80% ethanol, 2.5 ml of reagent A and 2.5 ml of reagent B. A set of standard ammonia solutions was also treated similarly. The values were expressed as micromole/l.

3.2.4.8 Determination of rate of ammonia excretion by *Oreochromis mossambicus*

Ammonia in the sample was estimated using the method of Boltz and Howel (1978).

Reagents

1. Reagent A: Dissolved 10 g of phenol with 50 mg of sodium nitroprusside in 500 ml of distilled water (This solution is stable for one month if kept in stoppered amber bottle in refrigerator).
2. Reagent B: Dissolved 5 g of sodium hydroxide in 10 ml of sodium hypochlorite and dilute to 500 ml with distilled water.
3. Standard ammonia solution: Dissolved 0.03819 g of anhydrous ammonium chloride in 100 ml of distilled water.
4. Working standard: Diluted 5 ml of Stock solution to 100 ml with distilled water

Procedure

Two litre water containing 0.92 mg/l, 1.84 mg/l and 3.06 mg/l cadmium chloride (which corresponds to 0.564 mg/l, 1.128 mg/l and 1.876 mg/l Cd^{++} respectively) each were taken in separate tanks. A tank containing 2 l tap water served as the control. Introduced one fish in each tank. (The fishes in the toxin containing tanks were treated with the same concentration of cadmium chloride for 7days). Immediately 1 ml of water sample was taken from each tank to determine the initial ammonia content in water. After 1 hour incubation 1 ml of sample was taken from each tank. To 1 ml of the 0 hour and 1 hour sample, 2.5 ml of reagent A was added. All the tubes were stoppered. The tubes were kept at room temperature for 5 min. To each tube 2.5 ml of reagent

B was added. The tubes were again kept for 5 min at room temperature. After 5 min the tubes were incubated at 37 °C for 20 minutes. The optical density was read after 30 minutes at 625 nm against a blank containing 1 ml of distilled water, 2.5 ml of reagent A and 2.5 ml of reagent B. A set of standard ammonia solutions was also treated similarly. Wet weights of the fishes were measured. From the data obtained, rate of ammonia excreted by the fish was calculated and expressed in μ moles of ammonia excreted/g/h.

3.2.4.9 Determination of rate of oxygen consumption by *Oreochromis mossambicus*

The oxygen concentration in the sample was estimated using the Winkler's method (1888).

Reagents

1. Winkler solution A- 480 g $MnSO_4$.$4H_2O$ dissolved in 800 ml of distilled water. Filtered and diluted to 1000 ml with distilled water
2. Winkler solution B- dissolved 700 g potassium hydroxide and 150 g potassium iodide in distilled water and dilute to 1000 ml
3. Sulphuric acid concentrated
4. Starch indicator – 1g starch dissolved in 100 ml warm water.
5. Paraffin Liquid
6. Sodium thiosulphate (0.01N)
7. Potassium iodate (0.01N)
8. Potassium iodide (5%)
9. Sulphuric acid (2N)

Procedure

a. Standardization of Sodium thiosulphate

Preparation of standard solution of potassium iodate: 0.09g of potassium iodate is weighed out accurately and transferred into a 250 ml standard flask. The solid is dissolved in water and then made up to the volume

Standardization of Sodium thiosulphate: 20 ml of the standard potassium iodate solution is pipetted out in to a conical flask and 10 ml of 5% Potassium iodide solution is added followed by 5 ml of 2N Sulphuric acid. The liberated iodine is titrated with the Sodium thiosulphate solution with constant stirring. When the colour of the solution has become pale yellow the solution is diluted to 100 ml with distilled water and 2 ml of starch solution added. The titration is continued until the colour changes from blue to colourless. The experiment is repeated till concordant values are obtained. The normality of sodium thio sulphate was calculated from the values.

b. Estimation of the rate of Oxygen consumption by fish:

Two litre water containing 0.92 mg/l, 1.84mg/l and 3.06 mg/l cadmium chloride (which corresponds to 0.564 mg/l, 1.128 mg/l and 1.876 mg/l Cd^{++} respectively) each was taken in separate tanks. A tank containing 2 litre tap water served as the control. One fish was introduced in to each tank. (The fishes in the toxin containing tanks were treated with the same concentration of cadmium chloride for 7 days). An even layer of liquid paraffin was poured over the water in the tanks to prevent further dissolution of atmospheric oxygen in to it. Water was siphoned immediately from each tank to the DO bottles taking all precautions to reduce contact of water with air to a minimum. Allowed the water to overflow the top of the sample bottle so that the water in contact with the air had been displaced and the volume of overflow was

measured. This sample was to determine the initial oxygen content in water. After 1 hour incubation siphon water from each tank to the DO bottles. 0.5 ml of winkler solution A followed by 0.5 ml of winkler solution B were added to all the sample bottles. The reagents were added to the bottom of the sample bottles so that 1 ml of water displaced from the top. The bottles were shaken well to distribute the precipitate in an even way. The precipitate was allowed to settle to a definite level. 1 ml of concentrated sulphuric acid was added gently just below the surface of the sample. The bottles were shaken vigorously till all the precipitate was dissolved. 20 ml of sample was pipetted out and titrated against the standardized sodium thiosulphate solution. When the colour of the solution has become pale yellow a few drops of starch solution were added. The titration was continued until the colour changes from blue to colourless. The experiment was repeated till concordant values were obtained. Wet weights of the fish were measured. Using the above data, the rate of oxygen consumed by the fish was calculated and expressed in µmoles of oxygen consumed/g/h.

3.2.4.10 Determination of ammonia quotient (AQ)

AQ is the mole to mole ratio of ammonia excreted to oxygen consumed.

$$\mathrm{AQ} = \frac{\text{Ammonia excreted } (\mu \text{ moles / g / h})}{\text{Oxygen consumed } (\mu \text{ moles / g / h})}$$

3.2.5 Statistical Analysis

The statistical analysis was carried out using the software SPSS 13.0 package. Two-way analysis of variance (ANOVA) was carried out to compare between different concentrations and also between tissues. If significant differences were revealed by the ANOVA test, Tukey's test was used to

further elucidate which tissues and concentrations were significantly different. One- way ANOVA followed by Tukey's test was carried out for the comparison between different concentrations in each tissue. For determining the significant difference between different concentrations in blood and serum parameters, one- way ANOVA followed by Tukey's test was done. Significance level (P value) was set at 0.05 in all tests.

3.3 Results

3.3.1 Lethal toxicity study

The calculated LC_{50} value for cadmium chloride exposure in *O. mossambicus* over periods of up to 96 hours is 9.2 mg/l. The LC_{50} levels and 95% confidence limits were calculated using Probit analysis (Finney, 1971). The results are presented in table 3.1

Table 3.1 96 h LC_{50} value for cadmium chloride in *O. mossambicus*

Acute Toxicity Range (mg l^{-1}) 95% confidence limit		Median LC_{50}(mg l^{-1})
9.11721	9.32946	9.2336

3.3.2 Total protein

In the present study the protein content in different tissues of *O. mossambicus* treated with different concentrations of cadmium chloride showed significant decrease ($P<0.05$) compared to control group (Fig 3.2 and Table 3.2) (One-way ANOVA followed by Tukey's test) .

Figure 3.1 Protein content in the various tissues exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

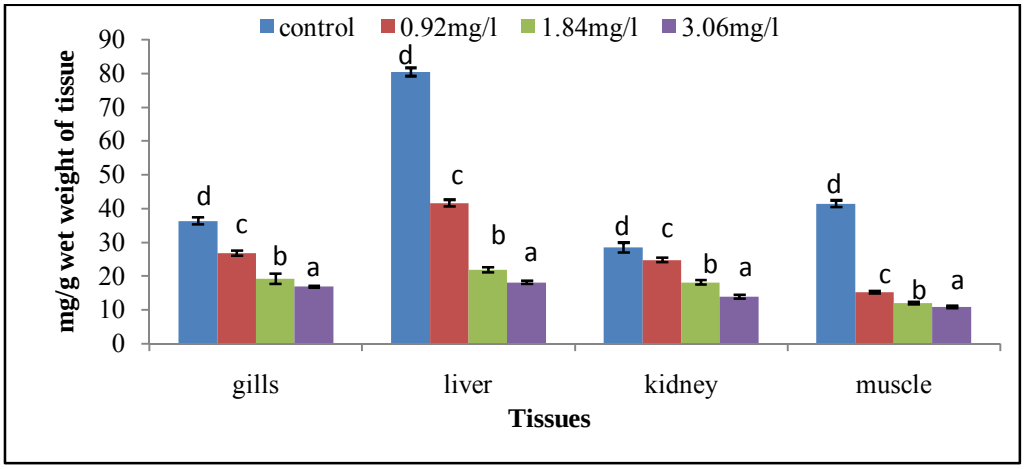

Table 3.2 Effect of exposure to different concentrations of cadmium chloride for 7 days on total protein content (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Total protein level			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	36.38 ± 1.02^{d}	26.79 ± 0.793^{c}	19.24 ± 1.491^{b}	16.93 ± 0.322^{a}
Liver	80.42 ± 1.23^{d}	41.61 ± 1.03^{c}	21.928 ± 0.703^{b}	18.214 ± 0.402^{a}
Kidney	28.55 ± 1.45^{d}	24.77 ± 0.663^{c}	18.23 ± 0.611^{b}	13.99 ± 0.585^{a}
Muscle	41.45 ± 0.99^{d}	15.26 ± 0.34^{c}	12.1 ± 0.42^{b}	10.96 ± 0.32^{a}

Values are expressed as mg protein /g wet weight of tissue.

Two factor ANOVA (Table 3.3) revealed that there was a significant decrease (P<0.05) in protein content in all the cadmium chloride treated experimental groups when compared to control group. Subsequent analysis by multiple comparison using Tukey's test (Table 3.14) revealed that there was significant difference (P<0.05) in protein content between control and all concentrations of cadmium chloride treated groups. Similarly multiple comparison using Tukey's test (Table 3.15) also revealed that there was a significant difference between tissues also.

Table 3.3 Two-Factor ANOVA for Total protein

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	7365.886	3	2455.295	3309.282	0.000
Between tissues	3230.097	3	1076.699	1451.190	0.000
concentration * tissues	2895.889	9	321.765	433.680	0.000
Error	23.742	32	0.742		
Total	47667.747	48			

df- degrees of freedom

3.3.3 Free amino acid content

In the present study the tissue free amino acid content in different tissues of *O. mossambicus* treated with cadmium chloride showed significant increase ($P<0.05$) compared with control group (Fig 3.3 and Table 3.4) (One-way ANOVA followed by Tukey's test) .

Figure3.2 Free amino acid content in the various tissues exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

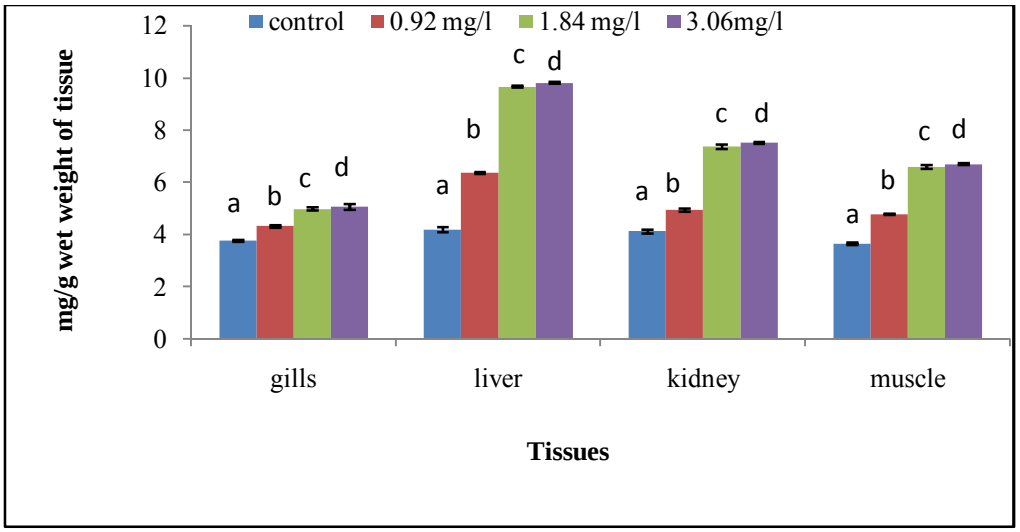

Table 3.4 Effect of exposure to different concentrations of cadmium chloride for 7 days on free amino acid content (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	Free amino acid			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	3.76 ± 0.039^{a}	4.32 ± 0.045^{b}	4.98 ± 0.06^{c}	5.06 ± 0.10^{d}
Liver	4.19 ± 0.1^{a}	$6.36\pm.0.03^{b}$	9.67 ± 0.036^{c}	9.82 ± 0.034^{d}
Kidney	4.12 ± 0.073^{a}	4.94 ± 0.065^{b}	7.37 ± 0.077^{c}	7.51 ± 0.036^{d}
Muscle	3.65 ± 0.04^{a}	4.78 ± 0.032^{b}	6.59 ± 0.074^{c}	6.7 ± 0.033^{d}

Values are expressed as mg / g wet weight of tissue.

Two factor ANOVA (Table 3.5) revealed that there was a significant increase ($P<0.05$) in free amino acid content in all the cadmium chloride treated experimental groups when compared to control group. Subsequent analysis by multiple comparison using Tukey's test (Table 3.14) revealed that there was significant difference ($P<0.05$) in free amino acid content between control and all concentrations of cadmium chloride treated groups. Similarly multiple comparison using Tukey's test (Table3.15) also revealed that there was a significant difference between tissues also. Among the tissues liver showed highest increase followed by kidney.

Table 3.5 Two-Factor ANOVA for tissue free amino acids

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	191.208	3	63.736	17789.874	0.000
Between tissues	112.832	3	37.611	10497.828	0.000
concentration * tissues	41.626	9	4.625	1290.947	0.000
Error	0.287	80	0.004		
Total	3645.458	96			

df-degrees of freedom

3.3.4 Activity of alanine aminotransferase

In the present study the activity of alanine aminotransferase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant

increase ($P<0.05$) compared with control group (Fig 3.4 and Table 3.6) in each tissue on different treatments (One-way ANOVA followed by Tukey's test) .

Fig 3.3 Effect of different concentrations of cadmium chloride on ALT activity in the various tissues of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

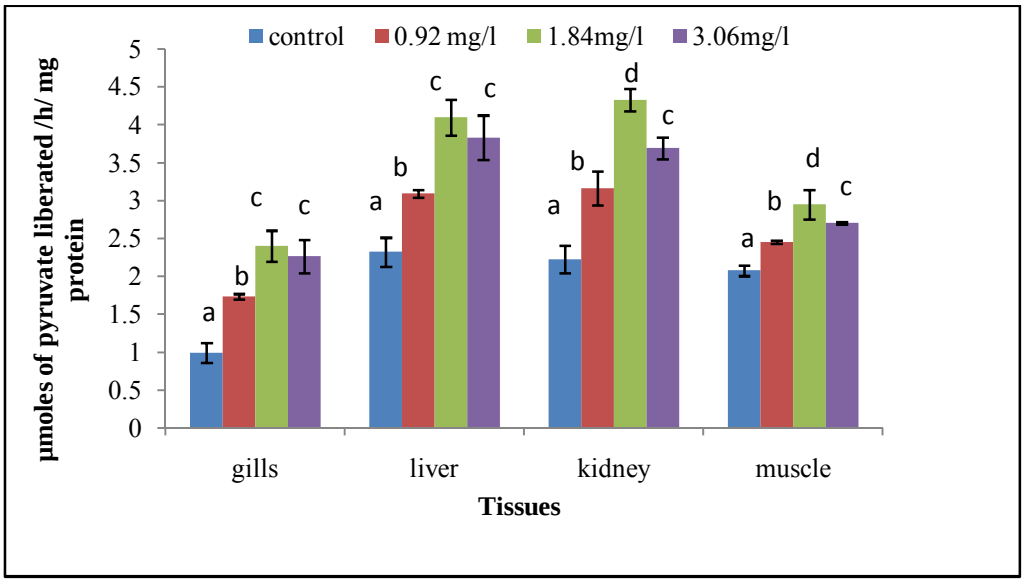

Table 3.6 Effect of exposure to different concentrations of cadmium chloride for 7 days on alanine aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	ALT activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.99 ± 0.13^{a}	1.73 ± 0.038^{b}	2.4 ± 0.20^{c}	2.26 ± 0.22^{c}
Liver	2.32 ± 0.19^{a}	3.09 ± 0.051^{b}	4.1 ± 0.24^{c}	3.83 ± 0.29^{c}
Kidney	2.22 ± 0.18^{a}	3.16 ± 0.23^{b}	4.33 ± 0.15^{d}	$3.69\pm0.0.14^{c}$
Muscle	2.08 ± 0.07^{a}	2.45 ± 0.023^{b}	2.95 ± 0.195^{d}	2.7 ± 0.02^{c}

Values are expressed as µmoles of pyruvate liberated /h/ mg protein

Two way ANOVA (Table 3.7) followed by comparisons with multiple comparison using Tukey's test (Table 3.14) revealed that all the three concentrations selected for the study differed significantly from one another and also with the control. Comparison between various tissues using Tukey's test (Table 3.15) indicated that liver tissue when compared with kidney exhibited no significant difference (NS), whereas tissues such as gills and muscle compared with liver and kidney exhibited significant difference (P<0.001).

Table 3.7 Two-Factor ANOVA for tissue ALT activity

Source of Variation	Sum of Squres	df	Mean Squre	F	Sig.
Between concentrations	16.250	3	5.417	189.396	0.000
Between Tissues	18.721	3	6.240	218.199	0.000
concentration×tissue	1.541	9	0.171	5.988	0.000
Error	0.915	32	0.029		
Total	405.063	48			

df- degrees of freedom

3.3.5 Activity of aspartate aminotransferase

There was a significant increase (P<0.05) in the activity of aspartate aminotransferase in different tissues of *O. mossambicus* treated with cadmium chloride compared to control (Fig 3.5 and Table 3.8). One-way ANOVA followed by Tukey's test revealed a significance at (P<0.05) in each tissue on different treatments

Figure 3.4 AST activity in the various tissues exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

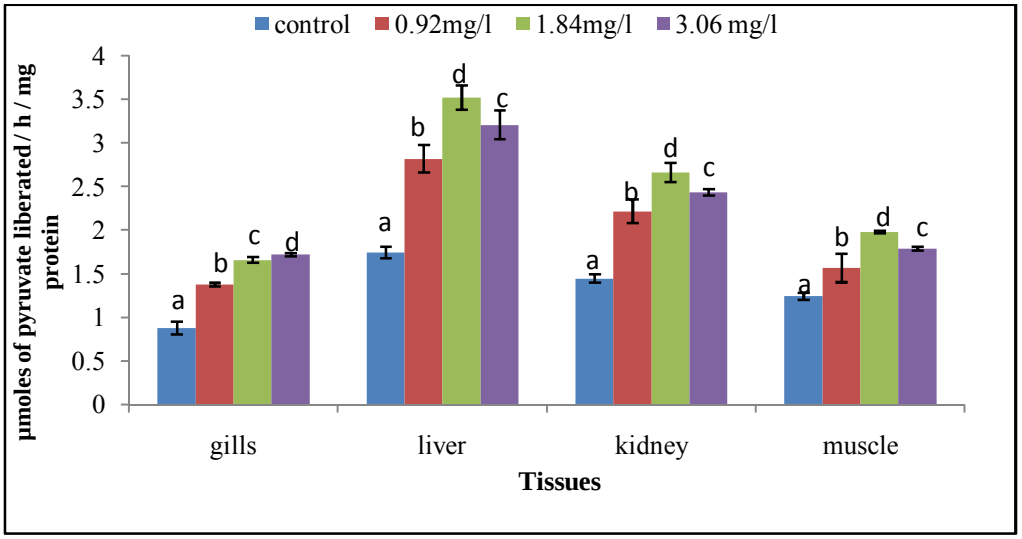

Table 3.8 Effect of exposure to different concentrations of cadmium chloride for 7 days on aspartate aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	AST activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.88 ± 0.075^{a}	1.38 ± 0.020^{b}	1.66 ± 0.033^{c}	1.72 ± 0.018^{d}
Liver	1.75 ± 0.066^{a}	2.82 ± 0.158^{b}	3.52 ± 0.140^{d}	3.21 ± 0.165^{c}
Kidney	1.45 ± 0.456^{a}	2.22 ± 0.134^{b}	2.66 ± 0.111^{d}	2.438 ± 0.036^{c}
Muscle	1.244 ± 0.039^{a}	1.57 ± 0.165^{b}	1.98 ± 0.016^{d}	1.79 ± 0.021^{c}

Values are expressed as µmoles of pyruvate liberated / h / mg protein

Tissue aspartate aminotransferase activity showed a significant increase ($P< 0.05$) between experimental groups of fishes and control group as observed by Two way ANOVA (Table 3.9). Further analysis by multiple comparison using Tukey's test (Table 3.14) revealed that there was a significant difference between all the three concentrations selected for the study from one another and

also with the control (P<0.001). Subsequent pair wise comparison between various tissues were carried out using Tukey's test (Table 3.15) which indicated significant difference (P<0.001). Among the tissues liver and kidney showed highest activity.

Table 3.9 Two-factor ANOVA for tissue AST activity

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	17.760	3	5.920	643.700	0.000
Between Tissues	28.589	3	9.530	1036.166	0.000
concentration×tissue	2.490	9	0.277	30.080	0.000
Error	0 .736	80	0.009		
Total	440.702	96			

df- degrees of freedom

3.3.6 Activity of glutamate dehydrogenase

In the present study the activity of glutamate dehydrogenase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant variations (P<0.05) compared with control group (Fig 3.6 and Table 3.10) (One-way ANOVA followed by Tukey's test). A statistically significant increase in glutamate dehydrogenase (P<0.05) was observed in liver, kidney and muscle of the treated groups compared to control. No significant variation was observed in gills of the treated groups compared to control.

Figure3.5 GDH activity in the various tissues exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

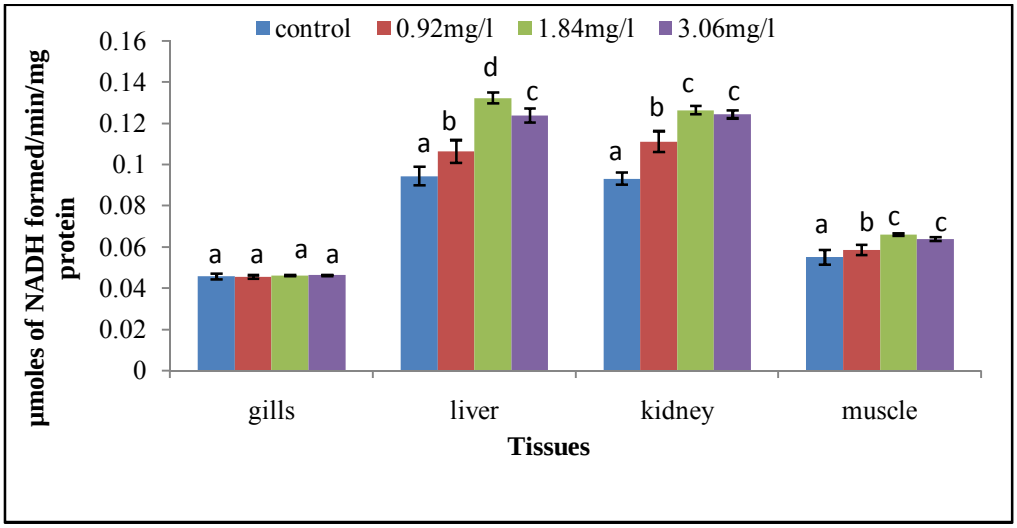

Table 3.10 Effect of exposure to different concentrations of cadmium chloride for 7 days on Glutamate dehydrogenase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	GDH activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.0456 ± 0.0014^{a}	0.0454 ± 0.0084^{a}	0.0461 ± 0.0031^{a}	0.0462 ± 0.0029^{a}
Liver	0.094 ± 0.005^{a}	0.106 ± 0.006^{b}	0.132 ± 0.003^{d}	0.124 ± 0.004^{c}
Kidney	0.093 ± 0.003^{a}	0.111 ± 0.005^{b}	0.126 ± 0.002^{c}	0.124 ± 0.0019^{c}
Muscle	0.055 ± 0.004^{a}	0.0585 ± 0.0023^{b}	0.0659 ± 0.0059^{c}	0.0638 ± 0.0089^{c}

Values are expressed in µmoles of NADH formed/min/mg protein

Two factor ANOVA (Table 3.11) revealed that there was a significant increase ($P<0.05$) in glutamate dehydrogenase activity in all the cadmium chloride treated experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table3.14) revealed that there was significant difference ($P<0.05$) between control and 0.92 mg/l, 1.84

mg/l, 3.06 mg/l cadmium chloride treated groups and between 0.92 mg/l and 1.84 mg/l, 0.92 mg/l and 3.06 mg/l. Between 1.84 mg/l and 3.06 mg/l there was a significance at P<005. Multiple comparison tests also (Table 3.15) revealed that there was a significant difference between tissues except between liver and kidney.

Table3.11 Two-factor ANOVA for tissue GDH activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	0.006	3	0.002	246.363	0.000
Between Tissues	0.091	3	0.030	3562.746	0.000
concentration * tissue	0.004	9	0.000	47.306	0.000
Error	0.001	80	8.51E-006		
Total	0.774	96			

df- degrees of freedom

3.3.7 Activity of arginase

In the present study the activity of arginase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant variations (P<0.05) compared with control group (Fig 3.7 and Table 3.12) (One-way ANOVA followed by Tukey's test). A statistically significant decrease in arginase activity (P<0.05) was observed in liver, kidney and gills of the treated groups compared to control. The muscle showed no or very little arginase activity.

Figure3.6 Arginase activity in the various tissues exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA)

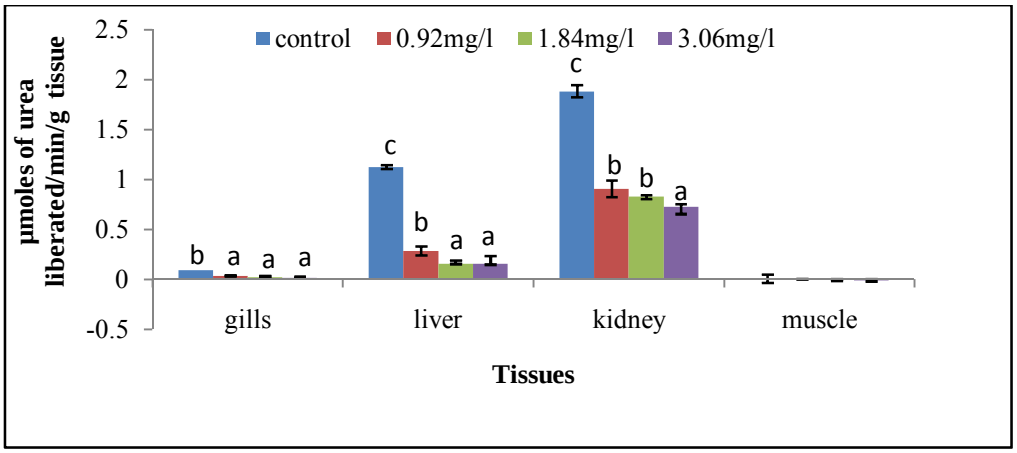

Table 3.12 Effect of exposure to different concentrations of Cadmium chloride for 7 days on arginase activity (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA)

Tissues	Arginase activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.096 ± 0.020^{b}	0.036 ± 0.0075^{a}	0.027 ± 0.0067^{a}	0.0198 ± 0.0086^{a}
Liver	1.13 ± 0.058^{c}	0.286 ± 0.045^{b}	0.163 ± 0.026^{a}	0.158 ± 0.078^{a}
Kidney	1.89 ± 0.041^{c}	0.909 ± 0.085^{b}	0.830 ± 0.014^{b}	0.73 ± 0.021^{a}
Muscle	0.0102 ± 0.0034^{b}	0.0044 ± 0.0010^{a}	0.00328 ± 0.0006^{a}	0.0024 ± 0.0003^{a}

Values are expressed in µmoles of urea formed/min/g tissue

Two factor ANOVA (Table 3.13) revealed that there was a significant decrease ($P<0.05$) in arginase activity in all the cadmium chloride treated experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 3.14) revealed that there was significant difference ($P<0.05$) between control and 0.92 mg/l, 1.84 mg/l, 3.06 mg/l cadmium chloride treated groups and between 0.92 mg/l and 1.84 mg/l, 0.92 mg/l and 3.06 mg/l. Between 1.84 mg/l and 3.06 mg/l exhibited no significant

difference. Multiple comparison tests also (Table 3.15) revealed that there was a significant difference between tissues except between gills and muscle.

Table3.13 Two-factor ANOVA for arginase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	2.431	3	0.810	578.200	0.000
Between tissues	9.106	3	3.035	2166.056	0.000
concentration * tissues	2.117	9	0.235	167.878	0.000
Error	0.045	32	0.001		
Total	21.123	48			

df-degrees of freedom

Table 3.14 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters					
	ALT	AST	GDH	Protein	Free amino acids	Arginase
Control Vs 0.92mg/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs 1.84mg/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs 3.06mg/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
0.92mg/l Vs 1.84mg/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.008[a]
0.92mg/l Vs 3.06mg/l	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
1.84mg/l Vs 3.06mg/l	0.000[a]	0.000[a]	0.002	0.000[a]	0.000[a]	0.287[d]

The values are significant at a=P<0.05 and not significant at d

Table 3.15 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters					
	ALT	AST	GDH	Protein	Free aminoacid	Arginase
Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.063[d]
Liver Vs Kidney	0.996[d]	0.000[a]	0.950[d]	0.000[a]	0.000[a]	0.000[a]
Liver Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Kidney Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.002[a]	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

3.3.8 Plasma ammonia

One-way ANOVA followed by Tukey's test showed that there was significant ($P<0.05$) increase in the plasma ammonia in all the treated groups compared to control (Fig 3.8 and Table 3.21). The ANOVA table is shown below (Table 3.16).

Figure3.7 Levels of Ammonia in the plasma of *O. mossambicus* exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

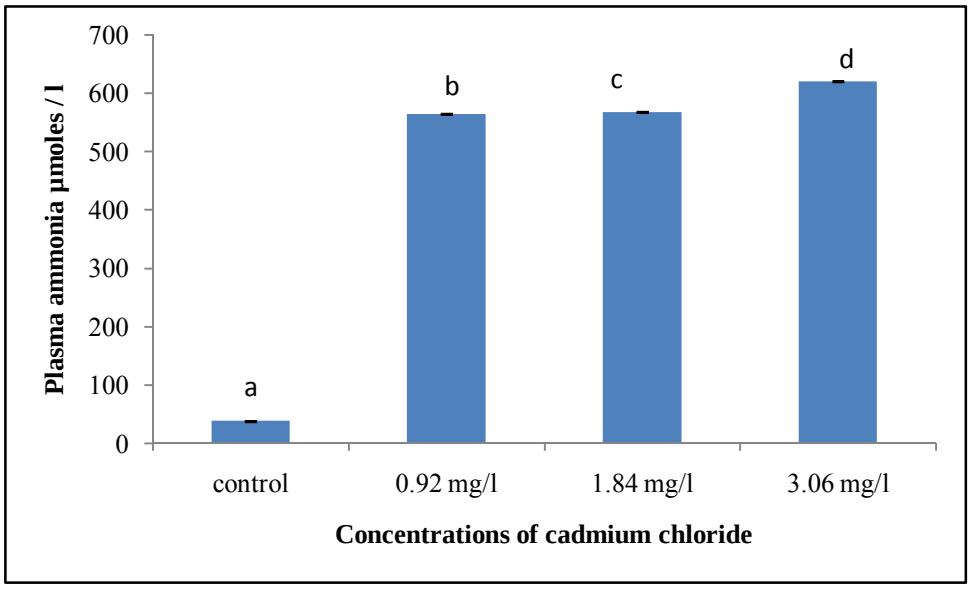

Table 3.16 One way ANOVA for plasma ammonia

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	3039.341	3	1013.114	7453.933	0.000
Within concentration	2.718	20	0.136		
Total	3042.060	23			

df-degrees of freedom

3.3.9 Ammonia excretion

O. mossambicus exposed to varying sub lethal concentration of cadmium chloride exhibited no significant ($P<0.05$) variations in the rates of excretion of ammonia compared to control (Fig 3.9 and Table 3.21). One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement and the table is shown below (Table 3.18).

Figure3.8 Rates of ammonia excretion by *Oreochromis mossambicus* exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

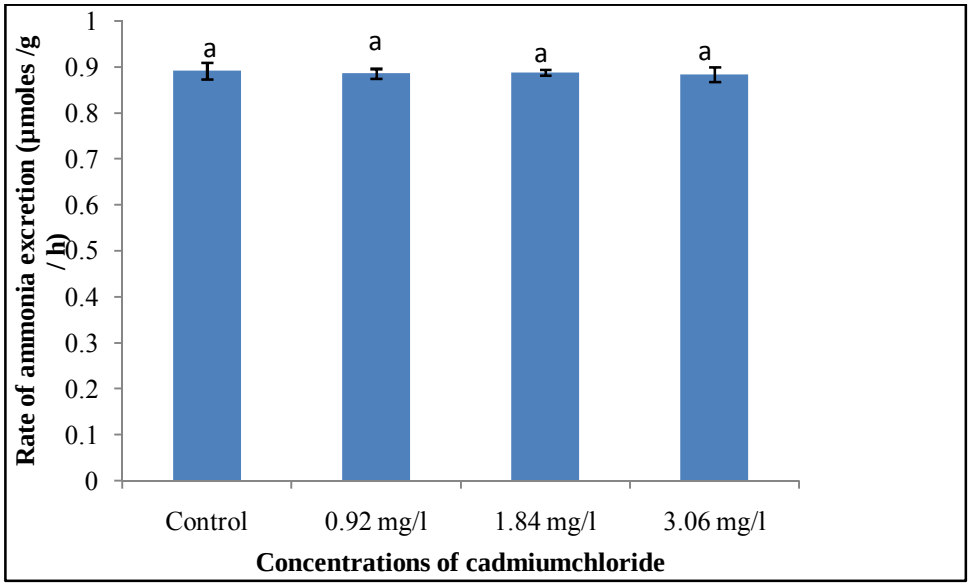

Table 3.17 One way ANOVA for ammonia excretion

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.000	3	0.000	0.319	0.812
Within concentration	0.004	20	0.000		
Total	0.004	23			

df-degrees of freedom

3.3.10 Oxygen consumption

One-way ANOVA followed by Tukey's test showed that there was significant decrease ($p<0.05$) in the rate of oxygen consumption of fish treated with cadmium chloride compared to control group (Fig 3.10 and Table 3.21). The ANOVA table is shown below (Table 3.19).

Figure3.9 Figure Rates of oxygen consumption by *Oreochromis mossambicus* exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA)

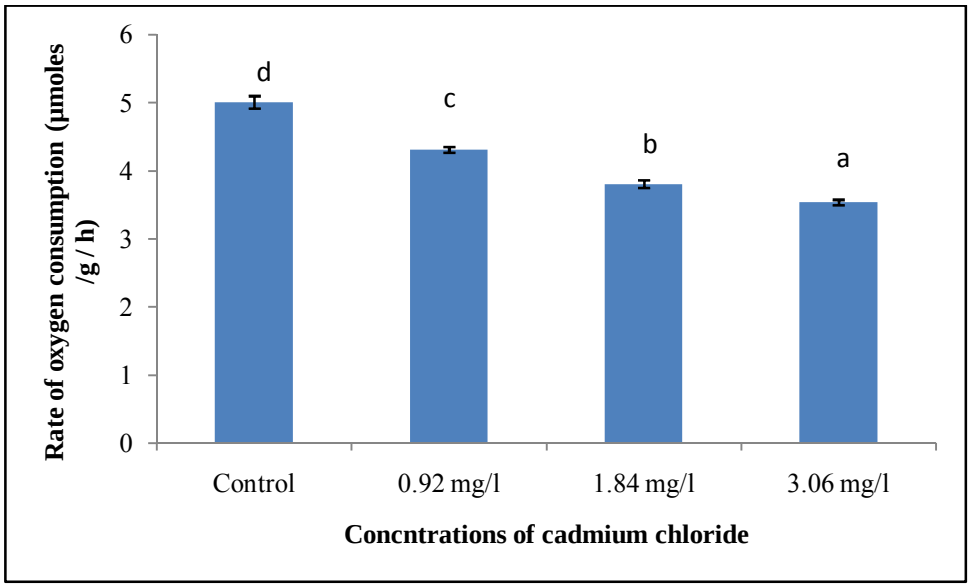

Table 3.18 One way ANOVA for rates of oxygen consumption by fish

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	7.532	3	2.511	672.580	0.000
Within concentration	0.075	20	0.004		
Total	7.607	23			

df- degrees of freedom

3.3.11 Ammonia quotient

O. mossambicus exposed to varying sub lethal concentration of cadmium chloride exhibited significant ($P<0.05$) increase in the ammonia quotient (Fig 3.11 and Table 3.21). One-way ANOVA followed by Tukey,s test has been carried out to ascertain the statement and the table is shown below (Table 3.20).

Figure 3.10 The Ammonia quotient of *Oreochromis mossambicus* exposed to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

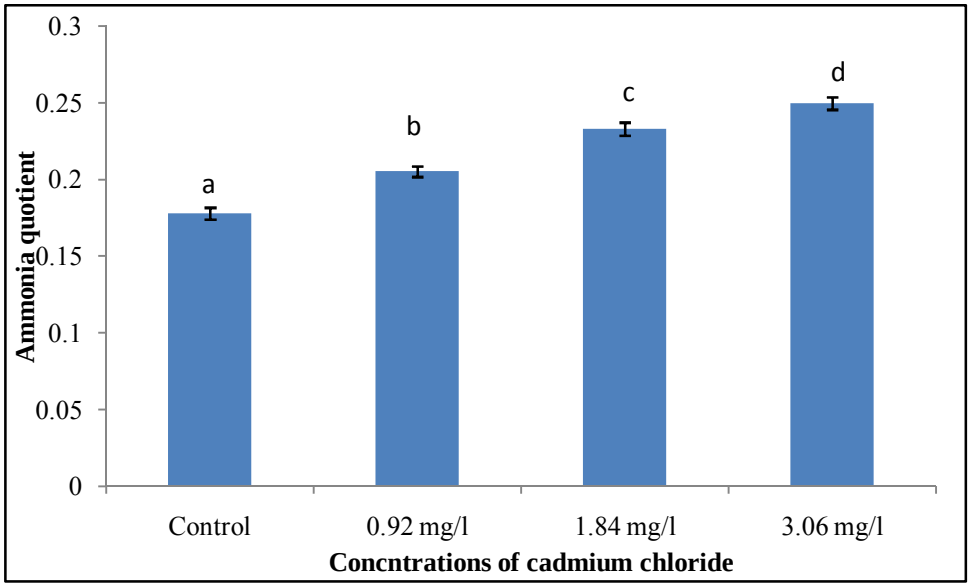

Table3.19 One way ANOVA for ammonia quotient

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.018	3	0.006	380.337	0.000
Within concentration	0.000	20	0.000		
Total	0.018	23			

df- degrees of freedom

Table 3.20 Effect of exposure to different concentrations of cadmium chloride for 7 days on plasma ammonia, ammonia excretion, oxygen consumption and ammonia quotient of *O.mossambicus*. Values in the same raw with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

Parameters	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06 mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Plasma ammonia (µmol/l)	38.22 ± 0.51^a	564.88 ± 0.44^b	568.68 ± 0.34^c	620.9 ± 0.42^d
Ammonia Excretion (µ moles/g/ h)	0.891 ± 0.018^a	0.8854 ± 0.011^a	0.8872 ± 0.0006^a	0.8835 ± 0.016^a
Oxygen Consumption (µmoles/g/h)	5.008 ± 0.093^d	4.311 ± 0.043^c	3.807 ± 0.055^b	3.537 ± 0.0041^a
Ammonia quotient	0.18 ± 0.004^a	0.21 ± 0.0035^b	0.23 ± 0.0043^c	0.25 ± 0.0041^d

3.4 Discussion

The present study demonstrated that the fish *Oreochromis mossambicus* exposed to sub-lethal concentrations of cadmium chloride (0.92 mg/l, 1.84 mg/l, and 3.06 mg/l (which corresponds to 1.876 mg/l, 1.128 mg/l and 0.564 mg/l Cd^{++} respectively) for 7 days displayed a significant decrease ($p<0.05$) in the level of protein in the gills, liver, kidney and muscle than the control. Proteins in an animal are being constantly degraded and re-synthesized from the free amino acid pool in tissue. A dynamic steady state always prevails between these two opposite processes of protein catabolism and anabolism. During stress conditions the balance between anabolism and catabolism will be impaired. Metabolism shifts towards a state of higher catabolism and the tissue proteins may undergo increased proteolysis. Reduction in the protein content in the tissues suggests its increased degradation in to amino acids. The amino acids may be fed in to the citric acid cycle through aminotransferases to cope up with the high energy demand posed by the heavy metal stress. The increased free amino acid pool (Bayne *et al*., 1981) can be used for ATP production by transamination reactions or gluconeogenic pathway. Tissue protein content has been suggested as an

indicator of xenobiotic-induced stress in aquatic organisms (Singh and Sharma, 1998). Under conditions of stress many organisms mobilize proteins as an energy source (Gilles, 1970). The result stands in good agreement with the observation reported by Almeida *et al*. (2001), who observed a reduction in the level of proteins in the liver and muscle of *Oreochromis niloticus* exposed to cadmium. Similar observations have been reported by Ramalingam and Ramalingam (1982). Additionally Kumari *et al*. (2010) observed a decrease in protein level in *Labeo rohita* treated with copper. A decrease in the protein content was also found in the hepatopancreas of edible crab *Scylla serrata* exposed to cadmium and the gills, liver, kidney, muscle, and intestine of the common carp exposed to mercury (Suresh *et al*., 1991; Reddy and Bhagyalakashmi, 1994).

A significant increase in the levels of free amino acids was observed when *Oreochromis mossambicus* exposed to sub-lethal concentrations of cadmium chloride for 7 days. Among the organs studied, liver showed highest increase because it is the major site of amino acid catabolism. The decline in total protein content and the simultaneous increase in free amino acids in the tissues studied indicate the activation of protein catabolism to counteract the Cd^{++} induced toxic stress. The free amino acids are mobilized in order to cope with the extra energy demands under stress conditions (De Smet and Blust, 2001). The increased free amino acids can be utilized for energy production by feeding them in to the citric acid cycle through aminotransferase reaction. This suggests that the increased protein breakdown is a functional response to deal with the extra energy requirements to cope with cadmium ion stress (Reddy and Bhagyalakashmi, 1994). De Smet and Blust (2001) also observed similar increase in free amino acids in common carp *Cyprinus carpio* exposed to cadmium.

The transaminases are known to play an important role in the utilization of amino acids for the oxidation and/or for gluconeogenesis (Rodwell, 1988) while

GDH, a mitochondrial enzyme, catalyzes the oxidative deamination of glutamate generating α-ketoglutarate, an important intermediate of the citric acid cycle. Significant increase ($p<0.05$) in the activities of alanine aminotransferase and aspartate aminotransferase were observed in the tissues of *O. mossambicus* exposed to cadmium chloride for 7 days. The alteration in aminotransferase activities indicates changes in energy metabolism in response to an enhanced energy demand to compensate the stress situation. De Smet and Blust (2001) indicated that elevated activities of AST and ALT in liver and kidney of *Cyprinus carpio* following cadmium exposures were due to increased protein breakdown to deal with the energy requirement. It was shown that these enzymes are influenced by metals before their accumulation in liver (Oner *et al*., 2009). Bakthavathsalam and Reddy (1984) reported significant increase in the levels of AST and ALT in the liver of heavy metal exposed fish, *Anabas testudineus* (Bloch). They suggested that the increase in the liver AST and ALT was due to elevated aminotransferase activity to overcome the stress. The increased activities of the two major aminotransferases AST and ALT in fish may thereby enhance transamination for the channeling of free amino acids into the citric acid cycle and/or to favour gluconeogenesis (Jurss and Bastrop, 1995). Similar increases in alanine and aspartate aminotransferases have also been found in heart, gills, and liver of cadmium-exposed stripped mullet *Mugil cephalus* (Hilmy *et al*., 1985), in gills, kidney and muscle of mercury-exposed carp (Suresh *et al*., 1991), in the hepatopancreas of cadmium exposed edible crab *S. serrata* (Reddy and Bhagyalakashmi, 1994) and in gills of copper-exposed carp (Karan *et al*., 1998).

In vertebrate cells, L-glutamate dehydrogenase (GDH; EC 1.4.1.3), primarily associated to the mitochondrial matrix, has a key regulatory function in cellular metabolism in controlling levels of ammonia and glutamate. It catalyses the reversible oxidative deamination of L-glutamate to α-ketoglutarate and

ammonia through reduction of NAD^+ or $NADP^+$ (Ciardiello, *et al*., 2000). In the present study an increased activity of GDH observed in the liver, kidney and muscle tissues may be because of an increase in glutamate oxidation, resulting in increase in ammonia production and α- ketoglutarate formation at the expense of NAD^+. The increase in the activity of GDH was found to be most prevalent in liver and kidney. This indicates significant role of these organs in the deamination. According to Almeida *et al*. (2001) cadmium acts as a stressor leading to metabolic alterations similar to those observed in starvation. Sanchez-Muros *et al.* (1998) reported that GDH activity increased in the liver during starvation, a fact which agrees with the enhanced availability of amino acids, of a tissue origin. During Cd^{++} exposure an elevation in the free amino acid content is observed. Similarly an enhancement in the activity of GDH due to carbofuran intoxication was observed in liver and muscle tissues of *C. batrachus* (Begum, 2004). Sastry and Subhadra (1985) also reported an increase in the activity of GDH in liver, kidney and muscle tissues of cadmium treated fish. Kumar *et al.* (2011) observed significant increase in the activity of GDH in *Channa punctatus* and *Clarias batrachus* on treatment with cypermethrin. The activity of GDH was found to be lower in gills than in the other tissues of fish (Walton and Cowey, 1977; Fields *et al.,* 1978; Hulbert *et al.,* 1978 a, b; Storey *et al.,* 1978).

The significant increase in the activities of ALT, AST and GDH in the tissues of fish on treatment with cadmium chloride as observed in the present investigation could be due to incorporation of keto acids into the TCA cycle. Cadmium chloride induces the generation of glutamate through tissue transamination followed by their conversion to α– ketoglutarate through oxidative deamination to favour gluconeogenesis or energy production (Prashanth and Neelagund, 2008).

Arginase is ubiquitous in fish tissues, with highest activities found in liver and kidney tissue (Cvancara, 1969; Portugal and Aksnes, 1983; Singh and Singh, 1988; Korte *et al*., 1997).

Arginase (L-arginine ureohydrolase) (EC 3.5.3.1) is a manganese-requiring enzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea (Christianson and Cox, 1999). In the present study a significant decrease ($P<0.05$) in arginase activity was observed in the tissues of *O.mossambicus* when exposed to cadmium chloride for 7 days. The decrease in arginase activity was mainly due to inhibition of the enzyme by cadmium ions. The decrease in arginase activity observed in the present study was supported by the findings of Tormanen (2006), who observed the inhibition of rat liver and kidney arginase by cadmium ion. Similarly the inhibition of rat liver and kidney arginase by copper and mercury ions has been reported by Tormanen (2001). Gonzalez *et al*. (2011) also observed a decrease in the arginase activity in the early stages of *Danio rerio* treated with pesticide malathion and a kraft pulp mill effluent.

Fish have a remarkable capacity to use proteins as an energy source (Van Waarde, 1983), and ammonia is the major end-product of nitrogen metabolism. A significant elevation of plasma ammonia was observed in fish exposed to sub lethal concentrations of cadmium chloride for 7 days. The reason for this seems to be a combined effect of elevated, stress induced, ammonia production and an unchanged excretion despite an elevated plasma-to-water gradient (Beaumont *et al*., 2000; Grosell *et al*., 2002; Taylor *et al*., 1996; Grosell *et al*., 2004). An increase in ammonia production as a result of metal induced stress together with an impaired ability to excrete ammonia across the gills is the typical response to metal exposure in freshwater fish and leads to elevated plasma ammonia levels (Grosell *et al*., 2004). Increased ammonia production can arise from a general corticosteroid-mediated stress response that includes increased protein catabolism

and gluconeogenesis (Freeman and Idler, 1973). The present finding was supported by Beaumont *et al*., 1995; Wang *et al*., 1998; Wilson and Taylor, 1993b, they too observed similar increase in plasma ammonia levels under exposure to copper. Lin and Randall, (1990); Booth *et al.* (1988) have also reported an increased plasma ammonia levels on exposure to high pH (pH 9.9) and increased aluminium levels.

No significant change in ammonia excretion was observed compared to control in *O.mossambicus* treated with cadmium chloride for 7 days. The reason for this seems an impaired ability to excrete ammonia across the gills. This is the typical response to metal exposure (Grosell *et al.,* 2004). De Boeck *et al*. (1995) observed similar results when treated with copper in the common carp, *Cyprinus carpio.*

Oxygen consumption of aquatic animals is a very sensitive physiological process and therefore, alteration in the respiratory activity is considered as an indicator of stress of animals exposed to heavy metals. The change in rate of oxygen consumption is a good index of the metabolic capacity of an organism to face environmental stress. A significant decrease in the oxygen consumption was observed when *Oreochromis mossambicus* exposed to sub-lethal concentrations of cadmium chloride for 7 days. The alteration in the normal respiratory metabolism is due to its intimate contact with water contaminated with Cd^{++} which decreases the oxygen diffusing capacity of the gills (Khan *et al*., 2000; Rao and Ramamurthy, 1996). Metals may induce various disturbances in fish gills. Excessive secretion and coagulation of mucus impair gas exchange across the secondary lamellae epithelium (Part and Lock, 1983; Handy and Eddy, 1989). Metal accumulated in the epithelium may also reduce oxygen uptake (Youson and Neville, 1987). Metal-induced gill lesions such as thickening and lifting of respiratory epithelium result in an increase of diffusion distance between the

water and blood which makes oxygen absorption difficult (Matthiessen and Brafield, 1973; Evans *et al*., 1988; Benedetti *et al*., 1989; De Boeck *et al*., 1995; Dalzell and MacFarlane, 1999). Jones *et al.* (1947) also reported decline of oxygen consumption in fish, *Gasterosteus aculeatus* when exposed to mercury chloride, copper sulphate and lead nitrate solution. Dutt *et al.* (1989) have reported mercuric chloride and methyl mercuric chloride have lowered oxygen consumption in cat fish, *Mystus vittatus*.

Proteins play a central role in the energy production during the stress caused by toxicants. Most of the nitrogenous end products of freshwater fish originate from protein catabolism, with ammonia as the principal end product. The contribution of protein catabolism to the total energy production of the fish can be assessed by determination of the ammonia quotient (AQ = mole to mole ratio of ammonia excreted to oxygen consumed (De Boeck *et al*., 1995; Brett and Zala, 1975; Kutty, 1972, 1978; Kutty and Peer Mohamed, 1975; Van Waarde, 1983). A significant increase in the ammonia quotient was observed in *O.mossambicus* when exposed to cadmium chloride for 7 days. This was supported by De Boeck *et al*. (1995) who also observed similar increase in ammonia quotient in common carp when treated with copper. Thus, although oxygen consumption is reduced by cadmium chloride exposure, protein catabolism appears to remain constant, or is at least less affected and becomes relatively more important. The increase in ammonia production may be the result of combined action of protein catabolism and purine nucleotide cycle.

The rate of protein breakdown is acute as evident in this study. This is supported by De Smet and Blust (2001) who reported that adult females of *Cyprinus carpio* exposed to either acute or chronic concentrations of cadmium showed an increase in protease activity, free amino acids, and aminotransferase activity in the liver and kidney. *C. carpio* exposed for 14 days to cadmium showed

a 60–98% increase in liver transaminase activities, thus indicating that cadmium might induce the activation of the amino acid catabolism (De la Torre *et al*., 1999).

In conclusion, the present study illustrates the impact of Cd^{++} on the catabolism of proteins and amino acids, in *Oreochromis mossambicus*. Proteins are known to play dominant role in accomplishing the immediate energy demand in recovering from the stress. The Cd^{++} toxicity in the fish *Oreochromis mossambicus* enhances the catabolism of proteins to handle the extra energy demand. The elevation in free amino acid content, ALT, AST, GDH and ammonia in plasma along with a reduction in total protein content of tissues indicate a boost in protein catabolism. The significant increase in AQ in treated fish indicates a marked increase in the catabolism of proteins during Cd^{++} induced stress.

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Chapter 4

Effect of Cadmium Ion (Cd^{++}) on the Purine Catabolism of *Oreochromis mossambicus* (Peters)

4.1 Introduction

Heavy metals have been used in many different areas for thousands of years. Lead (Pb) was one of the earliest metals discovered by the human race and was in use by 3000 B.C. The ancient Romans used lead (Pb) for making water pipes, building materials and pigments for glazing ceramics. In ancient Rome, lead acetate was used to sweeten old wine. Mercury was allegedly used by the Romans as a salve to alleviate teething pain in infants, and was later (from the 1300s to the late 1800s) employed as a remedy for syphilis. Cadmium pigments were discovered around 1820 and first commercialized for artists' use by the mid 1840's. Although adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues and is even increasing in some areas. Heavy metal input into the environment, either terrestrial or aquatic, is an important aspect of environmental pollution. Heavy metals such as cadmium, zinc, mercury, chromium, copper, etc., cause heavy pollution, particularly of the ponds, lakes, and riverine systems in zones

affected by effluents released from industries. Among animal species, fish are the inhabitants that cannot escape the detrimental effects of these pollutants (Basha and Rani, 2003).

Cadmium occurs naturally in ores together with zinc, lead and copper. Among divalent metals, cadmium (Cd) is one of the most hazardous bio toxics (Hellawell, 1986). EU cadmium usage has decreased considerably during the 1990s, mainly due to the gradual phase-out of cadmium products other than Ni- Cd batteries and the implementation of more stringent EU environmental legislation. Notwithstanding these reductions in Europe, however, cadmium production, consumption and emissions to the environment worldwide have increased dramatically during the 20^{th} century. Cadmium containing products are rarely re-cycled, but frequently dumped together with household waste, thereby contaminating the environment, especially if the waste is incinerated. Natural as well as anthropogenic sources of cadmium, including industrial emissions and the application of fertilizer and sewage sludge to farm land, may lead to contamination of soils and to increased cadmium uptake by crops and vegetables, grown for human consumption.

Most of the toxic metals are carcinogenic in nature. Among those metals known or suspected to have carcinogenic activity is cadmium, which has been reported to cause tumours in rats and mice (Lucis *et al*., 1972). It may have similar harmful consequences in man (Kipling and Waterhouse, 1967). Inhalation of high levels of cadmium oxide fumes or dust is intensely irritating to respiratory tissue and acute high level exposures can be fatal. Cadmium and certain cadmium compounds are listed by International Agency for Research on Cancer (IARC) as carcinogenic.

Cadmium is well known for its toxic effect on aquatic organisms (Pratap and Bonga, 1990). When present in the water column, cadmium readily accumulates in various tissues, especially in the gills, liver, kidneys and gonads of fish (Bentley, 1991), causing several physiological disturbances. The evaluation of the effects of cadmium on fish is of particular interest since fish are crucial components of aquatic ecosystems, playing a major role in the food chain. In fish the metal has adverse effect on growth and reproduction and causes osmoregulatory stress (Roch and Maly, 1979; Giles, 1984; Klaverkamp and Duncan, 1987; Pratap *et al*., 1989; verbost *et al*., 1987). Cadmium-exposed fish may show skeletal deformities, alterations in several enzymatic systems, including those involved in neurotransmission, transepithelial transport and intermediate metabolism, alteration of mixed function oxidase activities, abnormal swimming, changes in individual and social behaviour and metabolic disorders (Scott and Sloman, 2004; Wright and Welbourn, 1994). The exposure of *Cyprinus carpio* to sub lethal cadmium concentrations results in gill epithelium damage (Ferrari *et al*., 2009), which may lead to alterations in ion and gas exchange and energy balance. At the cellular level, heavy metals can cause a number of adverse effects, such as alterations in the communication between cells and in the interaction with intracellular signal transduction proteins, which may in turn lead to alterations in cell growth and differentiation (Goering, *et al.*, 1995). More recently, it has been shown that sub lethal cadmium also causes important changes in the swimming activity of *C. carpio* in captivity (Eissa *et al*., 2006, 2010). Among the processes that may be affected by the exposure to heavy metals are the metabolic rate, the excretion of ions (e.g., ammonium), respiration, food consumption and growth rates (Alves *et al*., 2006; Hashemi *et al*., 2008; Wilson *et al*., 1994).

To assess the effects of a toxic compound on an aquatic organism, responses to sub lethal levels of this compound should be studied. Sub lethal

concentrations of toxic compounds may cause biochemical, physiological, morphological and genetic changes, affecting the development, growth and reproduction. Before changes in viability occur, it is likely that changes in the energy status of the organism appear (De Boeck *et al*., 1995).

Although there is now a widespread knowledge of the harmful effects of the uptake of heavy metals by living organisms, the details of the biochemical aspects of such pollution are, as yet, limited. Reports on the effects of heavy metals on purine catabolism of fish are scarce. Purine metabolism is an essential biochemical pathway that is conserved across a wide-range of phyla and is considered a likely candidate for the most ancient metabolic pathway on the planet (Caetano-Anolles *et al*., 2007). In animals, the end products of purine degradation vary greatly from species to species. Purine catabolism is a way to excrete nitrogen from nucleic acids. In fishes small amount of urea is excreted through uricolytic pathway. The alterations in the catabolism of these compounds results in impaired excretion of nitrogen from purine nucleotides. The objective of this study was to investigate the effect of Cd^{++} on purine catabolism of *Oreochromis mossambicus*.

4.2 Materials and Methods

Collection, maintenance, acclimation, experimental design, Preparation of tissue and serum samples were same as explained in detail in chapter 3, section 3.2

4.2.1 Methods used for the biochemical analysis

4.2.1.1 Assay of AMP deaminase (EC 3.5.4.6)

AMP deaminase was assayed by the method of Pedersen and Berry (1977)

Reagents

1. 50 mmol Sodium citrate buffer pH 6.2
2. Ammonia stock standard (0.038 mmol)
3. Sodium AMP (19 mmol)
4. Phenol niroprusside solution-15.25 g of crystalline phenol and 708 mg of sodium nitroprusside in 250 ml water
5. Alkaline hypochlorite solution.
6. 1 mol/l KCl pH 7.4
7. 1 mmol/l 2- mercapto ethanol

Procedure

5% homogenates of gills, liver, muscle and 1% homogenate of kidney tissue were prepared in 50 mmol/l sodium citrate buffer pH 6.5, 1mmol/l KCl pH 7.4 at 4^{o}C and 1 mmol/l 2- mercapto ethanol. Then the homogenate were centrifuged at 10000 g for 5 minutes.

The supernatant obtained was used as the enzyme source. 1 ml of sodium citrate buffer pH 6.2 was pipetted out into test tubes labelled 'test' and 'control'. Added 0.2 ml of the sample into the test tube labelled 'test' and equilibrated the tubes at 37 ^{0} C for 10 minutes. Then the substrate AMP was added to the 'test' and 'control' tubes. The tubes were incubated at 27^{o}C for 30 minutes. After incubation, 1 ml phenol nitroprusside solution was added to both 'control' and 'test' tubes. Then 0.2 ml sample was added to the 'control tube'. 1ml of alkaline hypochlorite solution was added to each tube and kept at 37^{o}C for 10 minutes. The absorbance was measured at 630 nm in a spectrophotometer against a blank. The blank preparation was the same as that

of the test, except that the corresponding volumes of distilled water substitute the sample and the substrate. The AMP deaminase activities were expressed as μ moles of ammonia liberated / min / mg protein.

4.2.1.2 Assay of xanthine oxidase (EC 1.1.3.22)

Xanthine oxidase was assayed by the method of Bergmeyer *et al*. (1974).

Reagents

1. 50 mM potassium phosphate Buffer, pH 7.5
2. 0.15 mM xanthine solution-100 ml is prepared by dissolving xanthine in a minimal volume of sodium hydroxide. Add approximately 90 ml of distilled water. Adjusted pH to 7.5 at 25^{o}C with either 1M NaOH or 1M HCl. Diluted to a final volume of 100 ml.

Procedure

5% homogenates of gills, liver, muscle and 1% homogenate of kidney tissue were prepared in 0.33 M sucrose solution and centrifuged at 1000 g for 15 minutes. The supernatant obtained was used as the enzyme source. Pipetted out 1.9 ml phosphate buffer and 1.0 ml xanthine solution into the quartz cuvette and mixed by inversion and equilibrated to 25 ^{o}C. Then the absorbance at 290 nm was monitored until constant, using a spectrophotometer. Then 0.2 ml sample was added and immediately mixed by inversion and the increase in absorbance was recorded for approximately 5 minutes. The milli molar extinction coefficient of uric acid is 12.2 at 290 nm. The enzyme activity was calculated as μmoles of uric acid produced / minute / mg protein.

4.2.1.3 Assay of uricase (EC 1.7.3.3)

Uricase was assayed by the method of Mahler *et al*. (1955)

Reagents

1. Tris HCl buffer pH 8.0
2. 40 µM uric acid
3. 50 mmol sodium borate buffer, pH 9.0

Procedure

5% homogenate of liver tissue was prepared in Tris-HCl buffer pH 8.0 and centrifuged at 1000 g for 20 minutes. The supernatant obtained was used as the enzyme source. The reaction mixture consisted of 0.1 ml 40 µM uric acid, 0.2 ml enzyme source, 2.82 ml of 50 mmol sodium borate buffer, pH 9.0. Immediately mixed by inversion and recorded the decrease in absorbance at 293 nm with time for approximately 5 minutes. Millimolar extinction coefficient of uric acid at 293 nm is 12.6. The enzyme activity was calculated as micromoles of uric acid converted to allantoin / minute / mg protein.

4.2.1.4 Assay of allantoinase (EC 3.5.2.5)

Allantoinase was assayed by the method of Valentine *et al*. (1962)

Reagents

1. Tris HCl buffer pH 8.0
2. 15 mM allantoin
3. 0.5 N HCl
4. 15 mM phenyl hydrazine
5. 0.5 M potassium phosphate buffer

Procedure

5% homogenate of liver tissue was prepared in Tris HCl buffer pH 8.0 and centrifuged at 30,000 g for 30 minutes. The supernatant obtained was used as the enzyme source. The reaction mixture consists of 2 ml 50 mM Tris HCl buffer pH 8.0, 1.9 ml of 15 mM allantoin and 0.1ml of enzyme extract. The above mixture was incubated for 10 minutes at room temperature. After incubation 0.5 ml of 0.5 N HCl was added and kept it in a boiling water bath for 2 minutes to hydrolyse allantoic acid produced to glyoxylate. Then the tubes were cooled in an ice bath. 2ml aliquot was pipetted out in to a quartz cuvette containing 1ml of 15 mM phenyl hydrazine in 0.5 M potassium phosphate buffer (pH 7.0). The concentration of glyoxylate in the aliquot was measured from the initial rate of formation of the phenyl hydrazone. Absorption was measured at 322.5 nm. The enzyme activity was calculated as micromoles of glyoxylate produced / minute / mg protein.

4.2.1.5 Estimation of serum urea

The method employed was that of Varley (1976).

Reagents

1. Tri chloro acetic acid (TCA) (10%)
2. Stock Diacetyl monoxime (25 g/l)
3. Stock thiosemicarbazide (2.5g /l)
4. Acid ferric chloride solution: added 1.0 ml sulphuric acid to 100 ml of ferric chloride solution.
5. Acid reagent: added 10 ml of ortho phosphoric acid, 80 ml of sulphuric acid and 10 ml acid ferric chloride solution to one litre of water. The contents were then mixed well.

6. Colour reagent: To 300 ml of acid reagent, 200 ml of water, 10 ml stock diacetyl monoxime and 2.5 ml of thiosemicarbazide solution was added
7. Stock urea standard: 5 to 50 mmol/l of pure urea was taken which contained concentration ranging from 30- 300 mg/l

Procedure

In to a test tube 0.2 ml of serum, 1.0 ml of water and 1.0 ml of 10% TCA were added. The contents were mixed well and centrifuged. 0.2 ml of the supernatant was taken. To this added 3.0 ml of colour reagent. At the same time 0.2 ml of water as blank and 0.2 ml of standard urea solution were taken. All the tubes were heated in a boiling water bath for 20 minutes. The tubes were cooled to room temperature and the colour developed was then read at 520 nm within 15 minutes. The result was expressed as mg/dl of serum.

4.2.16 Estimation of uric acid in serum

Uric acid present in the serum was estimated by the method of Caraway (1963).

Reagents

1. Phospho tungstic acid reagent
2. 10% sodium carbonate
3. Standard uric acid: 100 mg uric acid and 60 mg lithium carbonate were taken. It was then dissolved in about 50 ml of distilled water. This was heated to about 60 °C to dissolve the uric acid completely. After cooling the solution was finally made up to 100 ml with water.
4. Working standard: Diluted 1ml of stock to 10 ml with water. 1ml of this solution contains 100 µg of uric acid.

Procedure

To 0.1 ml of the serum sample, 2.9 ml of water was added followed by 0.6 ml each of phosphotungstic acid and sodium carbonate. A blank was set up with 3.0 ml of distilled water. A set of standards were also treated in the same manner. The colour obtained was then read at 640 nm after 10 min. The values were expressed as mg/dl serum.

4.2.2 Statistical Analysis

The statistical analysis was carried out using the software SPSS 13.0 package. Two-way analysis of variance (ANOVA) was carried out to compare between different concentrations and also between tissues. If significant differences were revealed by the ANOVA test, Tukey's test was used to further elucidate which tissues and concentrations were significantly different. One-way ANOVA followed by Tukey's test was carried out for the comparison between different concentrations in each tissue. For determining the significant difference between different concentrations in blood and serum parameters One-way ANOVA followed by Tukey's test were done. Significance level (P value) was set at 0.05.

4.3 Results

4.3.1 Activity of AMP deaminase

The activity of AMP deaminase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant variations ($P<0.05$) compared with control group (Fig 4.1 and Table 4.1) (One-way ANOVA followed by Tukey's test). A statistically significant increase in AMP deaminase ($P<0.05$) was observed in gills, liver, kidney and muscle of the treated groups compared to control.

Figure 4.1 AMP deaminase activity in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA)

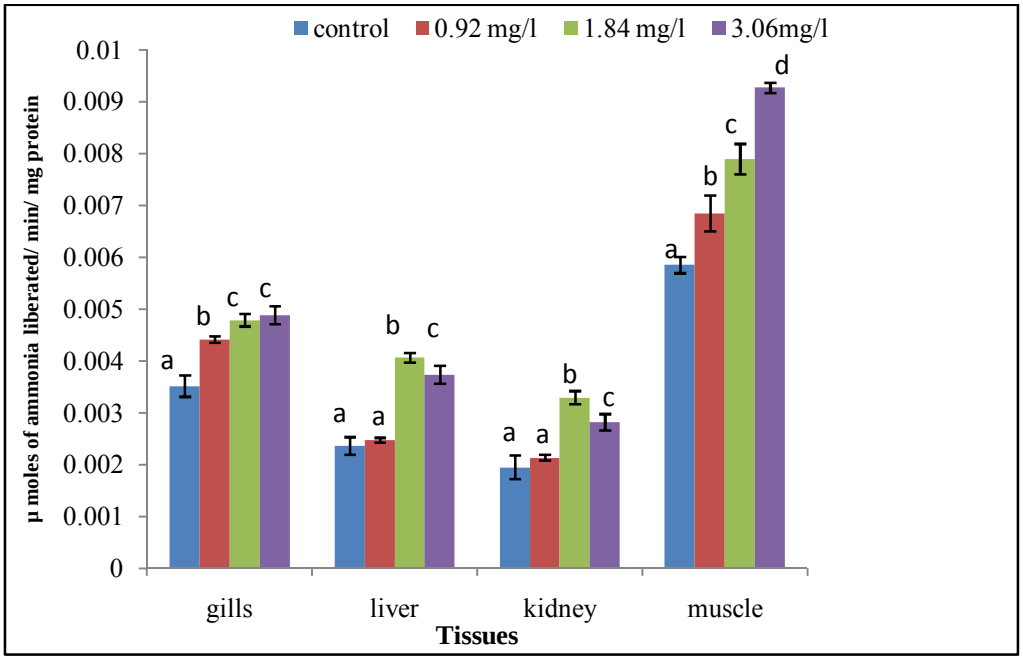

Table 4.1 Effect of exposure to different concentrations of Cadmium chloride for 7 days on AMP deaminase activity (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	AMP deaminase activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.0035 ± 0.0002^{a}	0.0044 ± 0.00006^{b}	0.005 ± 0.00012^{c}	0.0049 ± 0.00017^{c}
Liver	0.0024 ± 0.0002^{a}	0.0025 ± 0.00004^{a}	0.0041 ± 0.00009^{b}	0.004 ± 0.0002^{c}
Kidney	0.002 ± 0.00022^{a}	0.0021 ± 0.00006^{a}	0.0033 ± 0.00013^{b}	0.0028 ± 0.00016^{c}
Muscle	0.0059 ± 0.0002^{a}	0.007 ± 0.00035^{b}	0.0079 ± 0.0003^{c}	0.0093 ± 0.0001^{d}

Values are expressed as µ moles of ammonia liberated / min / mg protein.

Two factor ANOVA (Table 4.2) revealed that there was a significant increase ($P<0.05$) in AMP deaminase activity in all the cadmium chloride treated

experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 4.5) revealed that there was significant difference (P<0.05) between control and 0.92mg/l, 1.84mg/l, 3.06mg/l cadmium chloride treated groups and between 0.92mg/l and 1.84mg/l, 0.92mg/l and 3.06mg/l. Between 1.84mg/l and 3.06mg/l there was a significance at P<005. Multiple comparison tests also (Table 4.6) revealed that there was a significant difference between tissues. The highest activities were seen in muscle tissue followed by gills.

Table 4.2 Two-factor ANOVA for tissue AMP deaminase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	5.08E-005	3	1.69E-005	455.240	0.000
Between tissues	0.000	3	0.000	3083.532	0.000
concentration * tissues	1.51E-005	9	1.67E-006	44.940	0.000
Error	2.98E-006	80	3.72E-008		
Total	0.002	96			

df- degrees of freedom

4.3.2 Activity of xanthine oxidase

In the present study the activity of xanthine oxidase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant variations (P<0.05) compared with control group (Fig 4.2 and Table 4.3) (One-way ANOVA followed by Tukey's test). A statistically significant increase in xanthine oxidase activity (P<0.05) was observed in gills, liver, kidney and muscle of the treated groups compared to control.

Figure 4.2 xanthine oxidase activity in the various tissues exposed to different concentrations of Cadmium chloride. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

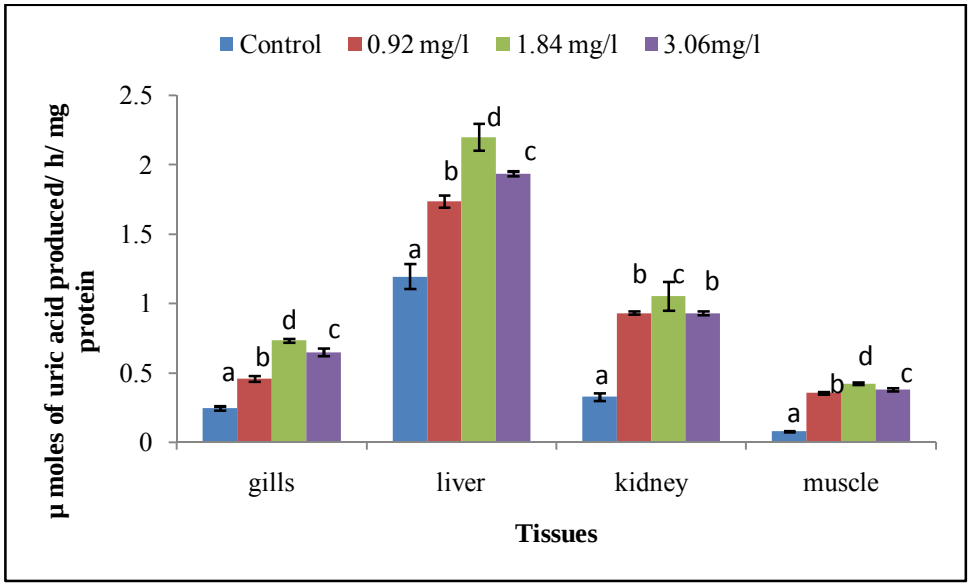

Table 4.3 Effect of exposure to different concentrations of Cadmium chloride for 7 days on xanthine oxidase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Xanthine Oxidase activity			
	Groups			
	Control	0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Gills	0.243±0.015^{a}	0.457±0.021^{b}	0.733±0.0141^{d}	0.648±0.027^{c}
Liver	1.194±0.091^{a}	1.735±0.044^{b}	2.199±0.096^{d}	1.935±0.017^{c}
Kidney	0.326±0.027^{a}	0.931±0.012^{b}	1.052±0.102^{c}	0.929±0.015^{b}
Muscle	0.078±0.005^{a}	0.355±0.009^{b}	0.422±0.0101^{d}	0.378±0.013^{c}

Values are expressed as µ moles of uric acid produced/mg protein/h

Two factor ANOVA (Table 4.4) revealed that there was a significant increase (P<0.05) in xanthine oxidase activity in all the cadmium chloride treated experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 4.5) revealed that there was significant difference (P<0.05) between control and 0.92mg/l, 1.84mg/l, 3.06mg/l cadmium chloride

treated groups and between 0.92mg/l and 1.84mg/l,0.92mg/l and 3.06mg/l. Between 1.84mg/l and 3.06mg/l there was a significance at P<005. Multiple comparison tests also (Table 4.6) revealed that there was a significant difference between tissues. The highest activities were seen in liver tissue followed by kidney.

Table 4.4 Two-factor ANOVA for tissue xanthine oxidase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	5.529	3	1.843	887.994	0.000
Between tissues	29.825	3	9.942	4789.709	0.000
concentration * tissues	0.954	9	0.106	51.074	0.000
Error	0.166	80	0.002		
Total	106.024	96			

df- degrees of freedom

Table 4.5 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters	
	AMP deaminase	xanthine oxidase
Control Vs 0.92mg/l	0.000[a]	0.000[a]
Control Vs 1.84mg/l	0.000[a]	0.000[a]
Control Vs 3.06mg/l	0.000[a]	0.000[a]
0.92mg/l Vs 1.84mg/l	0.000[a]	0.000[a]
0.92mg/l Vs 3.06mg/l	0.000[a]	0.000[a]
1.84mg/l Vs 3.06mg/l	0.019[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

Table 4.6 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters	
	AMP deaminase	xanthine oxidase
Gills Vs Liver	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]
Liver Vs Kidney	0.000	0.000[a]
Liver Vs Muscle	0.000[a]	0.000[a]
Kidney Vs Muscle	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

4.3.3 Activity of uricase

Uricase activity in the liver of *O. mossambicus* treated with cadmium chloride showed significant decrease ($P<0.05$) compared to control (Fig 4.3 and Table 4.7). One-way ANOVA followed by Tukey's test revealed a significant decrease at ($P<0.05$). A statistically significant decrease in uricase activity ($P<0.05$) was observed in 0.92mg/l, 1.84 mg/l and 3.06 mg/l concentrations compared to control.

Fig 4.3 Effect of different concentrations of cadmium chloride on uricase activity in the liver of *O. mossambicus*. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

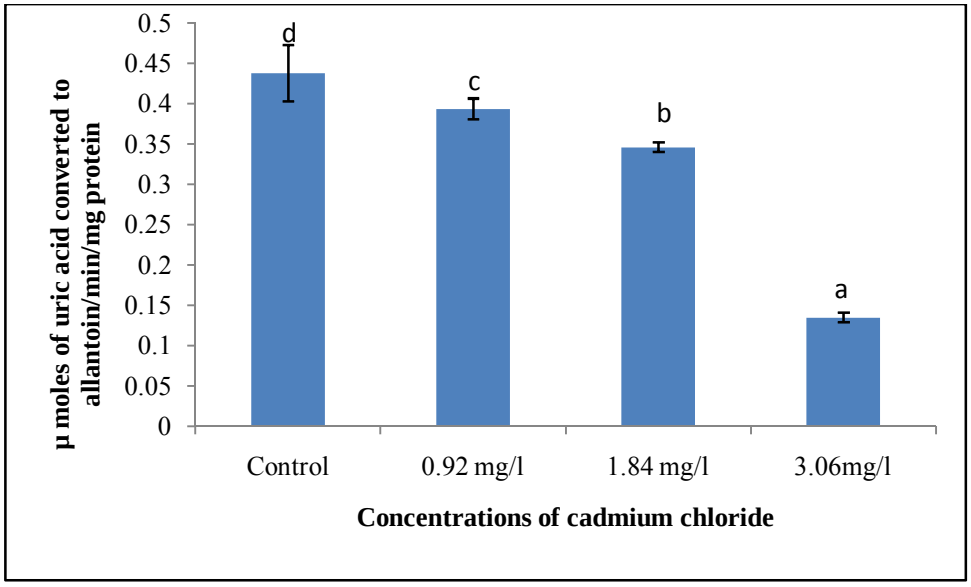

Table 4.7 Effect of exposure to different concentrations of cadmium chloride for 7 days on uricase activity (mean ± S.D) present in the liver of *O.mossambicus*. Values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

Group	Control	Concentrations of cadmium chloride		
		0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Uricase activity	$0.437759 \pm 0.034723^{d}$	$0.393592 \pm 0.012898^{c}$	$0.346142 \pm 0.006027^{b}$	$0.135045 \pm 0.006022^{a}$

Values are expressed as micromoles of uric acid converted to allantoin / minute / mg protein.

Table 4.8 One way ANOVA for uricase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	0.323	3	0.108	248.788	0.000
Within concentration	0.009	20	0.000		
Total	0.332	23			

df- degrees of freedom

4.3.4 Activity of allantoinase

In the present study the activity of allantoinase in the liver of *O. mossambicus* treated with cadmium chloride showed significant decrease ($P<0.05$) compared to control (Fig 4.4 and Table 4.9) One-way ANOVA followed by Tukey's test revealed a significant decrease at ($P<0.05$). A statistically significant decrease in allantoinase activity ($P<0.05$) was observed in 0.92mg/l , 1.84 mg/l and 3.06 mg/l concentrations compared to control.

Fig 4.4 Effect of different concentrations of cadmium chloride on allantoinase activity in the liver of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

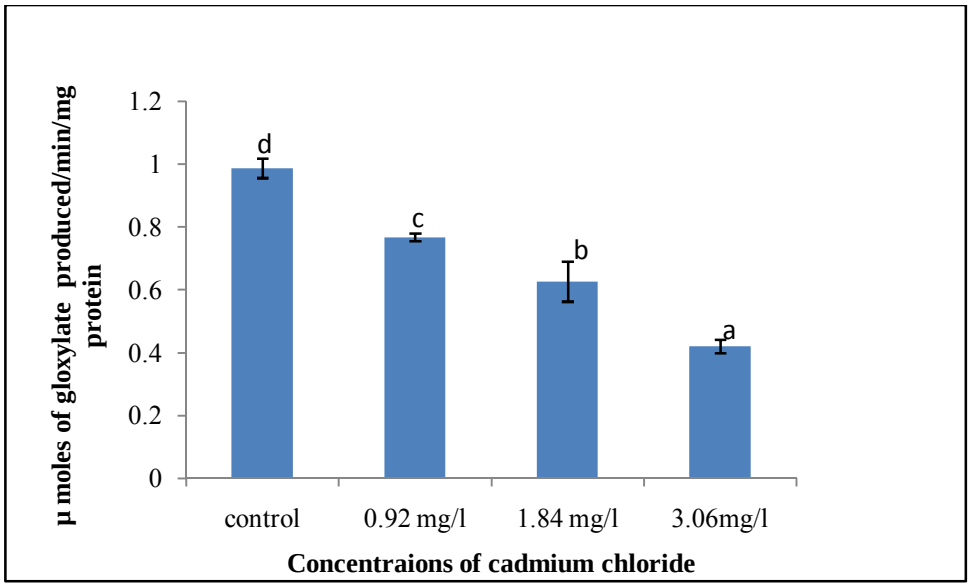

Table 4.9 Effect of exposure to different concentrations of cadmium chloride for 7 days on allantoinase activity (mean ± S.D) present in the liver of *O.mossambicus.* Values with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Group	Control	Concentrations of Cadmium chloride		
		0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Allantoinase activity	$0.986818 \pm 0.030859^{d}$	$0.767756 \pm 0.012232^{c}$	$0.626719 \pm 0.063915^{b}$	$0.420933 \pm 0.021285^{a}$

Values are expressed as micromoles of glyoxylate produced / minute / mg protein.

Table 4.10 One way ANOVA for allantoinase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	1.021	3	0.340	201.066	0.000
Within concentration	0.034	20	0.002		
Total	1.054	23			

df- degrees of freedom

4.3.5 Serum urea

Urea content in serum of *O. mossambicus* treated with different concentrations of cadmium chloride showed significant increase ($P<0.05$) compared to control group (Fig 4.5 and Table 4.13) (One-way ANOVA followed by Tukey's test). The ANOVA table is shown below (Table 4.11).

Figure 4.5 Levels of urea in the serum of *O.mossambicus* exposed for 7 days to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

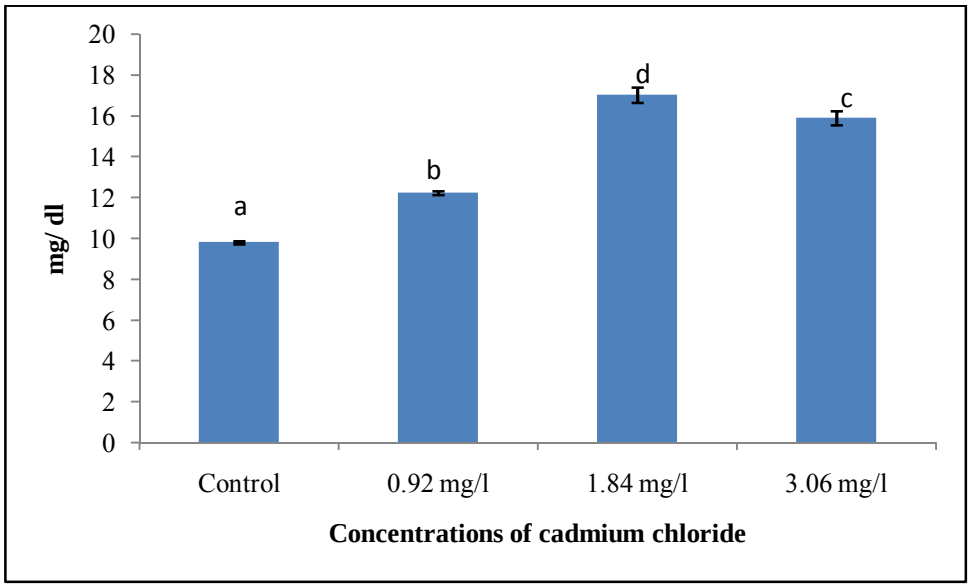

Table 4.11 One way ANOVA for serum urea

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	199.650	3	66.550	962.702	0.000
Within concentration	1.383	20	0.069		
Total	201.032	23			

df-degrees of freedom

4.3.6 Serum uric acid

One-way ANOVA followed by Tukey's test showed that there was significant (P<0.05) increase in the serum uric acid content in all the treated groups compared to control (Fig 4.7 and Table 4.13). The ANOVA table is shown below (Table 4.14).

Figure 4.7 Levels of uric acid in the serum of *O.mossambicus* exposed for 7days to different concentrations of cadmium chloride. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

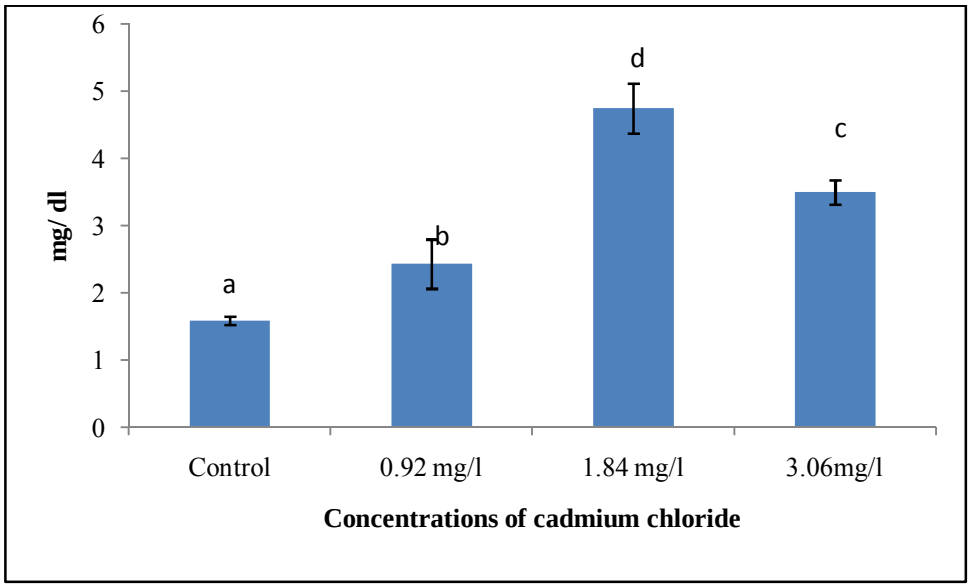

Table 4.12 One way ANOVA for serum uric acid

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentration	33.559	3	11.186	144.577	0.000
Within Concentration	1.547	20	0.077		
Total	35.107	23			

df-degrees of freedom

Table 4.13 Effect of exposure to different concentrations of cadmium chloride for 7 days on Serum parameters of *O.mossambicus.* Values in the same raw with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Serum parameters	Control	Concentrations of cadmium chloride		
		0.92 mg/l $CdCl_2$ (0.564 mg/l Cd^{++})	1.84 mg/l $CdCl_2$ (1.128 mg/l Cd^{++})	3.06mg/l $CdCl_2$ (1.876 mg/l Cd^{++})
Urea	9.83 ± 0.08^{a}	12.24 ± 0.1^{b}	17.05 ± 0.37^{d}	15.92 ± 0.35^{c}
Uric acid	1.59 ± 0.062^{a}	2.43 ± 0.37^{b}	4.75 ± 0.37^{d}	$3.5\pm.18^{c}$

Values are expressed as mg/dl

4.4 Discussion

In the present study, exposure of *O.mossambicus* to sub lethal concentrations of cadmium chloride resulted in significant alterations in the purine catabolizing enzymes. Purines are degraded in animals to waste nitrogenous substances; so the alterations in the catabolism of purines affect the homeostasis and the excretion of nitrogenous substances.

AMP deaminase is an important enzyme participating in the purine nucleotide cycle. The pathway is active in fish muscle, especially post exercise, when it is used to scavenge AMP produced by the hydrolysis of ATP during muscle contraction. This pathway directly liberates ammonia as NH_3. AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) plays an important role in the regulation of the adenylate energy charge (Chapman and Atkinson, 1973). (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al*. (1971) the value of energy charge $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$). High AMP deaminase activities have been detected in skeletal muscle (Conway and Cooke, 1939) and in gill tissue where it is associated with a very active purine metabolism (Leray *et al.*, 1979). But most of its activity is inhibited under normal physiological conditions in order to avoid excessive degradation of adenine nucleotides (Van den Berghe *et al.* 1977, 1980). In the present study an increased activity of AMP deaminase was observed in the gills, liver, kidney and muscle tissue of *O.mossambicus* exposed to sub lethal concentrations of cadmium chloride for 7 days. This may be due to the activation of AMP deaminase by limited proteolysis during stress. Similar increase in AMP deaminase were reported in Crab (*Scylla serrata*) exposed to cadmium chloride (Reddy and Bhagyalakshmi, 1994). The main inhibitors of

AMP deaminase seem to be GTP and inorganic phosphate (Van den Berghe *et al.,* 1977). Usually Stress leads to an increase of the phosphate concentration and this may decrease the AMP deaminase activity. But under conditions of environmental stress, the AMP deaminase activity is modified by limited proteolysis. This mechanism allows rapid adaptation to abrupt changes of external conditions. Limited proteolysis induces the change in the sensitivity of the enzyme to variations of intracellular pH and inorganic phosphate and increases the enzyme activity. Under such conditions; increased enzyme activity allows the preservation of the adenylate energy charge which can be considered as a vital parameter. Thus, modification of the AMP deaminase activity by limited proteolysis will preserve regulation of AMP deaminase by allosteric effectors (ATP, GTP) under conditions where accumulation of inorganic phosphate would otherwise result in almost complete inhibition of the enzyme (Raffin, 1986).

In the present experiment, the activity of xanthine oxidase was significantly increased in liver, kidney, muscle and gills. This was supported by Sastry and Subhadra (1985) who also observed similar increase in xanthine oxidase activity in kidney, intestine, ovaries and gills in freshwater catfish, *Heteropneustes fossilis*. The enhanced activity of xanthine oxidase suggests that metabolism of purines and formation of uric acid may increase in cadmium-treated fish. Basha and Rani, 2003 also reported that xanthine oxidase activities were increased during cadmium stress in the liver and muscle tissues of Tilapia. Similarly chronic ammonia exposures increased the xanthine oxidase activities in liver and white muscle of Nile tilapia (Hegazi *et al.*, 2010). Hegazi *et al*. (2010) explained the biochemical nature of the enzyme. According to him the enzyme (xanthine oxidoreductase; XOR) exists in separate but inter-convertible forms, with dehydrogenase (XD, EC 1.17.1.4) and oxidase (XO, EC 1.1.3.22) activity (Chung *et al*., 1997; Pritsos, 2000). This inter conversion occurs either

reversibly by the oxidation of sulfhydryl residues (Nishino *et al*., 2005) or irreversibly by proteolysis (Kuwabara *et al*., 2003). The two forms act on the same substrate, and convert hypoxanthine to xanthine and xanthine to uric acid. However, the native XD form, in contrast to XO form, utilizes NAD^+, producing NADH instead of superoxide anions ($O_2\bullet^-$). The oxidase form (XO) uses molecular oxygen instead NAD^+ as the electron acceptor and releases substantial amounts of superoxide anions ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2) (Nishino, 1994; Saugstad, 1996). The final end product of XD, uric acid, has a potential physiological function as a protective agent against oxidative damage. Uric acid acts as a scavenger of hydroxyl radicals, singlet oxygen, hypo chlorous acid, oxoheme oxidants and hydroperoxyl radicals. In alcohol consumption (Sultatos, 1988), tissue hypoxia, and ischemia-reperfusion (Nishino, 1994; Saugstad, 1996), glutathione depletion (Cighetti *et al*., 1993) and oxidizing agents such as hydrogen peroxide (Bindoli *et al*., 1988) promote the conversion of XD to XO. This mechanism was initially described in rat organs such as the liver, kidney (McKelvey et al., 1988) and brain (Martz et al., 1989). Therefore, the increase in the activity of XO may be attributed to an imbalance occurring in the physiological XD/XO ratio that augments XO formation due to stress. The induction of elevated levels of xanthine oxidase, in the fish shows a possible shift toward a detoxification mechanism.

Uricase (urate:oxygen oxidoreductase, (EC 1.7.3.3) is the enzyme responsible for the breakdown of the purine skeleton to allantoin in a variety of living systems (Osman *et al.,* 1989). Uricase participate in the purine breakdown pathway; catalyze the oxidation of uric acid in the presence of oxygen to allantoin and hydrogen peroxide (Fraisse *et al.,* 2002). Uricase enzyme is widely present in most vertebrates but is absent in humans (Schiavon *et al.,* 2000). Higher primates

(apes and humans) lack functional uricase and excrete uric acid as the end product of purine degradation (Friedman *et al.,* 1985, Yeldandi *et al.,* 1990).

The enzyme appears to be located in peroxisomes, as shown by several studies on rat, mouse and fish liver and it is actually used as a marker enzyme for this organelle (Tsukada *et al.,* 1968; Leighton *et al.,* 1969; Yokota, 1973; Noguchi *et al.,* 1979; Angermuller *et al.,* 1986, Osman *et al.,* 1989). In the present study a decrease in the activity of uricase was observed. The decrease in the activity may be due to the inhibition of uricase by cadmium chloride. Anderson and Vijayakumar (2011) reported that the metal ions like Co^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+} and Cu^{2+} reduced the uricase activity. The previous study also reported that the inhibition of uricase activity was noticed with Co^{2+}, Fe^{2+} and Zn^{2+} and enhanced with Ca^{2+} (Saeed *et al.,* 2004).

Allantoinase (allantoin amidohydrolase, E.C. 3.5.2.5) catalyzes the hydrolysis of allantoin to allantoic acid (Passino and Cotant, 1979). This reaction is part of the purinolytic pathway for degradation of uric acid to glyoxylic acid and urea. Although teleost fish are ammonotelic and the complete ornithine-urea cycle is absent (Brown and Cohen, 1960), the formation of urea by uricolysis has been demonstrated for both freshwater (Cvancara, 1969) and marine teleosts (Goldstein and Forster, 1965). Inhibition of allantoinase could result in the deposition of allantoin and in failure to excrete nitrogen from nucleic acids, proteins and amino acids that are normally eliminated through purinolytic and uricolytic pathways (Passino and Cotant, 1979). In the present study, a significant decrease in the activity of allantoinase was observed in the liver of *Oreochromis mossambicus* treated with different concentrations of cadmium chloride. Similarly Passino and Cotant, 1979 observed that mercury, lead and cadmium inhibited allantoinase. According to them the larger molecular sizes of mercury, lead and cadmium may result in steric hindrance of the active site of allantoinase so that

the enzyme is inhibited by these metals. Mercury and other metals usually inhibit enzymes by reacting with electron donor groups on these macromolecules (Eichhorn, 1975, Passino and Cotant, 1979).

On exposure to cadmium chloride, the levels of urea and uric acid were significantly enhanced in the blood of fish. The enhanced urea and uric acid content may be due to kidney failure and/or increased muscular tissue catabolism (Fruton and Simmonds 1958; Emmerson 1973). This result stands in good agreement with the observation reported by Adham *et al* (2002), who observed that levels of urea, uric acid and creatinine were significantly enhanced in fish grown in metal contaminated water. Similarly, serum urea concentration was elevated by copper exposure of *Heteropneustes fossilis* with increasing exposure time and this was associated with atrophy of excretory sites (Singh and Reddy, 1990). Grosell *et al*. (2004) also showed that plasma urea concentration increased approximately threefold after both acute (95h) and prolonged (30 d) copper exposure.

Blood urea nitrogen in teleost fish originates from uricolysis and arginolysis. In the present study the enzymes such as uricase, allantoinase and arginase were inhibited by cadmium chloride. So the elevation of urea content may be due to kidney dysfunction of the fish in response to the cadmium chloride exposure. Serum urea levels generally were enhanced by metal treatment (McDonald and Grosell, 2006).

Serum uric acid can be used as a rough index of the glomerular filtration rate (Hernandez and Coulson, 1967). Low values of uric acid have no significance but increasing values show disturbances in the kidney (Maxine and Benjamine, 1985). Similar increase in uric acid content in the serum was also observed when O*reochromis niloticus* were exposed to copper and lead (Al-Nagaawy, 2008). In

the present study the increased uric acid content may also be due to the elevated activity of xanthine oxidase. Uric acid acts as an antioxidant. So it increased due to the oxidative damage caused by the cadmium.

Thus it appears from the present investigation that the purine catabolism of the cadmium exposed fish is altered. Ammoniagenesis is triggered by increased deamination of purines via AMP deaminase and oxidative deamination of glutamate under cadmium-induced stress. This results in production and accumulation of ammonia. The activity of the enzyme xanthine oxidase is increased in the tissues of cadmium stressed fish. But the enzymes such as uricase and allantoinase were inhibited by the heavy metal. As a result of increased xanthine oxidase and decreased uricase and allantoinase activity the uric acid content increased in the blood. Uric acid acts as an antioxidant. Therefore the increase in uric acid content acts as a protective agent against the oxidative damage caused by cadmium chloride. Thus the organism is trying to attain homeostasis in cadmium-polluted habitats by increasing the activity of certain enzymes. In conclusion cadmium chloride is a potent toxic pollutant which caused the damage of kidney and increased the urea and uric acid in blood. It also affected the purine nucleotide cycle enzyme AMP deaminase and other purine catabolizing enzymes.

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Chapter 5

Effect of Acute Salinity Change on the Protein Catabolism of *Oreochromis mossambicus* (Peters)

5.1 Introduction

The aquatic environment is never static, it always varies. The organisms living in the aquatic environment have the ability to adjust physiologically to such changes. This ability allows the organism to survive the environmental fluctuations (Hoar, 1959; Kinne, 1963). Salinity is a major factor which affects the biological processes of aquatic organisms and the salinity of water varies throughout the year. Variations in water salinity cause osmotic stress in organisms. Fish live in environments with a wide variety of chemical characteristics (fresh, brackish and seawater, acidic, alkaline, soft and hard waters). From an osmoregulatory point of view, fish have developed several mechanisms to live in these different environments.

Euryhaline fish species travel between freshwater and seawater. The huge shifts in external salinity cause osmotic stress that is counteracted in several ways (Ballantyne, 2001). Successful salinity acclimation may require a metabolic reorganization to meet the increased energetic demands associated

with the exposure to the new environmental salinity. Euryhaline fish showed several metabolic changes and spent large amounts of energy, particularly in osmoregulatory (gills, intestine and kidney) and metabolic (liver) organs, to compensate for these salinity changes (Sangiao Alvarellos *et al*., 2005). Enhanced Na^+- K^+ ATPase activity in the gills plays an important role in whole body osmoregulation (McCormick and Saunders, 1987), but the individual cells in various tissues also make metabolic and osmotic adjustments (Ballantyne, 2001). Teleosts, inhabiting environments with various salinities, have complicated and sophisticated mechanisms of osmoregulation to maintain the internal osmotic and ionic homeostasis. This allows normal functioning of cellular and physiological processes and survival (Evans *et al*., 2005; Hwang and Lee, 2007). These osmoregulatory processes are achieved by various enzymes and transporters. The synthesis and operation of these transport related proteins are highly energy consuming. In general, timely additional energy is required particularly in a situation of a fluctuating environment to which an organism responds by synthesizing and activating related enzymes, transporters, and/or enzymes (Tseng and Hwang, 2008).

Measuring the oxygen consumed in the entire organism is a commonly used method to indirectly monitor its metabolic rate (Randall *et al*., 2002). Many earlier studies indicate that acclimating to different salinities causes changes in oxygen consumption. It is difficult to reconcile differences in those results obtained, likely due to differences in species, duration of acclimation, experimental design, and details of the measurement methodology (Febry and Lutz, 1987; Moser and Hettler, 1989; Morgan and Iwama, 1991; Aristizabal-Abud, 1992; Ron *et al*., 1995; Woo and Kelly, 1995; Haney and Nordlie, 1997; Morgan *et al*., 1997; Morgan and Iwama, 1998; Swanson, 1998; Plaut,

2000; Sardella *et al*., 2004; Gracia-Lopez *et al*., 2006; Wagner *et al*., 2006). Morgan and Iwama (1991) summarized five energy metabolism patterns from previous studies: (1) there is no change in the metabolic rate; (2) the metabolic rate is minimum in isosmotic salinity but increases in different salinities; (3) a linear relation exists between the metabolic rate and fluctuant salinity; (4) the metabolic rate increases in fresh water and is reduced in a condition of isosmotic salinity; and (5) the highest metabolic rate occurs in seawater

However, Morgan and Iwama (1991) concluded that: (1) in salmonids, the higher metabolic rate in higher water salinities reflect significant energetic costs, accompanied by declining growth rates, and these correlate very well with changes in oxygen consumption, (2) life habits appear, to a certain extent, to determine the type of metabolic response to salinity changes. i.e., the lowest metabolic rates are associated with the environment in which a species is most commonly found. As summarized by Boef and Payan (2001), 20%–68% of the total energy expenditure is estimated to be consumed by osmoregulation in different species. However, Morgan and Iwama (1991) stated that estimates of osmoregulation costs based on whole-fish oxygen consumption should consider the effects by other metabolic processes which respond to changes in salinity. During sea water acclimation, several hormones are known to affect different pathways of energetic metabolism (Sangiao-Alvarellos *et al*., 2006a,b). Other non-osmoregulatory organs (like brain) also show changes in energetic metabolism (Sangiao-Alvarellos *et al*., 2005; Polakof *et al*., 2006).

Amino acids play two key roles during metabolic adjustments to these different environments. (1) As important intracellular solutes, their levels must be adjusted to maintain cell volume (King and Goldstein, 1983b). (2) As metabolic energy sources, amino acids can be oxidized to provide ATP for

osmoregulation (Ballantyne and Chamberlin, 1988). Tissue amino acid level rises in response to elevated salinity in some teleost fishes (Assem and Hanke, 1983). Free amino acids in muscle of rainbow trout (Kaushik and Luquet, 1979), *T. mossambica* (Venkatachari, 1974), *Anguilla anguilla* (Huggins and Colley, 1971), flounder *Pleuronectes flesus* (Lange and Fugelli, 1965), and skate *Raja erinacea* (Forster and Hannafin, 1980) in seawater are elevated above those of freshwater controls. Other tissues such as gills, liver, heart, and kidney also display elevated free amino acid levels in seawater-acclimated fish, such as *T. mossambica* (Venkatachari, 1974).

The successful acclimation of *O.mossambicus* from freshwater to seawater depends on a substantial reorganization of many physiological systems over a relatively short period of time. Upon exposure to seawater, gill chloride cells become larger, more in number and their basolateral and apical membrane surface area is greatly increased to accommodate the placement of large numbers of newly synthesized ion transport proteins (e.g. Na^+-K^+ ATPase) (Eddy, 1982). The intestine is modified to enhance the uptake of sodium and chloride from ingested seawater to facilitate the passive absorption of water, and the kidney changes to produce more concentrated urine to conserve water (Eddy, 1982). Taken together, these modifications to the osmoregulatory organs suggest that the acclimation to seawater is an energetically expensive task. The high energy expenditure of osmoregulation is confirmed by reports of increased metabolic rate. Maxime et al. (Maxime *et al*., 1991) found a significant increase in oxygen consumption of rainbow trout in the first 24 hour of seawater acclimation. Similar studies with rainbow trout (Rao, 1968) and tilapia (Farmer and Beamish, 1969; Febry and Lutz, 1987) show that oxygen consumption rates are 27% higher in seawater than at isosmotic salinity (10%). Leray *et al*. (1981) found no change in rainbow trout

oxygen consumption, but did report an immediate decrease in ATP levels, ATP: ADP ratio and adenylate energy charge in rainbow trout gills following seawater transfer (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al*. (1971) the value of energy charge $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$).

The period immediately following seawater exposure is probably critical. During this time the osmoregulatory machinery is reorganized as the fish changes from actively accumulating sodium and chloride to actively secreting these ions. This period has been termed the 'initial crisis phase' and is characterized by increasing plasma ion concentrations as the fish struggles to osmoregulate (Gordon, 1959). Although the energy metabolisms of the osmoregulatory tissues are critical, the metabolism of the other 'supporting' tissues (e.g. liver, muscle) may be equally important. These 'support' tissues export substrates (e.g. amino acids, lipids) to the circulation, which can be picked up and oxidized by the gills and other osmoregulatory tissues for ATP production for the synthesis of macromolecules (e.g. proteins, membranes). Additionally, amino acids may be mobilized to serve as osmoregulatory intracellular solutes (Bystriansky *et al*., 2007).

Studies related to comprehensive analysis of the importance of protein catabolism during seawater acclimation of fish are rare. To assess this we monitored changes in activities of ALT, AST, GDH and arginase. We also assessed the tissue levels of protein, free amino acid and plasma ammonia. The rates of ammonia excretion, oxygen consumption and ammonia quotient were determined during the first 48 hour of salinity acclimation, a critical

period when many of the major physiological changes required for successful acclimation occur.

5.2 Materials and Methods

Collection, maintenance, acclimation, Preparation of tissue and serum samples, Methods used for biochemical analysis and statistical analysis were the same as explained in detail in chapter 3, section 3.2

5.2.1 Experimental design for the study of the effects of acute salinity change

For conducting biochemical studies, *O.mossambicus* (15±3g) were taken in three separate tubs and gradually acclimatized to 10ppt, 20 ppt and 30 ppt salinity respectively within 4 hours. Six replicates were kept for each experiment. The fishes were exposed for 48 hours. During the experimental period the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. The water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). A tub containing fresh water was kept as control.

5.3 Results

5.3.1 Total protein

In the present study the Protein content in different tissues of *O. mossambicus* exposed to 20 ppt and 30 ppt salinities showed significant decrease ($P<0.05$) compared to control group (Fig 5.1 and Table 5.1) (One-way ANOVA followed by Tukey's test) .

Figure 5.1 Protein content in the various tissues exposed to different salinities. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

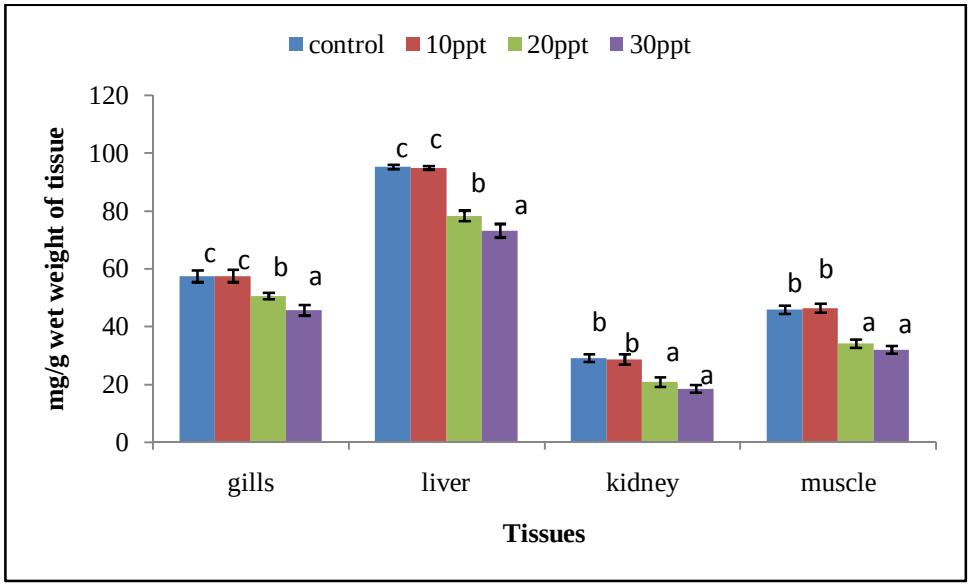

Table 5.1 Effect of exposure to acute salinity change for 48 hours on total protein content (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Tissue Protein Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	57.38 ± 2.015^{c}	57.53 ± 2.14^{c}	50.66 ± 1.15^{b}	45.72 ± 1.85^{a}
Liver	95.29 ± 0.80^{c}	94.95 ± 0.61^{c}	78.35 ± 1.84^{b}	73.21 ± 2.33^{a}
Kidney	29.13 ± 1.24^{b}	28.71 ± 1.82^{b}	20.77 ± 1.68^{a}	18.49 ± 1.36^{a}
Muscle	45.87 ± 1.46^{b}	46.37 ± 1.56^{b}	34.1 ± 1.43^{a}	32.02 ± 1.38^{a}

Values are expressed as mg/g wet weight of tissue

Two factor ANOVA (Table 5.2) revealed that there was a significant decrease (P<0.05) in protein content in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison using Tukey's test (Table 5.13) revealed that there was significant difference (P<0.05) in protein content between control and 20 ppt and 30 ppt salinity exposed groups. There is no significant difference between control and 10 ppt salinity exposed

groups. Similarly multiple comparison using Tukey's test (Table 5.14) also revealed that there was a significant difference between tissues also.

Table 5.2 Two-Factor ANOVA for totl protein

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	2025.397	3	675.132	261.450	0.000
Between tissues	24402.104	3	8134.035	3149.967	0.000
concentration * tissues	216.043	9	24.005	9.296	0.000
Error	82.632	32	2.582		
Total	149302	48			

df- degrees of freedom

5.3.2 Free amino acid content

In the present study the tissue free amino acid content in different tissues of *O. mossambicus* exposed to 20 ppt and 30 ppt salinities showed significant increase ($P<0.05$) compared with control and 10 ppt exposed group (Fig 5.2 and Table 5.3) (One-way ANOVA followed by Tukey's test) .

Figure5.2 Free amino acid content in the various tissues exposed to different salinities. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

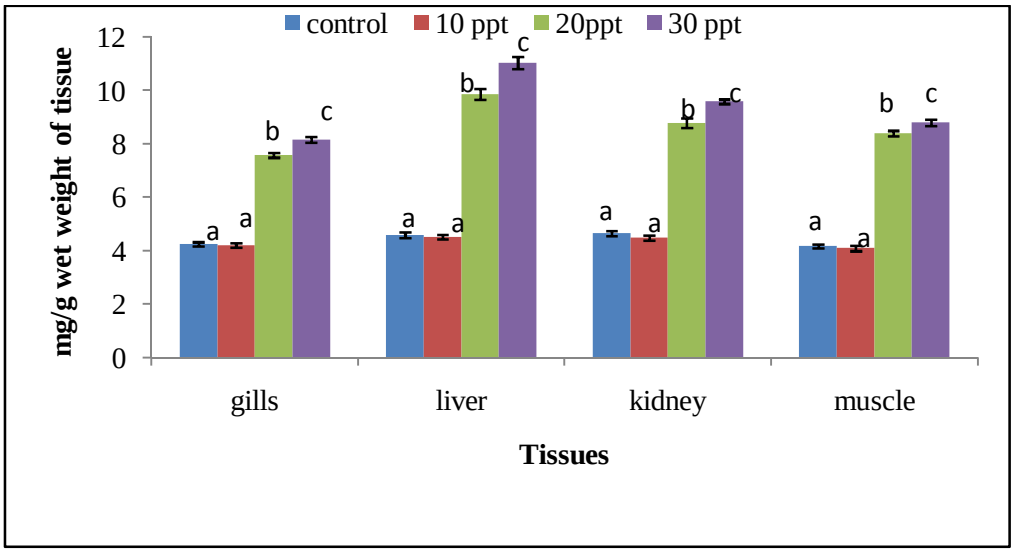

Table 5.3 Effect of acute exposure to different salinities on free amino acid content (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Free amino acid			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	4.24±0.08[a]	4.20±0.09[a]	7.58±0.089[b]	8.16±0.113[c]
Liver	4.59±0.1[a]	4.515±.0.09[a]	9.86±0.20[b]	11.04±0.24[c]
Kidney	4.65±0.094[a]	4.48±0.093[a]	8.79±0.18[b]	9.59±0.085[c]
Muscle	4.17±0.076[a]	4.093±0.105[a]	8.398 ±0.099[b]	8.801±0.124[c]

Values are expressed as mg/g wet weight of tissue.

Two factor ANOVA (Table 5.4) revealed that there was a significant increase (P<0.05) in free amino acid content in all the salinity treated experimental groups when compared to control group. Subsequent analysis by multiple comparison using Tukey's test (Table 5.13) revealed that there was significant difference (P<0.05) in free amino acid content between control and 30 ppt and 20 ppt salinity treated groups. But there was no significant difference between control and 10 ppt salinity exposed group. Similarly multiple comparison using Tukey's test (Table 5.14) also revealed that there was a significant difference between tissues also. Among the tissues liver showed highest increase followed by kidney.

Table 5.4 Two-Factor ANOVA for tissue free amino acids

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	527.914	3	175.971	11232.861	0.000
Between tissues	29.057	3	9.686	618.265	0.000
concentration * tissues	16.627	9	1.847	117.931	0.000
Error	1.253	80	0.016		
Total	4880.319	96			

df-degrees of freedom

5.3.3 Activity of alanine aminotransferase

In the present study the activity of alanine aminotransferase in different tissues of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant increase ($P<0.05$) compared with control and 10 ppt salinity exposed group (Fig 5.3 and Table 5.5) in each tissue on different treatments (One-way ANOVA followed by Tukey's test) .

Fig 5.3 Effect of acute salinity change on ALT activity in the various tissues of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

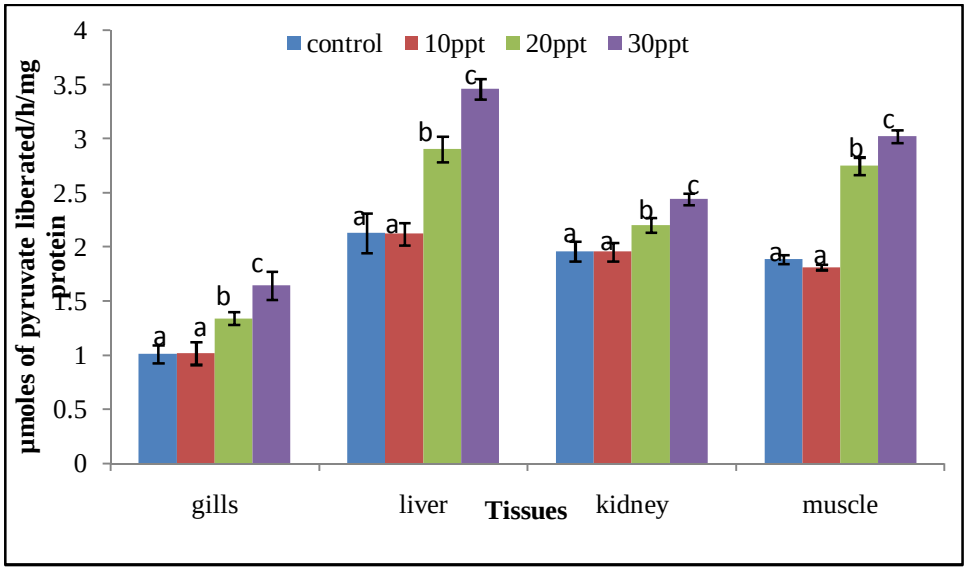

Table 5.5 Effect of acute salinity change on alanine aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA).

Tissues	ALT activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	1.01 ± 0.08^{a}	1.018 ± 0.105^{a}	1.34 ± 0.059^{b}	1.643 ± 0.1305^{c}
Liver	2.13 ± 0.18^{a}	2.12 ± 0.10^{a}	2.905 ± 0.118^{b}	3.46 ± 0.095^{c}
Kidney	1.96 ± 0.091^{a}	1.956 ± 0.086^{a}	2.20 ± 0.067^{b}	2.44 ± 0.054^{c}
Muscle	1.89 ± 0.041^{a}	1.813 ± 0.025^{a}	2.75 ± 0.081^{b}	3.023 ± 0.058^{c}

Values are expressed in µmoles of pyruvate liberated/h/mg protein

Two way ANOVA (Table 5.6) followed by comparisons with multiple comparison using Tukey's test (Table 5.13) revealed that all the three concentrations selected for the study differed significantly from one another and 20 ppt and 30 ppt groups differed significantly with the control. But there was no significant difference between control and 10 ppt exposed groups. Similarly multiple comparison tests using Tukey's test (Table 5.14) also revealed that there was a significant difference between tissues also. Among the tissues liver showed highest increase.

Table 5.6 Two-Factor ANOVA for tissue ALT activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	14.323	3	4.774	541.035	0.000
Between Tissues	26.363	3	8.788	995.824	0.000
concentration×tissue	2.593	9	0.288	32.644	0.000
Error	0.706	80	0.009		
Total	468.987	96			

df- degrees of freedom

5.3.4 Activity of aspartate aminotransferase

There was a significant increase ($P<0.05$) in the activity of Aspartate aminotransferase in different tissues of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity compared to control and 10 ppt salinity treated groups (Fig 5.4 and Table 5.7) (One-way ANOVA followed by Tukey's test)

Figure 5.4 AST activity in the various tissues exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

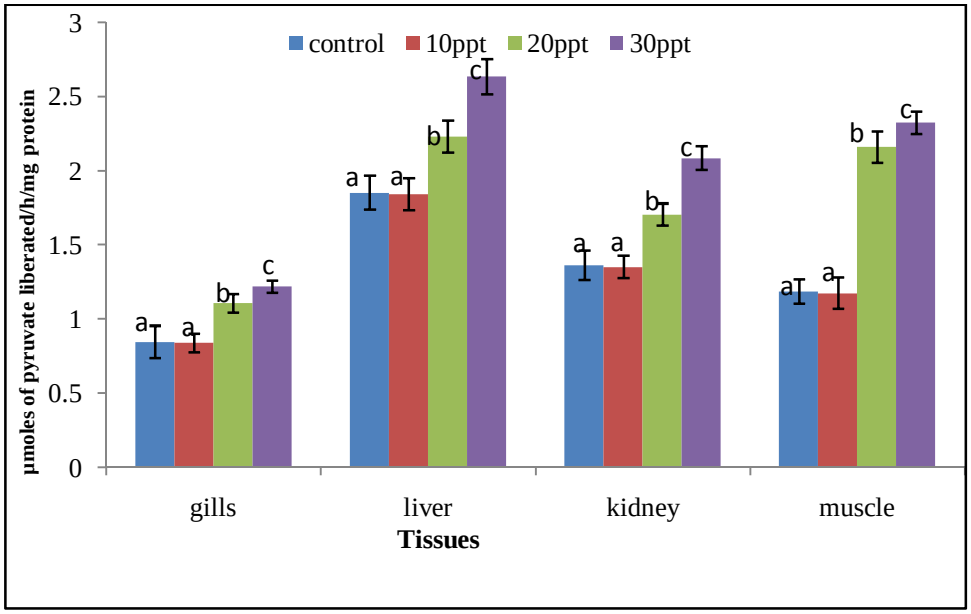

Table 5.7 Effect of exposure to acute salinity change on aspartate aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	AST activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	0.847±0.11[a]	0.84±0.032[a]	1.11±0.064[b]	1.22±0.0398[c]
Liver	1.854±0.114[a]	1.843 ±0.153[a]	2.231±0.108[b]	2.636±0.12[c]
Kidney	1.364±0.098[a]	1.352±0.0915[a]	1.706±0.075[b]	2.087±0.079[c]
Muscle	1.187±0.083[a]	1.176±0.075[a]	2.16±0.11[b]	2.326±0.075[c]

Values are expressed in µmoles of pyruvate liberated/h/mg protein

Tissue aspartate aminotransferase activity showed a significant increase (P< 0.05) between experimental groups of fishes and control group as observed by Two way ANOVA (Table 5.8). Further analysis by multiple comparison using Tukey's test (Table 5.13) revealed that there was a significant difference between all the three concentrations selected for the

study from one another. 20 ppt and 30 ppt salinity exposed groups showed significant difference with the control group (P<0.001). But there was no significant difference between 10 ppt exposed groups and control. Subsequent pair wise comparison between various tissues were carried out using Tukey's test (Table 5.14) which indicated significant difference (P<0.001). Among the tissues liver showed highest activity.

Table 5.8 Two-factor ANOVA for tissue AST activity

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	10.258	3	3.419	281.701	0.000
Between Tissues	15.837	3	5.279	434.898	0.000
concentration×tissue	1.961	9	0.218	17.953	0.000
Error	0.971	80	0.012		
Total	281.354	96			

df-degrees of freedom

5.3.5 Activity of glutamate dehydrogenase

In the present study the activity of glutamate dehydrogenase in different tissues of *O. mossambicus* treated with cadmium chloride showed significant variations (P<0.05) compared with control group (Fig 5.5 and Table 5.9) (One-way ANOVA followed by Tukey's test). A statistically significant increase in glutamate dehydrogenase (P<0.05) was observed in liver, kidney and gills of the 20 ppt and 30 ppt exposed groups compared to control and 10 ppt. A decreased activity (P<0.05) was found in muscle of the 20 and 30 ppt salinity exposed fish compared to control. No significant variation in the muscle GDH activity was observed between 10 ppt exposed fish and control. There was no statistically significant difference in the muscle GDH between 10 ppt exposed group and 20 ppt exposed group.

Figure5.5 GDH activities in the various tissues exposed acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

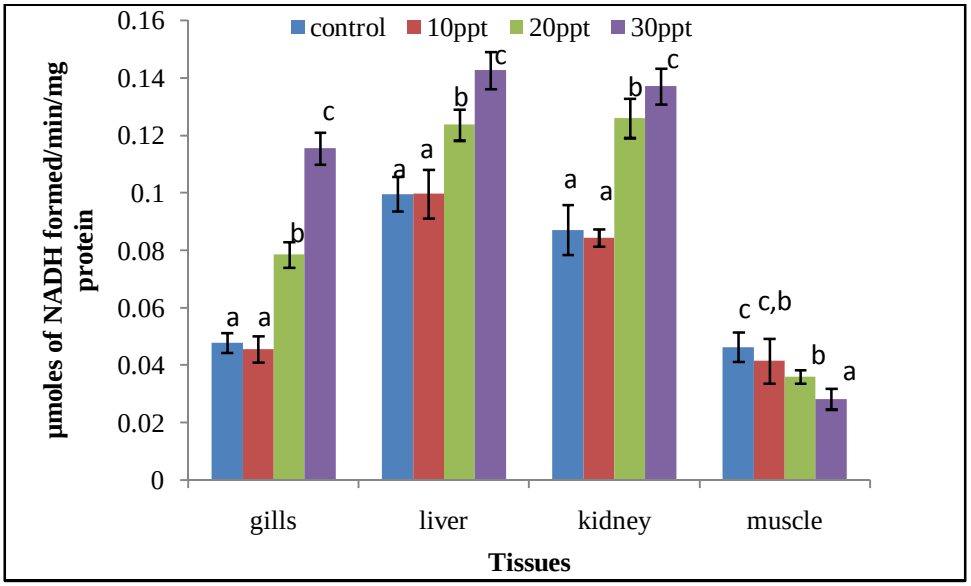

Table 5.9 Effect of exposure to acute salinity change to Glutamate dehydrogenase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	GDH activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	0.0478±0.0034[a]	0.04566±0.0046[a]	0.07854±0.0045[b]	0.1155±0.0056[c]
Liver	0.09956±0.006[a]	0.0996±0.008[a]	0.1236±0.005[b]	0.1425±0.006[c]
Kidney	0.087±0.009[a]	0.084±0.003[a]	0.1259±0.007[b]	0.137±0.006[c]
Muscle	0.046±0.005[c]	0.0415±0.008[c,b]	0.0359±0.0023[b]	0.028±0.0036[a]

Values are expressed in µmoles of NADH formed/min/mg protein

Two factor ANOVA (Table 5.10) revealed that there was a significant increase (P<0.05) in glutamate dehydrogenase activity in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 5.13) revealed that there was significant difference (P<0.05) between control and 20 ppt and 30 ppt salinity exposed groups and

between 10 ppt and 20 ppt, 30 ppt salinity exposed groups. Between 10 ppt and control there was no significance at P<005. Multiple comparison tests also (Table5.14) revealed that there was a significant difference between tissues also except between liver and kidney

Table 5.10 Two-factor ANOVA for tissue GDH activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Tissues	0.094	3	0.031	932.933	0.000
Between Concentrations	0.023	3	0.008	233.303	0.000
tissue*concentration	0.018	9	0.002	58.454	0.000
Error	0.003	80	3.35E-005		
Total	0.810	96			

df-degrees of freedom

5.3.6 Activity of arginase

In the present study the activity of arginase in gills and liver tissue of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant increase (P<0.05) compared with control and 10 ppt salinity exposed groups (Fig 5.6 and Table 5.11) (One-way ANOVA followed by Tukey's test). No significant variation was observed in arginase activity in kidney tissue of the fish exposed to acute salinity change compared to control group. The muscle showed no or very little arginase activity.

Figure 5.6 Arginase activity in the various tissues exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

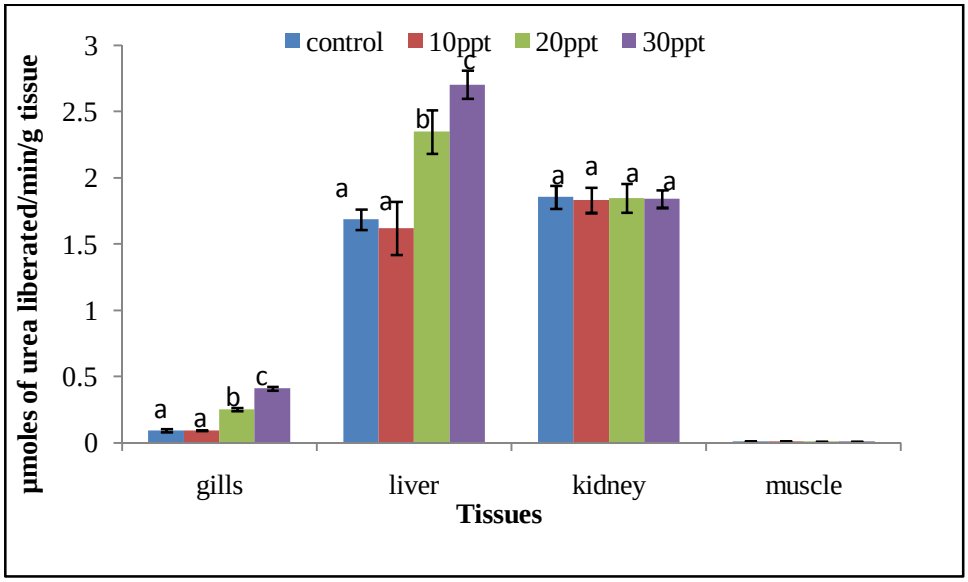

Table 5.11 Effect of exposure to acute salinity change on arginase activity (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Arginase activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	0.0943±0.013^{a}	0.0932±0.0027^{a}	0.254±0.011^{b}	0.411±0.014^{c}
Liver	1.69±0.077^{a}	1.621±0.201^{a}	2.35±0.165^{b}	2.71±0.106^{c}
Kidney	1.86±0.087^{a}	1.83±0.096^{a}	1.849±0.108^{a}	1.843±0.068^{a}
Muscle	0.0103±0.0014^{a}	0.01032±0.0018^{a}	0.0092±0.0014^{a}	0.009±0.0015^{a}

Values are expressed in µmoles of urea formed/min/g tissue

Two factor ANOVA (Table 5.12) revealed that there was a significant increase (P<0.05) in arginase activity in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table5.13) revealed that there was significant difference (P<0.05) between control and 20 ppt and 30 ppt salinity exposed groups and between 10 ppt and 20 ppt and 30 ppt. Between control and 10 ppt group exhibited no significant

difference. Multiple comparison tests also (Table 5.14) revealed that there was a significant difference between tissues.

Table 5.12 Two-factor ANOVA for tissue arginase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	2.055	3	0.685	92.474	0.000
Between tissues	83.994	3	27.998	3779.503	0.000
concentration * tissues	3.335	9	0.371	50.026	0.000
Error	0.593	80	0.007		
Total	193.791	96			

df-degrees of freedom

Table 5.13 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters					
	ALT	AST	GDH	Protein	Free amino acids	Arginase
Control Vs 10 ppt	0.886[d]	0.988[d]	0.465[d]	1.000[d]	0.076[d]	0.796[d]
Control Vs 20 ppt	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs 30 ppt	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
10 pptVs 20 ppt	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.008[a]
10 pptVs 30 ppt	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
20 pptVs 30 ppt	0.000[a]	0.000[a]	0.002	0.000[a]	0.000[a]	0.287[d]

The values are significant at a=P<0.05 and not significant at d

Table 5.14 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters					
	ALT	AST	GDH	Protein	Free aminoacid	Arginase
Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Liver Vs Kidney	0.000[a]	0.000[a]	0.950[d]	0.000[a]	0.000[a]	0.000[a]
Liver Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Kidney Vs Muscle	0.000[a]	0.043[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

5.3.7 Plasma ammonia

One-way ANOVA followed by Tukey's test showed that there was significant (P<0.05) increase in the plasma ammonia in 20 ppt and 30 ppt salinity exposed groups compared to control and 10 ppt (Fig 5.7 and Table 5.19).There was no significant difference between control and 10 ppt salinity exposed group. The ANOVA table is shown below (Table 5.15).

Figure 5.7 Levels of ammonia in the plasma of *O. mossambicus* exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

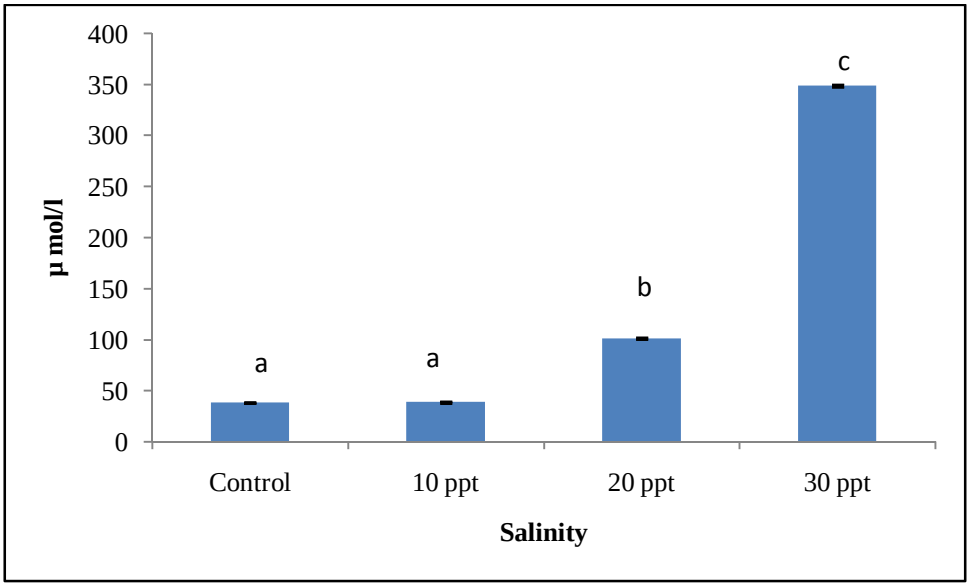

Table 5.15 One way ANOVA for plasma ammonia

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	392966.301	3	130988.767	158246.774	0.000
Within concentration	16.555	20	0.828		
Total	392982.856	23			

df-degrees of freedom

5.3.8 Ammonia excretion

O. mossambicus exposed to 20 ppt and 30 ppt salinity exhibited significant (P<0.05) increase in the rates of excretion of ammonia compared to control and 10 ppt exposed groups (Fig 5.8 and Table 5.19).There was no significant difference in the rates of excretion of ammonia between 10 ppt and control group. One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement and the table is shown below (Table 5.16).

Figure 5.8 Rates of ammonia excretion by *Oreochromis mossambicus* exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

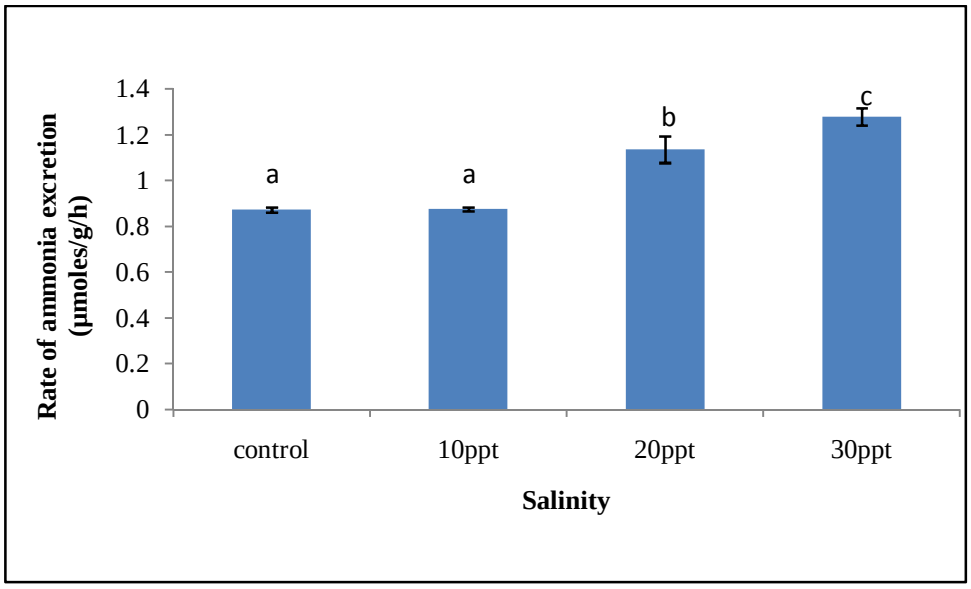

Table 5.16 One way ANOVA for ammonia excretion

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.725	3	0.242	196.276	0.000
Between groups	0.025	20	0.001		
Total	0.749	23			

df-degrees of freedom

5.3.9 Oxygen consumption

One-way ANOVA followed by Tukey's test showed that there was significant increase ($p<0.05$) in the rate of Oxygen consumption of fish exposed to 20 ppt and 30 ppt salinity compared to control and 10 ppt group. There was no statistically significant difference in the rate of Oxygen consumption between 20 ppt and 30 ppt salinity exposed fish. Similarly Oxygen consumption of fish exposed to 10 ppt salinity did not show any significant variation compared to control. (Fig 5.9 and Table5.19). The ANOVA table is shown below (Table 5.17).

Figure 5.9 Rates of oxygen consumption by *Oreochromis mossambicus* exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA)

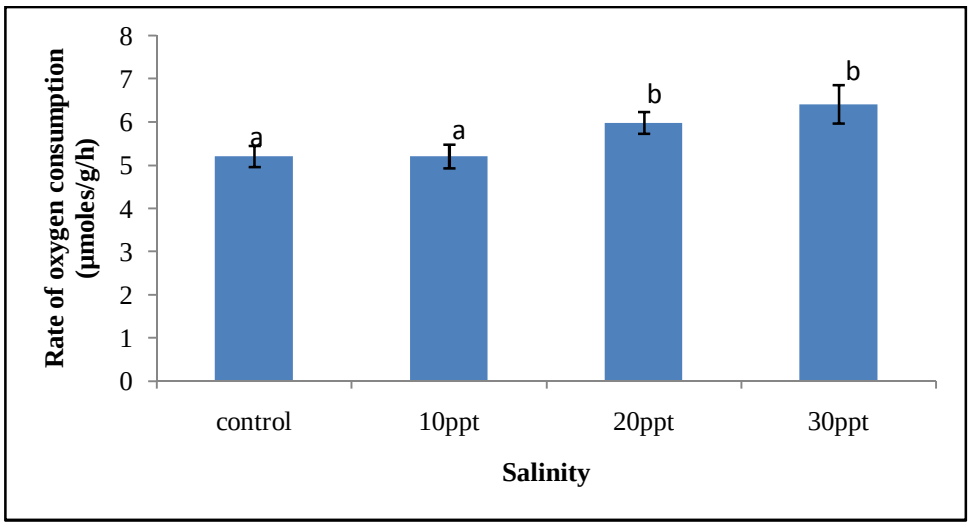

Table 5.17 One way ANOVA for rates of oxygen consumption by fish

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	6.488	3	2.163	21.771	0.000
Within Concentration	1.987	20	0.099		
Total	8.475	23			

df- degrees of freedom

5.3.10 Ammonia quotient

*O.*mossambicus exposed to 20ppt and 30 ppt salinity exhibited significant ($P<0.05$) increase in the ammonia quotient compared to control and 10 ppt salinity exposed group (Fig 5.10 and Table 5.19). No significant variation in ammonia quotient was observed between 20 ppt and 30 ppt salinity exposed groups. Similarly the AQ of control and 10 ppt exposed groups did not vary significantly. One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement and the table is shown below (Table 5.18).

Figure 5.10 The ammonia quotient of *Oreochromis mossambicus* exposed acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

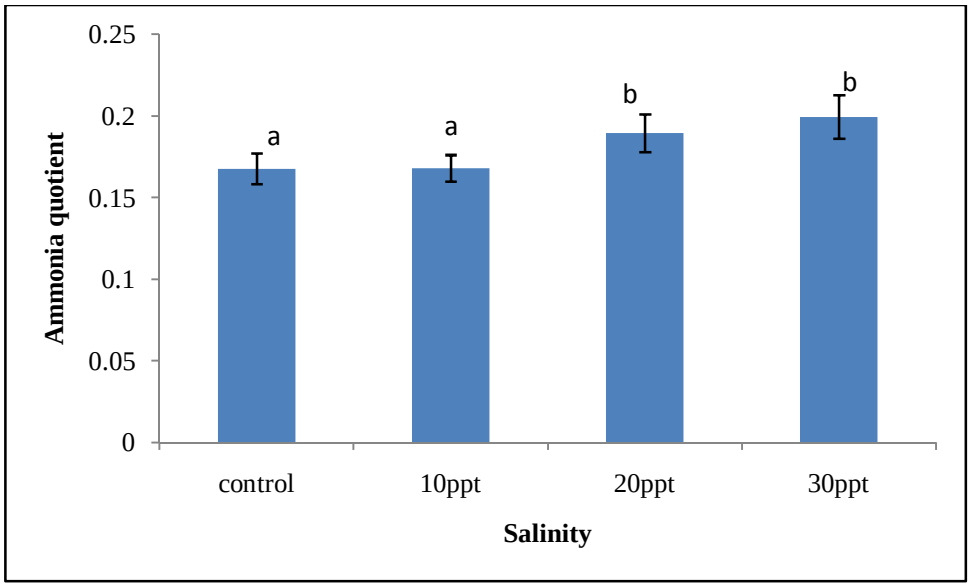

Table5.18 One way ANOVA for ammonia quotient

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	0.005	3	0.002	12.993	0.000
Within Concentrations	0.002	20	0.000		
Total	0.007	23			

df- degrees of freedom

Table 5.19 Effect of exposure to acute salinity change on plasma ammonia, ammonia excretion, oxygen consumption and ammonia quotient of *O.mossambicus*. Values in the same raw with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

Parameters	Control	10 ppt	20 ppt	30 ppt
Plasma ammonia (µmol/l)	38.22 ± 0.514^{a}	38.8 ± 0.676^{a}	101.37 ± 0.865^{b}	348.97 ± 1.15^{c}
Ammonia excretion (µmole/g/h)	0.871 ± 0.011^{a}	0.874 ± 0.0077^{a}	1.133 ± 0.057^{b}	1.276 ± 0.0377^{c}
Oxygen consumption (µmole/g/h)	5.21 ± 0.25^{a}	5.212 ± 0.27^{a}	5.99 ± 0.25^{b}	6.42 ± 0.45^{b}
Ammonia quotient	0.1675 ± 0.0094^{a}	0.1679 ± 0.0081^{a}	0.1895 ± 0.0115^{b}	0.1994 ± 0.0135^{b}

5.4 Discussion

Protein is a major component in fish feeds because it provides the essential and nonessential amino acids to synthesize body protein and in part provides energy for maintenance. Philips (1969) suggested that 70% of dietary calories of feed in trout (*O.mykiss*) are from protein; thus a greater percentage of dietary protein is metabolized for energy demands than is utilized for body protein synthesis (Tseng and Hwang, 2008).Therefore, the metabolism of proteins and the resulting metabolites serve as energy sources that are important for fish acclimating to severe environments (Aragao *et al*., 2004; Cara *et al*., 2007, Tseng and Hwang, 2008). In the present study the acute exposure of *O.mossambicus* to 20 ppt and 30 ppt salinity showed a significant decrement in the levels of proteins in different tissues compared to control and 10 ppt. The decrease in the levels of protein suggests the existence of protein hydrolysis due to increased energy needs during salinity stress. This result stands in good agreement with the observation reported by Venkatachari (1974), who observed that salinity exposure significantly reduced the protein content of *Oreochromis mossambicus*.

There was a significant increase in the levels of free amino acids in different tissues of *Oreochromis mossambicus* exposed to 20 ppt and 30 ppt salinity compared to control and 10 ppt. The increased protein hydrolysis would

lead to an increase in the free amino acid pool as reported by Venkatachari (1974). The amino acids may be utilized for ATP production in two different ways. They could be converted to keto acids via transaminase and then fed in to the citric acid cycle. Alternatively they could be channeled in to gluconeogenic pathway. Frick and Wright (2002) have found that non-essential amino acids, such as proline and taurine, are responsible for the increase of free amino acids at high salinities in the tissues of the mangrove killifish, *Rivulus marmoratus.* Similar increase in amino acids was reported in other teleosts acclimated to sea water (Huggins and Colley, 1971; Lasserre and Gilles, 1971; Colley *et al*., 1974; Ahokas and Sorg, 1976).According to Walton and Cowey (1982), the transdeamination of non-essential amino acids like aspartate and alanine are important for energy production in fish. Chang *et al*. (2007a) observed accumulation of aspartate and alanine in muscle of climbing perch (*Anabas testudineus*) after six days of acclimation to 30 ppt sea water.

The fish liver depends mainly on amino acid catabolism for its own energy requirements (Ballantyne, 2001). The main pathway for amino acid catabolism in fish liver is through transdeamination of several amino acids to form glutamate and its further deamination by GDH (Ballantyne, 2001). The alanine amino transferase and aspartate aminotransferase activities increased in all the tissues of fish acclimated to 20 ppt and 30 ppt compared to control. There is no difference in the activities of ALT and AST in tissues of fish acclimated to 10 ppt salinity compared to control. The oxidation of aspartate and alanine by their respective aminotransferases can lead to the accumulation of glutamate, which is deaminated via glutamate dehydrogenase or being fed in to the citric acid cycle. The increase in alanine amino transferase and aspartate aminotransferase activities suggest peripheral proteolysis and amino acid catabolism in 20 ppt and 30 ppt sea water acclimating fish. Vijayan *et al*.

(1996b) observed that cortisol mobilizes amino acids and increases amino acid catabolism in *O.mossambicus*. Kammerer *et al.* (2010) observed that cortisol increased rapidly by 3 hour and remained elevated for 3 days in response to salinity challenge in tilapia. These two observations coupled together suggest a significant role for cortisol in the amino acid mobilization process. This increase in the activities of aspartate and alanine aminotransferases stands in good agreement with the observation reported by Assem and Hanke (1983), who reported increases in liver ALT and AST activities in tilapia acclimating to seawater. Aas-Hansen *et al*. (2005) reported that increased liver ALT and AST activities during downstream migration of Arctic char prior to seawater exposure, suggesting that the observed changes in amino acid metabolism are an important preparation for life in seawater. Similar increase in the activity of ALT and AST was also observed during the sea water acclimation of arctic char (Bystriansky *et al.*, 2007).

There is no significant difference in the activities of ALT and AST in tissues of fish acclimated to 10 ppt salinity compared to control. This indicates that there was no excessive substrate mobilization for energy purposes in 10 ppt acclimatizing fish. The decreased hepatic ALT and AST activities in the fish in 10 ppt salinity suggest a lower hepatic potential for amino acid catabolism, implying a lower energy demand. This metabolic organization is consistent with the theory that the energetic cost of osmoregulation is lowest in an isosmotic environment, where the ionic gradients between blood and water would be minimal. The transdeamination of non-essential amino acids, like aspartate and alanine was known to be important pathways for energy production in fish (Walton and Cowey, 1982). Studies on climbing perch (*Anabas testudineus*) showed significant increases of both aspartate and alanine in muscles after six days of acclimation to sea water of 30 ppt salinity

(Chang *et al*., 2007a). Aspartate aminotransferase activity increased in gills following salinity change, suggesting an enhanced ability to utilize aspartate (Bystriansky *et al*., 2007). In addition, Bystriansky *et al*. (2007) also observed increases in alanine concentrations in gills and white muscles, and a decrease in the plasma after acclimation to sea water for 96 h. Mommsen *et al*. (1980) suggested that alanine may be the preferred carrier of amino acid nitrogen for inter tissue transport, as several amino acids can be converted to alanine, released to the blood, and used as a fuel source in other tissues.

Glutamate dehydrogenase (GDH) activity significantly increased in the gills, liver and kidney of animals acclimatizing to 20 ppt and 30 ppt compared to control and 10 ppt. Jurss *et al*. (1986) showed elevated GDH in liver of rainbow trout exposed to increasing salinity. Jurss *et al*. (1985) speculated that metabolic adaptation to elevated salinity may involve regulation by metabolite activation (e.g., activation of GDH by leucine). Thus, as amino acid levels (including leucine) rise in tissues as part of the osmotic adaptation, the activity of GDH would be enhanced. This would allow increased catabolism of amino acids to provide the energy needed for osmoregulation. Similarly Kultz and Jurss (1993) reported increased GDH activity in the gills and kidney tissue. Since GDH is activated by AMP and ADP (Hayashi *et al*., 1982) the observed increase in the activity of GDH in the gills and kidneys of fish exposed to 20 and 30 ppt salinity suggests that there is an increase in the concentrations of ADP and AMP due to the high activity of Sodium potassium ATPase. Muscle showed much decreased activity in all the groups. This suggests that glutamate dehydrogenase activity is either absent or virtually absent in skeletal muscle (Lowenstein, 1972). There is no significant difference in the activities of glutamate dehydrogenase of 10 ppt acclimatizing fish compared to control. This indicates that the oxidative deamination is minimum in isotonic salinity.

The arginase activity was significantly increased in liver and gills of fishes acclimatizing to 20 ppt and 30 ppt salinity. But arginase activity in the kidney and muscle of animals acclimatizing to 20 ppt and 30 ppt salinity showed no significant difference compared to control and 10 ppt. In the same way arginase activities increased in liver, but not in kidney or white muscle of rainbow trout in 20% seawater as reported by Jurss *et al*. (1987) and Ballantyne (2001). Vijayan *et al*. (1996a) reported that hepatic arginase activity was increased by cortisol in the ammoniotelic sea raven, *Hemipterus americanus.* Kammerer *et al*. (2010) reported that cortisol increased rapidly by 3h and remained elevated for 3 days in response to salinity stress in Tilapia. So the increased activity of arginase may be due to an elevation in the level of cortisol during acute salinity exposure.

The results of the present study show that oxygen consumption and ammonia excretion are influenced by salinity. In the present study the oxygen consumption rate of *O. mossambicus* at 30 ppt and 20 ppt was significantly higher than in control or 10 ppt salinity. There was no difference in the oxygen consumption rate of Tilapia between control and 10 ppt. Similar results have been reported by Morgan *et al*. (1997). No difference in the oxygen consumption rate of *O. mossambicus* was observed between control and 10 ppt in this acute study, suggesting that acclimation to 10 ppt does not impose an energetic demand on the Tilapia. Increased oxygen consumption at higher salinities could be attributed to the increase of the cost of osmoregulation and changes in the metabolism (Gracia-Lopez *et al*., 2006). Also, these results agree with those of Gracia-Lopez *et al*. (2006), Plaut (2000), Moser and Hettler (1989), who found that oxygen consumption, was directly related to salinity. Aristizabal-Abud (1992) found minimum oxygen consumption at iso-osmotic zone. These results are consistent with the theory that the energetic

cost of osmoregulation is lowest in an isosmotic environment, where the ionic gradients between blood and water would be minimal.

The plasma ammonia content was significantly increased in *Oreochromis mossambicus* acclimatizing to 20 ppt and 30 ppt salinity. The increase in plasma ammonia may be due to the increased catabolism of aminoacids and due to the elevated activity of AMP deaminase. Similar results were reported by Lee and Chen (2003) who observed that an increase in salinity caused elevated hemolymph ammonia in *Marsupenaeus japonicus.*

In freshwater and marine teleosts, ammonia excretion constitutes 60%-95% of nitrogen wastes, (Campbell and Anderson, 1991; Wood, 1993; Wright, 1993). In the present study, an increase was observed in the ammonia excretion of *Oreochromis mossambicus* at 20 ppt and 30 ppt salinity compared to control. But there was no significant variation in ammonia excretion at 10 ppt salinity compared to control. The increase in the ammonia excretion rate and plasma ammonia levels may be due to the increase in protein catabolism during acute changes in salinity. There is no significant difference in the plasma ammonia content and ammonia excretion rate of 10 ppt acclimatizing fish compared to control. This shows that the protein catabolism is minimum in isotonic salinity. Similar increase in ammonia excretion was observed in *Rivulus marmoratus* exposed to 15% and 30% sea water compared to those exposed to 0% salinity (Frick and Wright, 2002). An increase in metabolism due to stress has been documented to cause increased ammonia production (Mommsen *et al*., 1999), which could have played a role here. During stress, production of ammonia is stimulated by an elevation of catecholamines and cortisol (WendelaarBonga, 1997) and the consequent alteration of hepatic metabolism (Beaumont *et al*., 2003).

A significant increase in the ammonia quotient was observed in *O.mossambicus* when exposed to acute salinity change, which indicates increased protein degradation. Thus it is clear from the present study that proteins are very important fuels for energy production during stress conditions in fish

In conclusion, the present work signifies that the time immediately after salinity acclimation is critical. Lots of energy is required for osmoregulation during this critical phase. Proteins act as metabolic energy sources. Increased protein catabolism is evident in the present study. The proteins are catabolized and provide energy for osmoregulation. The study also indicates that the protein catabolism is minimum in isosmotic salinity. The results of this study provide additional information on the physiological changes in the oxygen consumption and nitrogen excretion of *Oreochromis mossambicus* during acute salinity change.

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Chapter 6

Effect of Acute Salinity Change on the Purine Catabolism of *Oreochromis mossambicus* (Peters)

6.1 Introduction

Any environmental disturbance can be considered as a potential source of stress, as it prompts a number of responses in the animal to deal with the physiological changes triggered by exterior changes (Martinez-Alvarez *et al*., 2002).These responses can be detected in fish and in other vertebrates in the form of changes in hormonal or substrate concentrations in the plasma or alterations in erythrocyte parameters, such as cell volume or enzyme activities (Donaldson, 1981). The internal perturbation of the fish, either directly or as a result of alterations of the environment affects the physiological mechanism of the animal. This will lead to adaptation to new conditions. Sometimes due to these physiological changes, survival can be threatened and death can result.

Estuarine euryhaline teleosts, normally experience rapid salinity change. When a euryhaline teleost goes from hyperosmotic (salt water) to hypo-osmotic (fresh water) media, it tends to gain water followed by loss of osmolytes and water. Alternatively, upon exposure to an increase in salinity, the initial effect is a loss in water followed by accumulation of osmolytes until the intracellular and extracellular osmolalities match one another.

The capacity to regulate plasma ions in the face of changing external salinity is an obvious necessity for fish that live in estuaries or that move between fresh water and seawater as part of their normal life cycle (Mccormick, 2001). The need to respond to salinity change may be rapid, such as during tidal cycles or rapid movements through estuaries, or slow, such as in the seasonal or ontogenetic acquisition of salinity tolerance in anadromous fish. (Mccormick, 2001) The former requires the rapid activation of existing mechanisms (transport proteins and epithelia), whereas the second requires the differentiation of transport epithelia and synthesis of new transport proteins.

Adaptation to sea water in euryhaline teleosts is thought to involve the development of ion secretion pathways in the mitochondria-rich cells of the gill epithelium under the influence of cortisol, growth hormone and insulin-like growth factors (Bern and Madsen, 1992; Jacob and Taylor, 1983; Mancera and McCormick, 1998). In a euryhaline freshwater teleost, the *Oreochromis mossambicus*, cortisol and growth hormone levels are elevated 1^{st} and 4^{th} day after transfer to sea water but not when these animals are transferred to brackish water or fresh water (Morgan *et al*., 1997). Nakano *et al*. (1998) observed an increase in growth hormone levels after transfer of tilapia to 70 % sea water, but at 12 h only. Transfer of tilapia through brackish water to full-strength sea water increases the size and number of mitochondria-rich cells in the opercular epithelium with a concomitant increase in Cl^- secretion rate (Foskett *et al*., 1981, 1983). Hence, fresh water resident teleosts with euryhaline capability, such as the Tilapia, readily adapt to salinity change over days if intermediate salinities are provided (Foskett *et al*., 1983; Nakano *et al*., 1998). When tilapia was transferred directly to sea water (35 ppt), they died within 4 hour. However, they survived after transfer to 25 ppt sea water (Hwang *et al*., 1989). The organs involved in osmoregulation in teleosts include the opercular membrane, gills, gut, kidney, and urinary bladder. The

gill is the primary organ that responds to the critical problem of salinity changes in teleostean fish.

Physiological response of fish to environmental stressors such as salinity evokes primary and secondary stress responses. Primary responses, which involve the initial neuroendocrine responses, include the release of catecholamines from chromaffin tissue (Barton, 2002; Randall and Perry, 1992; Reid *et al*., 1998a), and the stimulation of the hypothalamic-pituitary-interrenal (HPI) (Mccormick, 2001) axis culminating in the release of corticosteroid hormones into circulation (Donaldson, 1981; WendelaarBonga, 1997; Mommsen *et al.*, 1999). Secondary responses include changes in plasma and tissue ion and metabolite levels, hematological features, and heat shock or stress proteins (HSPs), all of which relate to physiological adjustments in metabolism, respiration, acid-base status, hydro mineral balance, immune function and cellular responses (Pickering, 1981; Iwama *et al.*, 1998; Mommsen *et al.*, 1999).

Numerous studies have investigated the effect of salinity changes in teleosts at a physiological level (quantification of osmoregulation, enzyme activity and hormone levels, urine volume measurement etc.) and in terms of the variations in the expression of specific mRNA (Hirose *et al*., 2003). The activity of ion-transporters is particularly well studied in seawater challenged fishes.

The studies on the purine catabolism of fish exposed to stress are rare. Purines are major components of nucleic acids and nucleotides. They are continuously formed and degraded in the biosphere. The purine nucleotides are synthesized de novo from phosphoribosyl pyrophosphate, amino acids, CO_2, and formate. When nucleotides are degraded to nucleobases and nucleosides, they may be reutilized via purine salvage pathways (Nygaard, 1983; Schultz, 2001) or further degraded. The ability to degrade purine compounds have been found in all kingdoms and can occur either aerobically or anaerobically, but by separate pathways (Vogels and van der Drift, 1976; DeMoll and Auffenberg,

1993). In the aerobic pathway, the committed step in the degradation of purine bases is the oxidation of hypoxanthine and xanthine to uric acid, catalyzed by xanthine dehydrogenase. The various purine-degradative pathways are unique and differ from other metabolic pathways because they may serve quite different purposes, depending on the organism or tissue. The purines or their immediate degradation products, which are subjected to further degradation, are abundant in nature. They arise from decaying tissue or organisms or are excreted by living cells. While some organisms degrade the naturally occurring purines to CO_2 and ammonia, other organisms contain only some of the steps of the purine degradation pathways, resulting in partial degradation of purines or certain intermediary compounds of the degradation pathway. In human, anthropoid apes, birds, uricotelic reptiles, and almost all insects, uric acid is the end product (Keilin, 1959; Wu *et al*., 1989), and it is subsequently excreted. Allantoin is the end product in uricolytic organisms such as most mammals, some insects, and gastropods (Fujiwara and Noguchi, 1995; Keilin, 1959). Fish, amphibians, and lamellibranchs completely degrade purines to urea, ammonia, and CO_2 (Hayashi *et al*., 1994; Keilin, 1959; Mommsen and Walsh, 1992).

The present study was conducted as an attempt to find out the changes in the purine catabolism of *O.mossambicus* exposed to acute salinity change. The present study examines the possible consequences that acute salinity change can cause on the activities of enzymes such as AMP deaminase, xanthine oxidase, uricase and allantoinase. In addition the plasma urea and uric acid levels were also estimated during acute salinity change.

6.2 Materials and Methods

Collection, maintenance, acclimation, experimental design, preparation of tissue and serum samples were the same as explained in detail in chapter3,

section 3.2. Methods used for the biochemical analysis and statistical analysis were the same as explained in chapter 4, section 4.2.1 and 4.2.2.

6.3 Results

6.3.1 Activity of AMP deaminase

The activity of AMP deaminase in different tissues of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant variations ($P<0.05$) compared with control group (Fig 6.1 and Table 6.1) (One-way ANOVA followed by Tukey's test). A statistically significant increase in AMP deaminase activity ($P<0.05$) was observed in gills, liver, kidney and muscle of the 20 ppt and 30 ppt exposed groups compared to control. No significant variation in the activity of AMP deaminase was observed in fish exposed to 10 ppt salinity compared to control group.

Figure 6.1 AMP deaminase activity in the various tissues exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA)

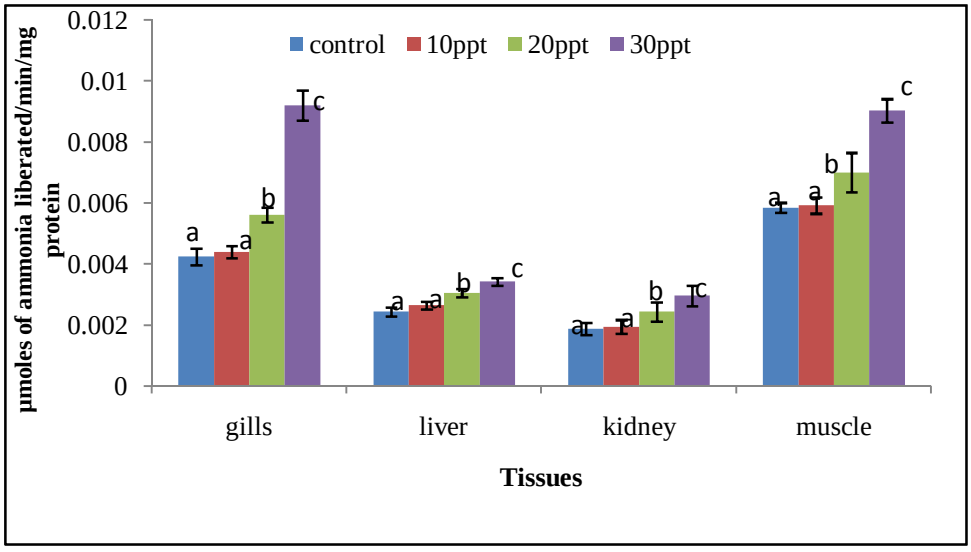

Table 6.1 Effect of exposure to acute salinity change on AMP deaminase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	AMP deaminase activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	0.00424±0.00027[a]	0.0044±0.0002[a]	0.0056±0.00024[b]	0.0092±0.0005[c]
Liver	0.0024±0.00014[a]	0.00264±0.00013[a]	0.00305±0.00014[b]	0.00342±0.00012[c]
Kidney	0.00188±0.0002[a]	0.00194±0.0000226[a]	0.00243±0.0003[b]	0.00296±0.00034[c]
Muscle	0.00585±0.00016[a]	0.00592±0.00027[a]	0.007±0.00065[b]	0.00904±0.00038[c]

Values are expressed as µ moles of ammonia liberated / min / mg protein.

Two factor ANOVA (Table 6.2) revealed that there was a significant increase (P<0.05) in AMP deaminase activity in all the salinity treated experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 6.5) revealed that there was significant difference (P<0.05) between control and 20 ppt and 30 ppt salinity treated groups and between 10 ppt and 20 ppt, 30 ppt groups. Between 20 ppt and 30 ppt there was a significant difference at P<005. There was no significant difference between 10 ppt and control group. Multiple comparison tests also (Table 6.6) revealed that there was a significant difference between tissues. The highest activities were seen in muscle tissue followed by gills.

Table 6.2 Two-factor ANOVA for tissue AMP deaminase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	9.94E-005	3	3.31E-005	320.741	0.000
Between Tissues	0.000	3	0.000	1184.804	0.000
concentration * Tissue	4.44E-005	9	4.94E-006	47.769	0.000
Error	8.27E-006	80	1.03E-007		
Total	0.002	96			

df- degrees of freedom

6.3.2 Activity of xanthine oxidase

In the present study the activity of xanthine oxidase in the liver tissue of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant increase ($P<0.05$) compared with control group (Fig 6.2 and Table 6.3) (One-way ANOVA followed by Tukey's test). No significant variation was observed in the xanthine oxidase activity in the liver tissue of 10 ppt exposed fish compared to control group. No statistically significant variation in xanthine oxidase activity was observed in gills, kidney and muscle of the acute salinity exposed fishes compared to control.

Figure 6.2 Xanthine oxidase activity in the various tissues exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

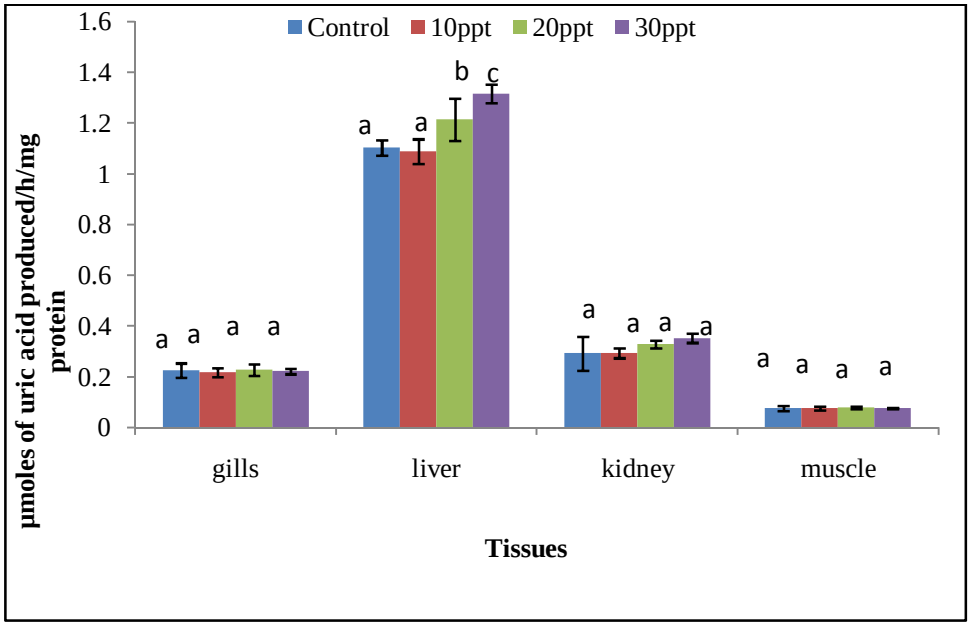

Table 6.3 Effects of exposure to acute salinity change on xanthine oxidase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Xanthine Oxidase activity			
	Groups			
	Control	10 ppt	20 ppt	30 ppt
Gills	0.225±0.028[a]	0.217±0.0185[a]	0.227±0.0232[a]	0.221±0.0111[a]
Liver	1.102±0.03[a]	1.088±0.05[a]	1.213±0.083[b]	1.315±0.037[c]
Kidney	0.292±0.067[a]	0.2935±0.02[a]	0.327±0.0154[a]	0.352±0.018[a]
Muscle	0.075±0.0098[a]	0.076±0.00757[a]	0.0776±0.0053[a]	0.0754±0.0015[a]

Values are expressed as µmoles of uric acid produced/h /mg protein

Two factor ANOVA (Table 6.4) revealed that there was a significant variation (P<0.05) in xanthine oxidase activity in acute salinity exposed experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 6.5) revealed that there was significant difference (P<0.05) between control and 20 ppt and 30 ppt salinity exposed groups and between 10 ppt, 20 ppt and 30 ppt salinity exposed groups. Between control and 10 ppt no significant variation was observed. Multiple comparison tests also (Table 6.6) revealed that there was a significant difference between tissues. The highest activities were seen in liver tissue.

Table 6.4 Two-factor ANOVA for tissue xanthine oxidase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between tissues	17.798	3	5.933	5030.474	0.000
Between concentrations	0.084	3	0.028	23.685	0.000
tissue * concentration	0.136	9	0.015	12.783	0.000
Error	0.094	80	0.001		
Total	37.425	96			

df- degrees of freedom

Table 6.5 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters	
	AMP deaminase	Xanthine Oxidase
Control Vs 10 ppt	0.513[d]	0.962[d]
Control Vs 20 ppt	0.000[a]	0.001[a]
Control Vs 30 ppt	0.000[a]	0.000[a]
10 ppt Vs 20 ppt	0.000[a]	0.000[a]
10 ppt Vs 30 ppt	0.000[a]	0.000[a]
20 ppt Vs 30 ppt	0.019[a]	0.019[a]

The values are significant at a=P<0.05 and not significant at d

Table 6.6 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters	
	AMP Deaminase	Xanthine Oxidase
Gills Vs Liver	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]
Liver Vs Kidney	0.000[a]	0.000[a]
Liver Vs Muscle	0.000[a]	0.000[a]
Kidney Vs Muscle	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

6.3.3 Activity of uricase

Uricase activity in the liver of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant increase (P<0.05) compared to control (Fig 6.3 and Table 6.7). One-way ANOVA followed by Tukey's test revealed a significant increase at (P<0.05). No statistically significant variation in uricase activity (P<0.05) was observed in 10 ppt salinity exposed group compared to control.

Fig 6.3 Effects of acute salinity change on uricase activity in the liver of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

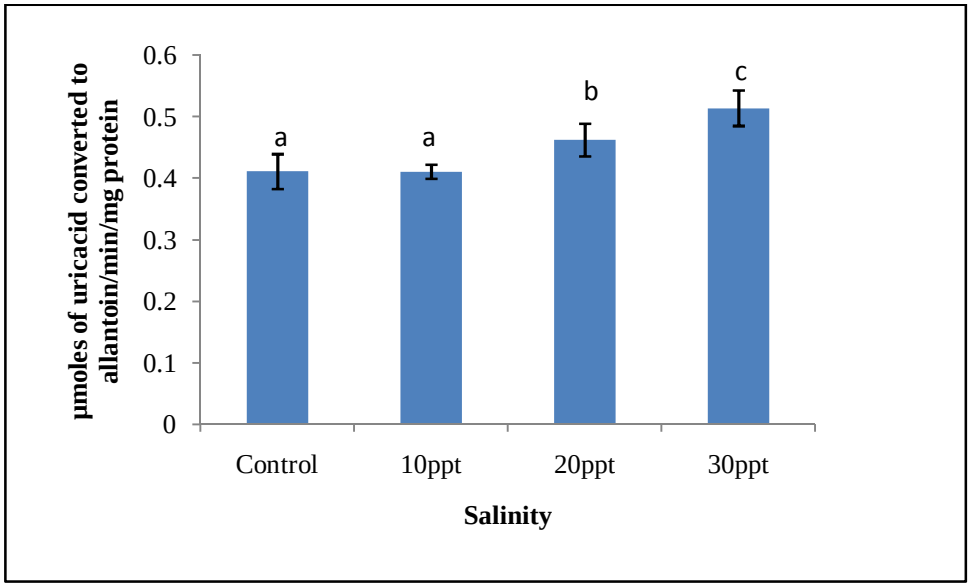

Table 6.7 Effects of exposure to acute salinity change on uricase activity (mean ± S.D) present in the liver of *O.mossambicus.* Values with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Group	Control	Salinity		
		10 ppt	20 ppt	30 ppt
Uricase activity	0.411±0.028[a]	0.41 ±0.0114[a]	0.462±0.027[b]	0.514±0.029[c]

Values are expressed as micromoles of uric acid converted to allantoin / minute / mg protein.

Table 6.8 One way ANOVA for uricase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	0.044	3	0.015	23.598	0.000
Within Concentration	0.012	20	0.001		
Total	0.056	23			

df- degrees of freedom

6.3.4 Activity of allantoinase

In the present study the activity of allantoinase in the liver of *O. mossambicus* exposed to 20 ppt and 30 ppt salinity showed significant increase

(P<0.05) compared to control (Fig 6.4 and Table 6.9) (One-way ANOVA followed by Tukey's test). No significant variation in allantoinase activity was observed in the liver of 10 ppt salinity exposed fish compared to control group.

Fig 6.4 Effects of acute salinity change on allantoinase activity in the liver of *O. mossambicus*. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

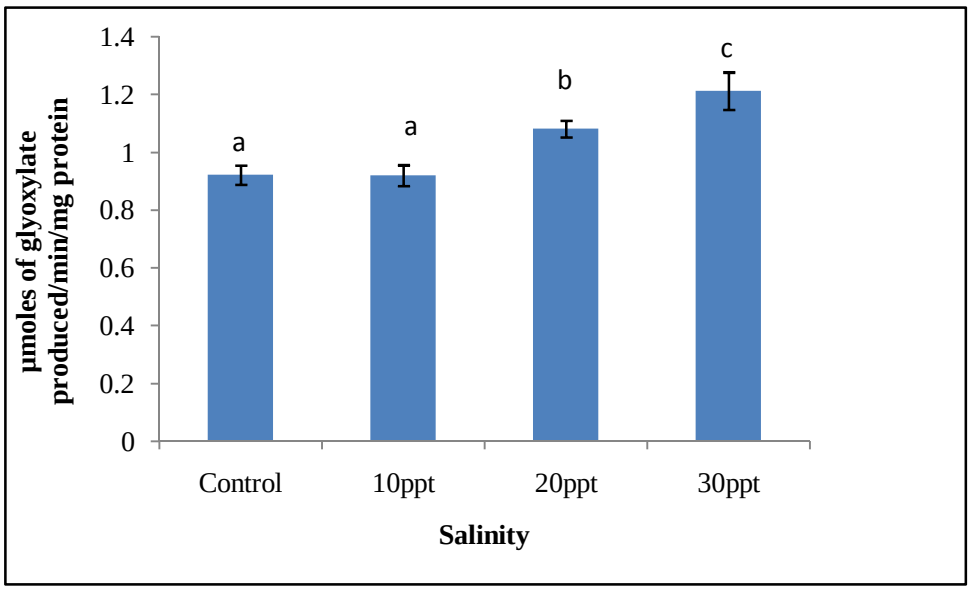

Table 6.9 Effects of exposure to acute salinity change on allantoinase activity (mean ± S.D) present in the liver of *O.mossambicus*. Values with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Group	Control	Salinity		
		10 ppt	20 ppt	30 ppt
Allantoinase activity	0.9213±0.033[a]	0.9198±0.04[a]	1.081±0.03[b]	1.21±0.065[c]

Values are expressed as micromoles of glyoxylate produced / min / mg protein.

Table 6.10 One way ANOVA for allantoinase activity

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.358	3	0.119	53.324	0.000
Within concentration	0.045	20	0.002		
Total	0.403	23			

df- degrees of freedom

6.3.5 Serum urea

Urea content in serum of *O. mossambicus* treated with 20 ppt and 30 ppt salinity showed significant increase ($P<0.05$) compared to control group (Fig 6.5 and Table 6.13) (One-way ANOVA followed by Tukey's test) . There was no significant variation observed in serum urea content of 10 ppt salinity exposed fish compared to control group. The ANOVA table is shown below (Table 6.11).

Figure 6.5 Levels of urea in the serum of *O.mossambicus* exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

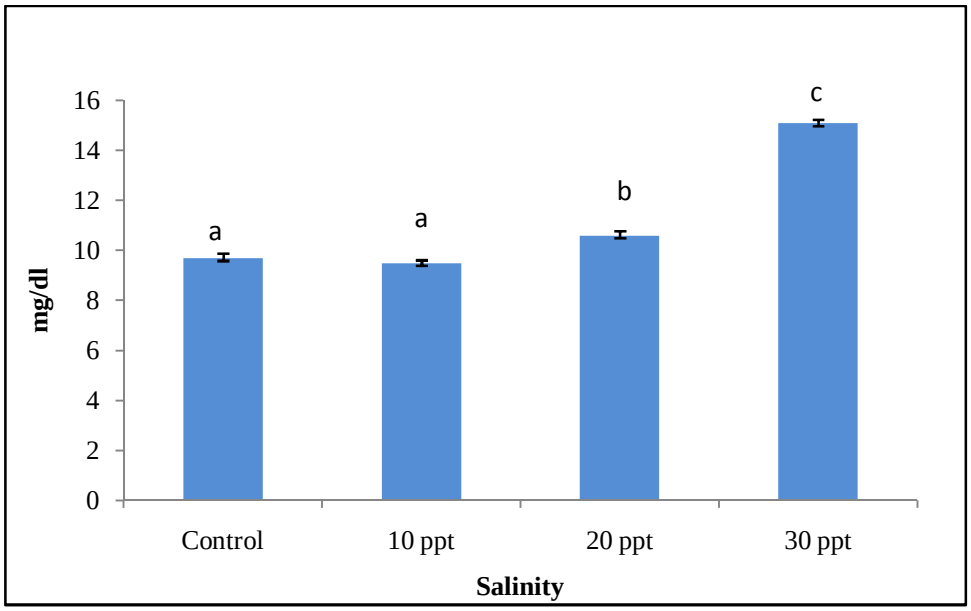

Table 6.11 One way ANOVA for serum urea

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	120.052	3	40.017	1648.494	0.000
Within concentration	0.486	20	0.024		
Total	120.537	23			

df-degrees of freedom

6.3.6 Serum uric acid

One-way ANOVA followed by Tukey's test showed that there was significant ($P<0.05$) increase in the serum uric acid content in 20 ppt and 30 ppt salinity exposed fish compared to control (Fig 6.7 and Table 6.13). The ANOVA table is shown below (Table 6.12). There was no significant variation observed in the serum uric acid content of fish exposed to 10 ppt salinity compared to control. No significant difference was observed between 20 ppt and 30 ppt salinity exposed groups.

Figure 6.6 Levels of uric acid in the serum of *O.mossambicus* exposed to acute salinity change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

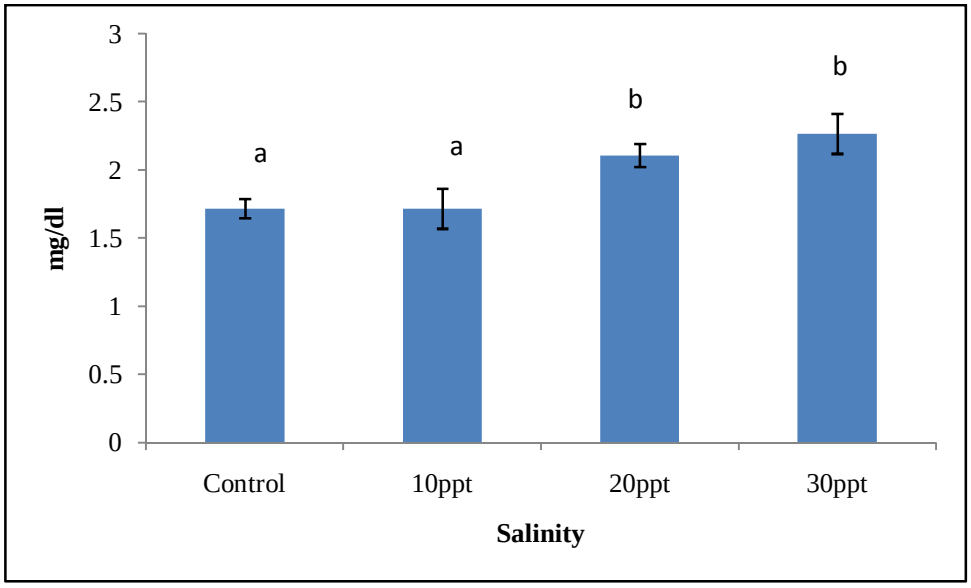

Table 6.12 One way ANOVA for serum uric acid

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	1.401	3	0.467	28.310	0.000
Within concentration	0.330	20	0.016		
Total	1.730	23			

df-degrees of freedom

Table 6.13 Effects of exposure to acute salinity change on serum parameters of *O.mossambicus.* Values in the same raw with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Serum parameters	Control	Salinity		
		10 ppt	20 ppt	30 ppt
Urea	9.7±0.17[a]	9.5±0.1[a]	10.6 ±0.16[b]	15.1±0.12[c]
Uric acid	1.718 ± 0.072[a]	1.716 ±0.15[a]	2.11 ±0.084[b]	2.27 ± 0.15[b]

Values are expressed as mg/dl

6.4 Discussion

Adaptive mechanisms occurring during acute salinity exposure are energetically expensive. The additional energy requirement of osmoregulation during the initial crisis period causes biochemical changes in the adenylate pool (Leray *et al*., 1981). A progressive increase in the chloride cells together with the buildup of an efficient Na+-K+ ATPase activity (Thomson and Sargent, 1977; Bouef *et al*., 1978) was seen during initial periods of salinity acclimation. These metabolic responses result in a drop of circulating ion concentration which is further supported by a significant fall in gill adenylate energy charge (Leray *et al*., 1981). Conditions of stress cause a decrease in the value of energy charge (Atkinson, 1977) (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al*. (1971) the value of energy charge $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$). To stabilize energy charge AMP deaminase gets activated. The activation of AMP deaminase when energy charge falls might be a useful means of limiting the extent of sudden drops in energy charge (Atkinson, 1977). The AMP deaminase converts AMP in to IMP. This has a short term benefit by effectively maintaining a high intra muscular ATP/ADP ratio. However IMP can be further degraded to hypoxanthine and then to uric acid. Uric acid then converts to urea via the uricolytic pathway in fishes.

In the present study an increased activity of AMP deaminase was observed in the gills, liver, kidney and muscle tissue of *O.mossambicus* exposed to 20 ppt and 30 ppt salinity for 48 hours. Raffin (1986) reported an increased activity of AMP deaminase, when trout were transferred to sea water or during reverse transfer to fresh water. According to Dorge *et al*. (1981), variations of the salinity of the environment may induce perturbations in the intracellular concentrations of cations. The AMP deaminase isoenzymes from trout gill were activated by sodium and potassium ions, sodium being the most efficient (Raffin, 1984). Thus the increase in the activity of AMP deaminase observed in the present study may due to the activation by sodium or potassium ions due to the increase in the salinity.

According to Raffin (1986) the salinity stress, impose a high energy demand. In this case, a fall in the adenylate energy charge (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al*. (1971) the value of energy charge $\left(EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}\right)$ is observed. AMP deaminase may stabilize the adenylate energy charge (Chapman and Atkinson, 1973; Solano and Coffee, 1978). Limited proteolysis will result in increased AMP deaminase activity under conditions of increased energy demand, where the concentration of inorganic phosphate is dramatically increased. Therefore, regulation of AMP deaminase by limited proteolysis can be considered as a safety mechanism, allowing preservation of the adenylate energy charge under conditions where energy demand exceeds the capacities of glycolysis and/or cellular oxidation. So the limited proteolysis of AMP deaminase, inducing increased enzymatic activity, could be a response to stress.

In the present study the activity of xanthine oxidase was increased in fish exposed to 20 ppt and 30 ppt salinity compared to control. There was no difference in the activity of xanthine oxidase in fish exposed to 10 ppt salinity compared to control group. Cheng *et al*. (2004) observed activities of xanthine dehydrogenase and xanthine oxidase in hepatopancreas increased directly with salinity level in kuruma shrimp *Marsupenaeus japonicus.* Increased activity of xanthine oxidase in fish exposed to hyper-osmotic conditions suggests an enhanced degradation of purine nucleotides leading to uricogenesis.

An increased uricase and allantoinase activity was observed in fish exposed to 20 ppt and 30 ppt salinity. This increased activity may be due to an elevation in the cortisol during acute salinity exposure. Kammerer *et al*. (2010) reported that cortisol increased rapidly by 3h and remained elevated for 3days in response to salinity stress in tilapia. In support of our data, Vijayan *et al*. (1996a) observed that exposure to exogenous cortisol triggers an increase in allantoicase activity in the ammonotelic sea raven, *Hemipterus americanus.* Rodela and Wright (2006) have reported that cortisol increases uricolysis in the mangrove killifish *Rivulus marmoratus.*

The serum urea and uric acid content were increased in *O.mosambicus* exposed to 20 ppt and 30 ppt salinity compared to control group. In *O.mossambicus* urea is mainly formed through the hydrolysis of arginine (argininolysis), and degradation of uric acid occurs through the uricolytic pathway. Uric acid is formed by the degradation of nucleic acids (Claybrook, 1983; Regnault, 1987), and is also formed by de novo synthesis from excess dietary nitrogen and glutamine, asparate and glycine (Linton and Greenaway, 1997a). The increase in urea content is due to the elevated activities of arginase and uricolytic enzymes such as uricase and allantoinase. The elevated uric acid content may be due to the elevated activity of xanthine oxidase.

Marsupenaeus japonicus reared in a salinity of 42% shows significant increases in hemolymph urea levels and urea excretion (Lee and Chen, 2003). Increases of hemolymph urea and uric acid were observed in *Marsupenaeus japonicus* when salinity increased from 18% to 34% and 42% (Lee and Chen, 2003). Tiger shrimp (*Penaeus monodon*), which have an isosmotic point of 26.5 ppt, show higher hemolymph urea levels when placed in a salinity of 45 ppt (Fang *et al*., 1992). Higher urea excretion and hemolymph urea have also been observed in mud crab (*Scylla serrata*) reared in a salinity of 40 ppt (Chen and Chia, 1996).

O.mossambicus exposed to acute salinity change showed significant increase in the activities of enzymes related to purine catabolism. This results in the elevation of urea and uric acid content in the serum. In conclusion the acute salinity exposure increased the urea production of *O.mossambicus* from purines.

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Chapter 7

Effect of Acute Changes in Temperature on the Protein Catabolism of *Oreochromis mossambicus*

7.1 Introduction

Temperature is one of the essential and unstable environmental factors. It penetrates into every region of the biosphere and profoundly influences all forms of life by increasing or decreasing some of the vital activities of the organism. It is frequently a limiting factor for the growth or distribution of animals and plants. An animal's response to wide ranges in temperature is influenced by its physiology. All invertebrates, lower chordates, cyclostomes, fishes, amphibians and reptiles have no internal mechanism for thermal regulation, and their body temperatures vary with the surrounding environmental temperatures. Such animals are called cold blooded, ectothermic or poikilothermic organisms. Temperature directly affects the rate of all biological processes, such as food intake, metabolism and nutritional efficiency (Brett, 1979; Burel *et al*., 1996).

Water temperature is one of the most important factors affecting survival, growth, feeding, and other physiological performances in aquatic ectotherms (Van Weerd and Komen, 1998; Martinez-Alvarez et al., 2005). Generally, growth

increases with rising temperature. Fish are ectothermic vertebrates that inhabit an aquatic environment with high temperature conductivity. As a result, temperature is an important factor influencing their biogeographic distribution over evolutionary time. In addition, daily and seasonal temperature fluctuations have an important impact during the lifetime of individual fish. Therefore, fish is a convenient model to study the effects of thermal stress in the intact organism on both short and long time scales (Basu *et al*., 2002). However, an increase in the water temperature beyond the optimum limits of a particular species accelerates the metabolic rate and subsequently increases oxygen consumption in fish. This adversely affects fish health and even results in death (Van Weerd and Komen, 1998). The increase in tissue oxygen consumption may also stimulate the production of reactive oxygen species (ROS), which may cause oxidative stress (Lesser, 2006). As an adaptive response to challenging environmental conditions, the cells subjected to high temperature increase their antioxidant defenses, particularly the antioxidant and associated enzymes (Martinez-Alvarez *et al*., 2005). Oxygen consumption is a widely studied indicator of metabolic rate, and temperature has a profound influence on metabolic processes in poikilothermic animals such as fish (Brett and Groves, 1979). Metabolic rate is reported frequently to overshoot following a temperature increase, and this is followed usually by a decline to an intermediate level, i.e. partial compensation (Alderdice, 1976; Cossins and Bowler, 1987; Evans, 1990).

Acclimation to colder temperatures has been shown to greatly affect physiological and biochemical homeostasis (Alsop *et al*., 1999). Cellular alterations in the activities of key oxidative enzymes (Sidell, 1980; Johnston and Dunn, 1987) and increased density of mitochondria and lipid droplets have been observed with lower acclimation temperature (Egginton and Sidell, 1989). Also, changes at the organ level occur, such as decreased cardiac output (Farrell,

1997), decreased blood flow to all organs except red muscle (Taylor *et al*., 1993, 1996; Wilson and Egginton, 1994) and increased amounts of red muscle (Sidell, 1980). Whole-animal effects such as altered behaviour (Crawshaw and O'Connor, 1997) and decreased swimming performance (Beamish, 1978; Johnston and Ball, 1997) at lower temperature have also been observed.

Bullock (1955) shows that several poikilotherms can acclimatize to the temperature change by suitable compensation in their metabolism. Houston and DeWilde (1968) observed that the red blood cells, the packed cell volume and the hemoglobin vary directly with temperature in *Cyprinus carpio*. Concentration of cortisol increases in *Oncorhyncus tshawytscha* and *Salmo clarkiclarki* (Strange *et al*., 1977). In *Sarotherodon mossambicus*, a temperature rise triggers more phosphorylase activity, enhances hepatic glycogenesis and increases the glucose concentration in blood (Radhakrishnaiah and Parvatheswararao, 1984). Zaragoza *et al*. (2008) studied the effect of thermal stress on hematological parameters in *O. mossambicus*. Rao and Ramachandra (1961) reported that the osmotic pressure and the content of chloride and free amino acids of the blood undergo systematic change during acclimatization to high temperature in the freshwater field crab, *Paratelphusa*sp and the freshwater mussel, *Lamellidens marginali*. Amino acid incorporation and polypeptide chain elongation rates were determined in toadfish at the upper and lower ends of their range of temperature tolerance (Haschemeyer and Mathews, 1982).

Proteins are essential in all living organisms, performing roles ranging from structural to catalytic. The synthesis and degradation of proteins is therefore a fundamental physiological process, and an animal's protein pool is in a continual state of flux, with new proteins entering the pool via protein synthesis and being removed via protein degradation. Protein synthesis is energetically expensive, accounting for 11–42% of basal metabolism in a

range of ecto- and endotherms, and is therefore a major component of overall animal energetics (Houlihan *et al*., 1995a). The continual synthesis and degradation of proteins is not only vital for tissue maintenance and animal growth but is also important in allowing animals to adapt to changing environmental conditions, to replace denatured or damaged proteins, to mobilize amino acids and to allow metabolic regulation (Hawkins, 1991). Ammonia excretion by fish is an indicator of the intensity of protein catabolism (Brett and Zala, 1975; Kutty, 1978; Wood, 1993), Studies of ammonia excretion are less frequent than those of oxygen consumption, and the influence of temperature on ammonia excretion has been studied for relatively few fish species.

However, the effects of temperature on protein catabolism of fish have rarely been studied. It is, important to have information about the effects of temperature on the protein catabolism of fish in order to gain a better insight into metabolic adaptations occurring during adverse conditions.

7.2 Materials and Methods

Collection, maintenance, acclimation, preparation of tissue and serum samples, methods used for biochemical analysis and statistical analysis were the same as explained in detail in chapter3, section 3.2.

7.2.1 Experimental design for the study of the effects of acute changes in temperature

For conducting biochemical studies *O.mossambicus* (15±3g) were taken in two separate tubs containing tap water. They were gradually acclimatized to 15°C and 40°C respectively by slowly raising the water temperature within 12 hours. Six replicates were kept for each experiment. The experimental animals were

exposed for 72 hours. During the experimental period the animals were fed on the same diet so as to avoid the effects of starvation on normal physiological processes and antioxidant stress. The water was changed daily to maintain the dissolved oxygen concentration at optimum level (USEPA, 1975). Suitable controls were maintained at 25°C. Feeding was stopped 24 hour prior to the sacrifice.

7.3 Results

7.3.1 Total Protein

In the present study the Protein content in different tissues of *O. mossambicus* exposed to 15°C and 40°C showed significant decrease ($P<0.05$) compared to control group (Fig 7.1 and Table 7.1) (One-way ANOVA followed by Tukey's test) .

Figure 7.1 Protein content in the various tissues exposed to different temperatures. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

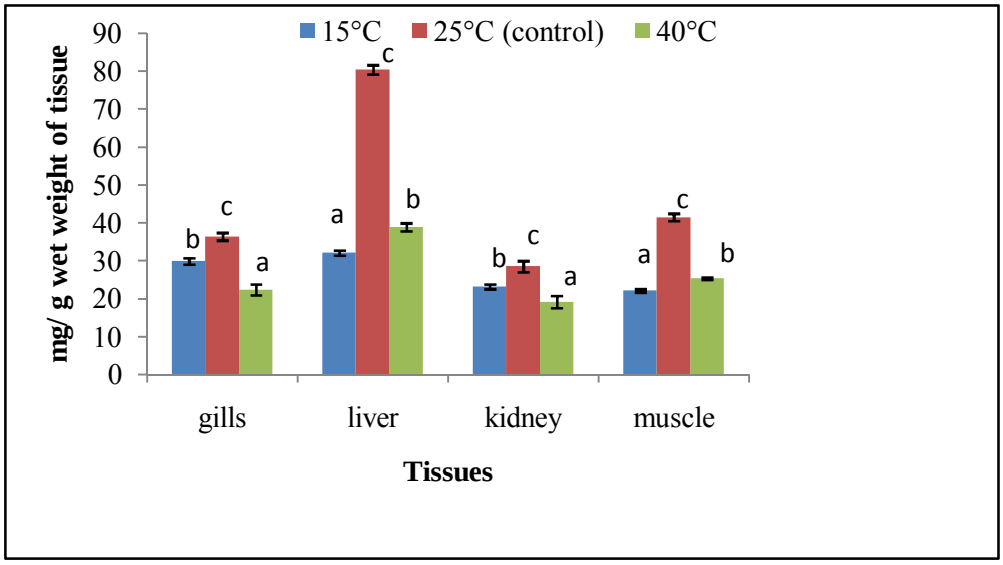

Table 7.1 Effects of exposure to acute temperature change for 72 hours on total protein content (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Tissue Protein		
	Groups		
	15°C	25°C (control)	40°C
Gills	29.89±0.79^{b}	36.381±1.02^{c}	22.34±1.49^{a}
Liver	32.1±0.70^{a}	80.42±1.23^{c}	38.9±1.03^{b}
Kidney	23.12±0.61^{b}	28.5±1.45^{c}	19.13±1.56^{a}
Muscle	22.09±0.42^{a}	41.44±0.998^{c}	25.26±0.34^{b}

Values are expressed as mg/g wet weight of tissue

Two factor ANOVA (Table 7.2) revealed that there was a significant decrease (P<0.05) in protein content in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison using Tukey's test (Table 7.13) revealed that there was significant difference (P<0.05) in protein content between control and 15°C and 40°C temperature exposed groups. There is no significant difference between 15°C and 40°C temperature exposed groups. Similarly multiple comparison using Tukey's test (Table 7.14) also revealed that there was a significant difference between all the tissues except gill and muscle.

Table 7.2 Two-Factor ANOVA for Total Protein

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	3227.287	2	1613.643	1471.017	0.000
Between tissues	3754.746	3	1251.582	1140.958	0.000
concentration * tissues	1953.416	6	325.569	296.793	0.000
Error	26.327	24	1.097		
Total	48879.712	36			

df- degrees of freedom

7.3.2 Free amino acid content

In the present study the free amino acid content in different tissues of *O. mossambicus* exposed to 15°C and 40°C showed significant increase (P<0.05)

compared with control (Fig 7.2 and Table 7.3) (One-way ANOVA followed by Tukey's test) .

Figure7.2 Free amino acid content in the various tissues exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

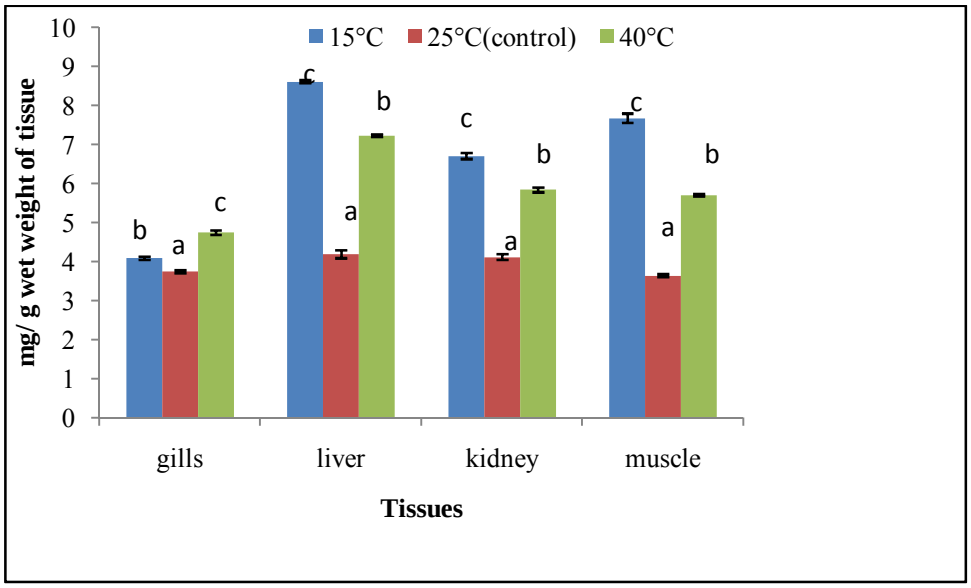

Table 7.3 Effects of acute change in temperature on free amino acid content (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Tissue free aminoacid		
	Groups		
	15°C	25°C(control)	40°C
Gills	4.09±0.045^{b}	3.755±0.039^{a}	4.75±0.061^{c}
Liver	8.614±0.036^{c}	4.192±0.099^{a}	7.23±0.029^{b}
Kidney	6.71±0.077^{c}	4.12±0.073^{a}	5.85±0.065^{b}
Muscle	7.68±0.12^{c}	3.65±0.038^{a}	5.7±0.032^{b}

Values are expressed as mg/g wet weight of tissue.

Two factor ANOVA (Table 7.4) revealed that there was a significant increase (P<0.05) in free amino acid content in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison

using Tukey's test (Table 7.13) revealed that there was significant difference ($P<0.05$) in free amino acid content between control and 15°C and 40°C temperature exposed groups. Similarly multiple comparison using Tukey's test (Table 7.14) also revealed that there was a significant difference between tissues also. Among the tissues liver showed highest increase followed by muscle and kidney.

Table 7.4 Two-Factor ANOVA for tissue free amino acids

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	101.636	2	50.818	11705.378	0.000
Between tissues	56.001	3	18.667	4299.745	0.000
concentration * tissues	32.437	6	5.406	1245.231	0.000
Error	0.260	60	0.004		
Total	2390.780	72			

df-degrees of freedom

7.3.3 Activity of alanine aminotransferase

In the present study the activity of alanine aminotransferase in different tissues of *O. mossambicus* exposed to 40°C showed significant increase ($P<0.05$) compared with control group. The gill and liver tissues of 15°C exposed groups showed a decreased activity compared to control group. But the muscle tissue of the group exposed to 15°C showed an increased activity compared to control. The kidney tissue of the group exposed to 15°C did not show any significant difference in the ALT activities compared with the control. The alanine aminotransferase activity in different tissues of *O. mossambicus* exposed to 15°C showed significant difference ($P<0.05$) compared with 40°C exposed groups. (Fig 7.3 and Table7.5) (One-way ANOVA followed by Tukey's test).

Fig 7.3 Effect of acute temperature change on ALT activity in the various tissues of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

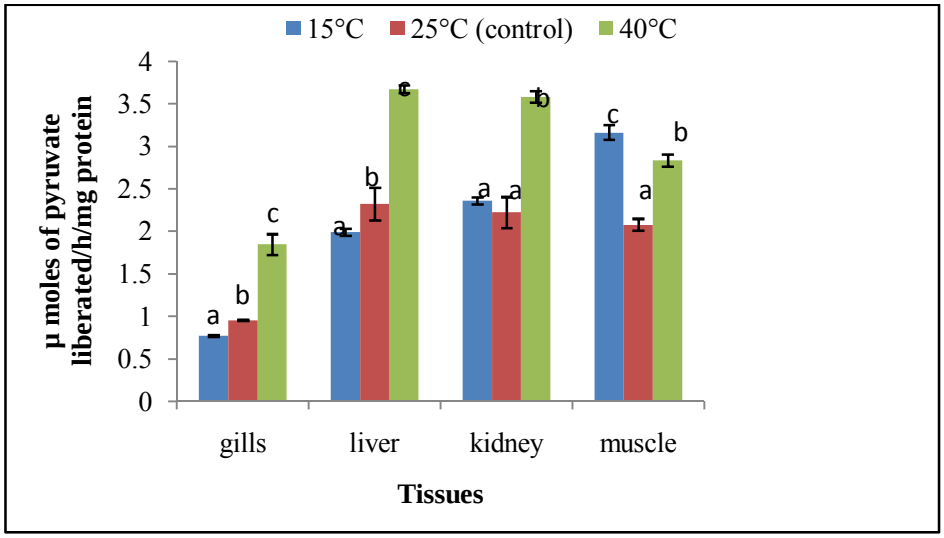

Table 7.5 Effects of acute change in temperature on alanine aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	ALT activity		
	Groups		
	15°C	25°C (control)	40°C
Gills	0.766±0.012^{a}	0.952±0.0076^{b}	1.84±0.121^{c}
Liver	1.987±0.04^{a}	2.32±0.191^{b}	3.67±0.046^{c}
Kidney	2.357±0.04^{a}	2.2±0.182^{a}	3.58±0.07^{b}
Muscle	3.16±0.09^{c}	2.075±0.071^{a}	2.83±0.072^{b}

Values are expressed in µmoles of pyruvate liberated/h/mg protein

Two way ANOVA (Table 7.6) followed by comparisons with multiple comparison using Tukey's test (Table 7.13) revealed that all the concentrations selected for the study differed significantly from one another. Similarly multiple comparison using Tukey's test (Table 7.14) also revealed that there was a significant difference between gill and liver or gill and kidney or gill and muscle. But there was no significant difference between liver and kidney or liver and muscle or kidney and muscle.

Table 7.6 Two-Factor ANOVA for tissue ALT activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	8.207	2	4.103	440.799	0.000
Between Tissues	15.240	3	5.080	545.712	0.000
concentration×tissue	3.772	6	0.629	67.536	0.000
Error	0.223	24	0.009		
Total	220.084	36			

df- degrees of freedom

7.3.4 Activity of aspartate aminotransferase

There was a significant increase ($P<0.05$) in the activity of Aspartate aminotransferase in different tissues of *O. mossambicus* exposed to 15°C and 40°C compared to control (Fig 7.4 and Table 7.7) (One-way ANOVA followed by Tukey's test)

Figure 7.4 AST activity in the various tissues exposed to acute changes in temperature. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different treatments (One-way ANOVA).

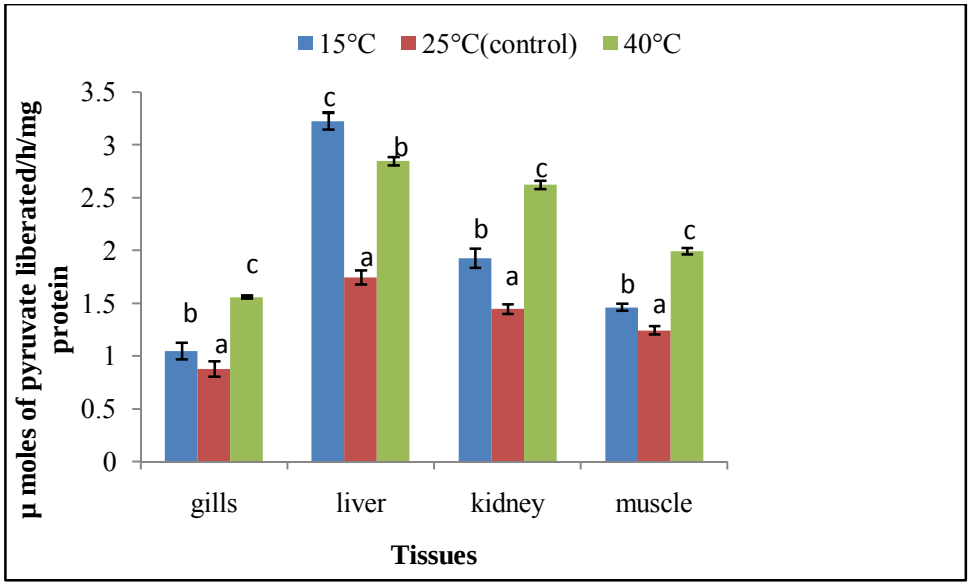

Table 7.7 Effects of exposure to acute changes in temperature on aspartate aminotransferase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	AST activity		
	Groups		
	15°C	25°C (control)	40°C
Gills	1.05±0.079[b]	0.88±0.075[a]	1.56±0.015[c]
Liver	3.23±0.08[c]	1.75±0.066[a]	2.85±0.04[b]
Kidney	1.93±0.09[b]	1.45±0.046[a]	2.62±0.042[c]
Muscle	1.46±0.035[b]	1.24 ± 0.039[a]	2.0±0.029[c]

Values are expressed in µmoles of pyruvate liberated/h/mg protein

Two way ANOVA (Table 7.8) followed by comparisons with multiple comparison using Tukey's test (Table 7.13) revealed that all the concentrations selected for the study differed significantly from one another. Subsequent pair wise comparison between various tissues were carried out using Tukey's test (Table 7.14) which indicated significant difference (P<0.001). Among the tissues liver showed highest activity.

Table 7.8 Two-factor ANOVA for tissue AST activity

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	10.570	2	5.285	1586.839	0.000
Between Tissues	20.653	3	6.884	2067.110	0.000
concentration×tissue	4.032	6	0.672	201.781	0.000
Error	0.200	60	0.003		
Total	278.041	72			

df-degrees of freedom

7.3.5 Activity of glutamate dehydrogenase

In the present study the activity of glutamate dehydrogenase in different tissues of *O. mossambicus* exposed to 15°C and 40°C showed significant variations (P<0.05) compared with control group (Fig 7.5 and Table 7.9) (One-way ANOVA followed by Tukey's test). A statistically significant increase in

glutamate dehydrogenase (P<0.05) was observed in liver, kidney, gills and muscle of the 40°C exposed groups compared to control. A decreased activity (P<0.05) was found in different tissues of *O. mossambicus* exposed to 15°C compared to control.

Figure7.5 GDH activities in the various tissues exposed acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

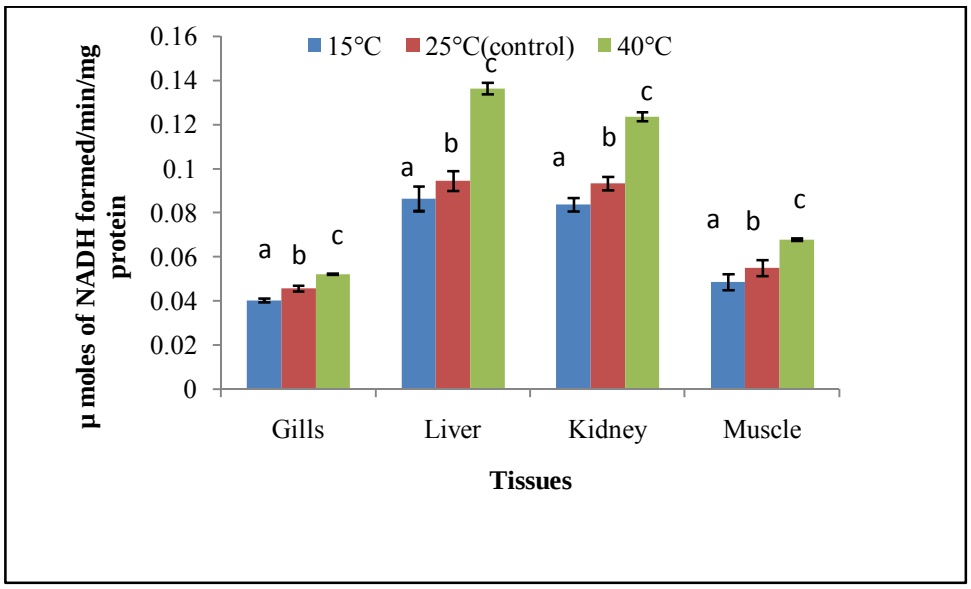

Table 7.9 Effects of exposure to acute change in temperature to glutamate dehydrogenase activity (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	GDH activity		
	Groups		
	15°C	25°C (control)	40°C
Gills	0.0404±0.00084[a]	0.046±0.0014[b]	0.052±0.00031[c]
Liver	0.086±0.0055[a]	0.094±0.0046[b]	0.136±0.0026[c]
Kidney	0.0837±0.003[a]	0.0932±0.0031[b]	0.124±0.002[c]
Muscle	0.0486±0.004[a]	0.055±0.0036[b]	0.068±0.0006[c]

Values are expressed in µmoles of NADH formed/min/mg protein

Two factor ANOVA (Table 7.10) revealed that there was a significant variation ($P<0.05$) in glutamate dehydrogenase activity in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table7.13) revealed that there was significant difference ($P<0.05$) between control and 15°C or 40°C exposed groups and between 15°C and 40°C exposed groups. Multiple comparison tests also (Table 7.14) revealed that there was a significant difference between tissues also.

Table 7.10 Two-factor ANOVA for tissue GDH activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Concentrations	0.012	2	0.006	651.667	0.000
Between Tissues	0.049	3	0.016	1771.710	0.000
concentration * tissue	0.003	6	0.001	63.350	0.000
Error	0.001	60	9.17E-006		
Total	0.495	72			

df-degrees of freedom

7.3.6 Activity of arginase

In the present study the activity of arginase in gills, liver and kidney of *O. mossambicus* exposed to 15°C showed significant decrease ($P<0.05$) compared with control group (Fig 7.6 and Table 7.11) (One-way ANOVA followed by Tukey's test). Significant increase ($P<0.05$) was observed in arginase activity in gills, liver and kidney tissue of the fish exposed to 40°C compared to control group (Fig 7.6 and Table 7.11) (One-way ANOVA followed by Tukey's test). The Muscle showed no or very little arginase activity.

Figure 7.6 Arginase activity in the various tissues exposed to acute changes in temperature. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

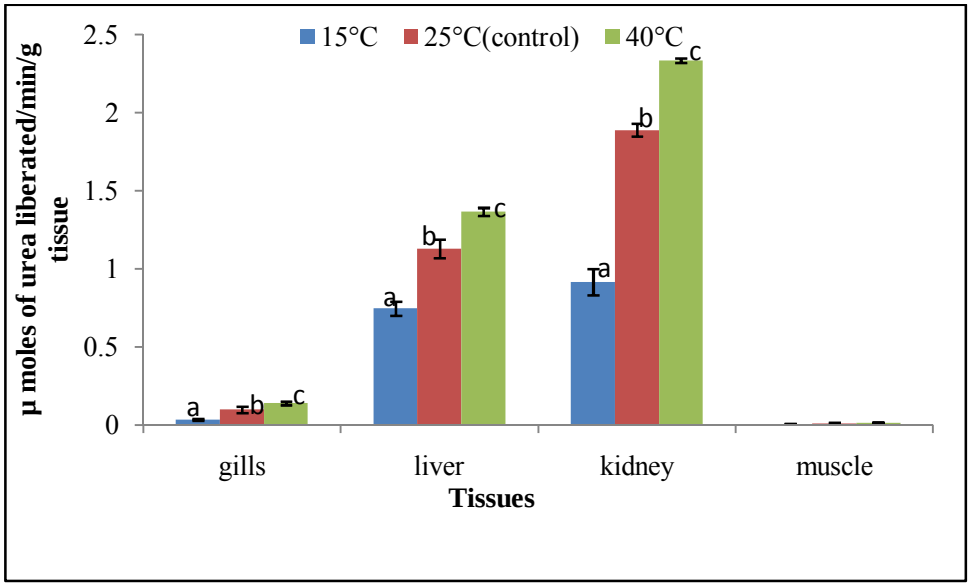

Table 7.11 Effects of exposure to acute temperature change on arginase activity (mean ± S.D) present in different tissues of *O.mossambicus*. Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Tissues	Arginase activity		
	Groups		
	15°C	25°C (control)	40°C
Gills	0.033±0.008[a]	0.096±0.020[b]	0.137±0.011[c]
Liver	0.745±0.045[a]	1.126±0.059[b]	1.363±0.026[c]
Kidney	0.913±0.085[a]	1.89±0.041[b]	2.331±0.014[c]
Muscle	0.005±.001[a]	0.01±0.0034[a]	0.0133±0.0006[a]

Values are expressed in µmole urea formed/min/g tissue

Two factor ANOVA (Table 7.12) revealed that there was a significant variation (P<0.05) in arginase activity in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 7.13) revealed that there was significant difference (P<0.05) between control and 15°C and 40°C exposed groups and between 15°C and 40°C

exposed groups. Multiple comparison tests also (Table 7.14) revealed that there was a significant difference between tissues.

Table 7.12 Two-factor ANOVA for tissue arginase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	1.790	2	0.895	676.768	0.000
Between tissues	18.103	3	6.034	4561.976	0.000
concentration * tissues	1.962	6	0.327	247.273	0.000
Error	0.032	24	0.001		
Total	40.638	36			

df-degrees of freedom

Table7.13 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters					
	ALT	AST	GDH	Protein	Freeamino acids	Arginase
Control Vs15° C	0.000[a]	0.988[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Control Vs40° C	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
15° C Vs40° C	0.000[a]	0.000[a]	0.000[a]	0.634[d]	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

Table 7.14 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters					
	ALT	AST	GDH	Protein	Free aminoacid	Arginase
Gills Vs Liver	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]	0.000[a]	0.999[d]	0.000[a]	0.001[a]
Liver Vs Kidney	0.560[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Liver Vs Muscle	0.915[d]	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Kidney Vs Muscle	0.907[d]	0.043[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

7.3.7 Plasma ammonia

One-way ANOVA followed by Tukey's test showed that there was significant (P<0.05) increase in the plasma ammonia in 40°C exposed groups

compared to control. The plasma ammonia content decreased significantly (P<0.05) in 15°C exposed groups compared to control (Fig 7.7 and Table 7.19).The ANOVA table is shown below (Table 7.15).

Figure 7.7 Levels of ammonia in the plasma of *O. mossambicus* exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

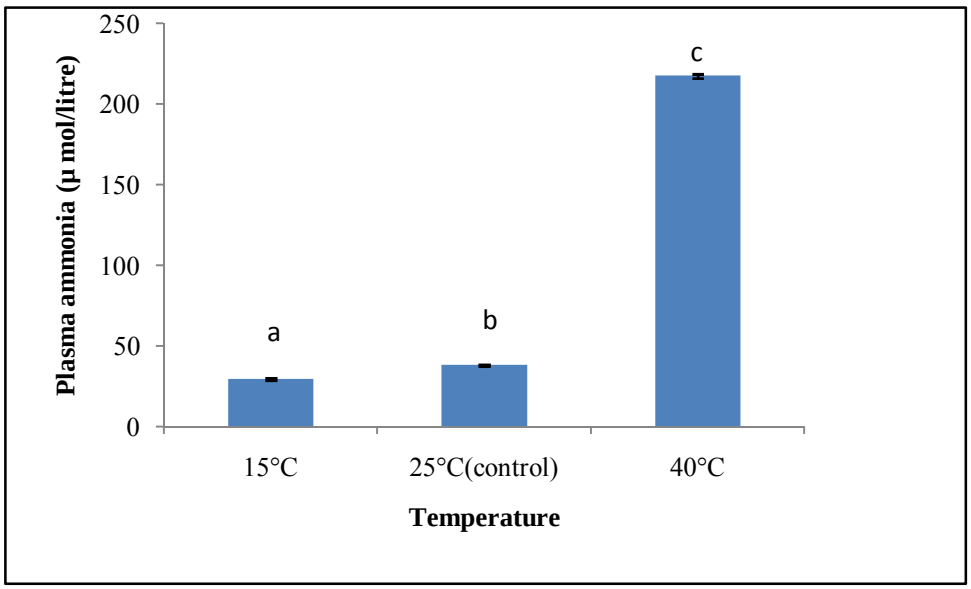

Table 7.15 One way ANOVA for plasma ammonia

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	134806.863	2	67403.432	78274.953	0.000
Within concentrations	12.917	15	0.861		
Total	134819.780	17			

df-degrees of freedom

7.3.8 Ammonia excretion

O. mossambicus exposed to 15°C temperature exhibited significant (P<0.05) decrease in the rates of excretion of ammonia compared to control group. There was significant increase in the rates of excretion of ammonia in fishes exposed to 40°C compared to control group (P<0.05). (Fig 7.8 and

Table 7.19). One-way ANOVA followed by Tukey's test has been carried out to ascertain the statement and the table is shown below (Table 7.16).

Figure 7.8 Rates of ammonia excretion by *Oreochromismossambicus* exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

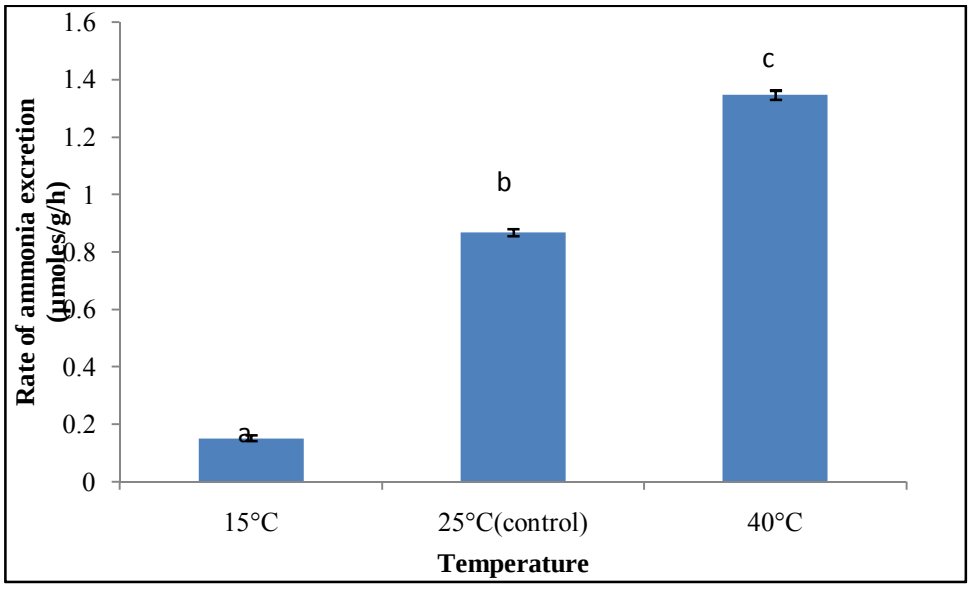

Table 7.16 One way ANOVA for ammonia excretion

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	4.331	2	2.166	12320.260	0.000
Between groups	0.003	15	0.000		
Total	4.334	17			

df-degrees of freedom

7.3.9 Oxygen consumption

One-way ANOVA followed by Tukey's test showed that there was significant increase ($p<0.05$) in the rate of Oxygen consumption of fish exposed to 40°C compared to control group. There was statistically significant decrease ($p<0.05$) in the rate of Oxygen consumption of fish exposed to 15° C

compared to control. (Fig 7.9 and Table 7.19). The ANOVA table is shown below (Table 7.17).

Figure 7.9 Rates of oxygen consumption by *Oreochromis mossambicus* exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

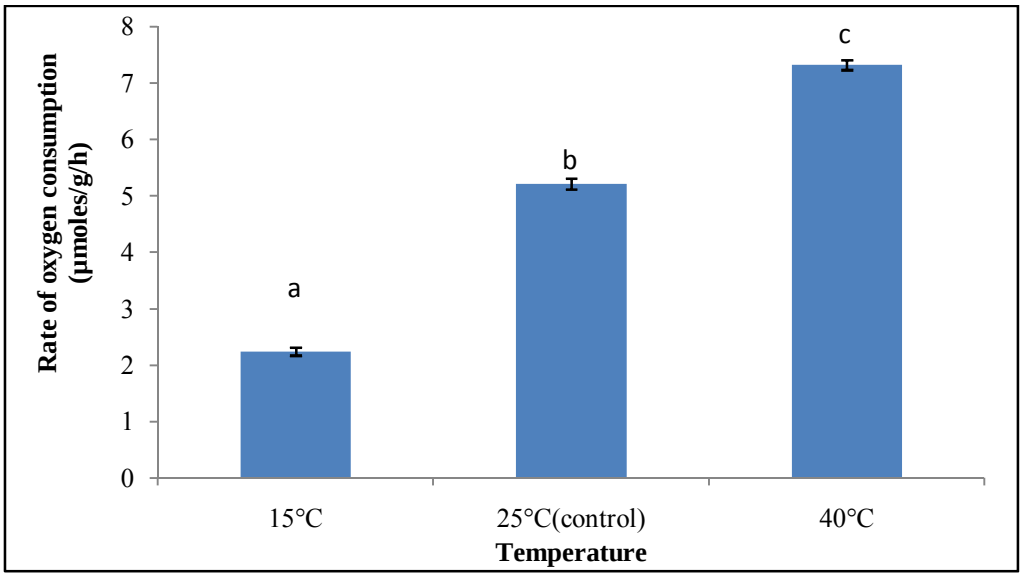

Table 7.17 One way ANOVA for rates of Oxygen consumption by fish

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentration	78.111	2	39.055	5440.318	0.000
Within concentration	0.108	15	0.007		
Total	78.219	17			

df- degrees of freedom

7.3.10 Ammonia quotient

O.mossambicus exposed to 15°C temperature exhibited significant (P<0.05) decrease in the rates of ammonia quotient compared to control group. There was significant increase in the ammonia quotient in fishes exposed to 40°C compared to control group (P<0.05). (Fig 7.10 and Table 7.19). One-way

ANOVA followed by Tukey's test has been carried out to ascertain the statement and the table is shown below (Table 7.18).

Figure7.10 The Ammonia quotient of *Oreochromis mossambicus* exposed acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA).

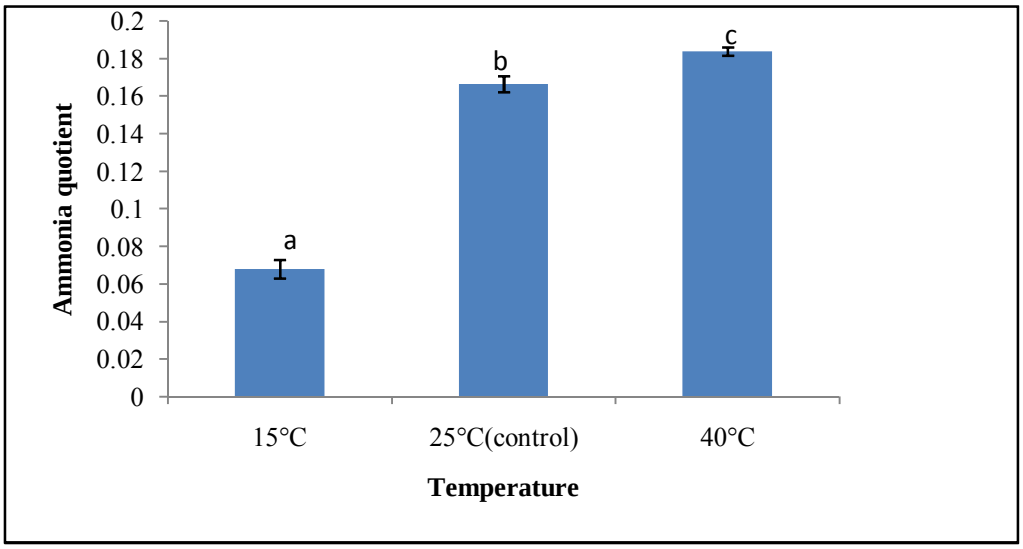

Table7.18 One way ANOVA for ammonia quotient

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	0.047	2	0.023	1454.058	0.000
Within Groups	0.000	15	0.000		
Total	0.047	17			

df- degrees of freedom

Table 7.19 Effects of exposure to acute change in temperature on Plasma ammonia, ammonia excretion, oxygen consumption and ammonia quotient of *O.mossambicus* . Values in the same raw with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Parameters	15 °C	25°C (control)	40 °C
Plasma ammonia (µmole/l)	29.5±0.79^{a}	38.2±0.56^{b}	217.3±1.28^{c}
Ammonia excretion (µmole/g/h)	0.1523±0.01^{a}	0.868±0.0125^{b}	1.346±0.0163^{c}
Oxygen consumption (µmole/g/h)	2.24±0.0701^{a}	5.21±0.095^{b}	7.318±0.086^{c}
Ammonia quotient	0.06805±0.005^{a}	0.17±0.00434^{b}	0.184 ±0.0022^{c}

7.4 Discussion

Temperature is the most important environmental parameter for aquatic life. Increase in temperature adversely affects the health of aquatic animals by increasing metabolic rates and subsequent oxygen demand and the invasiveness and virulence of bacteria and other pathogens (Wedemeyer *et al*., 1999; Krishnamoorthy *et al*., 2008). As body temperature decreases changes in the physical chemistry of the cell produce a reduction in the metabolic activity. The activities of aerobic enzymes remain much lower at cold than warm temperatures (Johnston and Dunn, 1987). Members of genus *Oreochromis* exist in a wide range of water temperatures (Alsop *et al*., 1999). They have been found to thrive at temperatures hotter than that of the mammalian body (Coe, 1966; Narahara *et al*., 1996; Alsop *et al*., 1999) yet can also survive temperatures as low as 11°C (Kindle and Whitmore, 1986; Alsop *et al*., 1999).

Environmental stress causes a variety of detectable and recognizable physiological change in fishes. Protein is one of the most important biochemical constituents of fish. Fish can be subjected to great and sometimes rapid changes in ambient temperature which directly affect the protein content in fishes. In the present study a significant decrease in protein content was observed in *O. mossambicus* exposed to acute change in temperature compared to control group. Jagtap and Mali (2011) observed that at higher temperature of exposure, *Channa punctatus* showed decreased protein content in the muscle. In addition DeLong *et al*. (1958) states that the dietary protein requirement of fish increases at higher temperatures and it has been suggested that this is due to increased oxidation of amino acids. Several studies on the effects of temperature on protein turnover in fish have found that, providing food is not limiting, fractional rates of protein synthesis and degradation are

significantly greater at higher water temperatures (Fauconneau and Arnal, 1985; Watt *et al*., 1988; Mathers *et al*., 1993). Even in the presence of unlimited ration, once a certain critical optimum temperature for protein synthesis is exceeded, then protein accretion rates drop precipitously, largely through an increase in protein degradation rates (Reid *et al.,* 1995, 1997, 1998). In Atlantic wolf fish (*Anarhichas lupus*), when the temperature increased to the upper thermal limit protein degradation increased while retention efficiency and growth decreased (McCarthy *et al*., 1999).

Protein turnover refers to the continuous degradation and renewal of intracellular proteins. These are hydrolyzed to their component amino acids and usually replaced by an equal amount of freshly synthesized protein (Hawkins, 1991). Positive growth occurs when the rate of synthesis surpasses the rate of degradation (Houlihan *et al*., 1995a; Sugden and Fuller, 1991). Conversely, in the case of negative growth, protein degradation surpasses the rate of protein synthesis (Hawkins, 1991). Factors that have an effect on growth necessarily affect (directly or indirectly) protein synthesis, protein degradation or both. Although little is known about protein degradation, there is a strong indication that it is a very important process in the determination of growth rate (Dobly *et al*., 2004; Fraser and Rogers, 2007; Houlihan *et al*., 1995a). El-sherif and El-feky (2009) studied the influence of different water temperatures on growth performance of Nile tilapia and the results showed that growth performance for tilapia was significantly decreased at 15°C and 20°C. This observation signifies that protein degradation surpasses the rate of protein synthesis during exposure to low temperature. A higher level of oxidative modification of proteins could perhaps explain the higher rate of protein degradation at cold temperature. High rate of protein degradation at low temperature is suggested as an adaptive response to keep the steady-state

concentration of oxidatively modified proteins at an acceptable level (Lamarre *et al*., 2009).

Free amino acid content was found to be significantly elevated ($P<0.05$) in all tissues exposed to acute temperature change compared to control. The increased free amino acid pool can be used for ATP production by transamination reactions or by gluconeogenic pathway. Increase in free amino acid levels may be the result of breakdown of protein for energy and impaired incorporation of amino acids required in protein synthesis. Tantarpale *et al*. (2011) observed an increase in muscle free amino acid content in *Channa striatus* with a rise in temperature. High temperature creates higher free amino acid mobilization, as higher enzyme activities of transaminases; ALT and AST were observed in *C. carpio* with increasing temperature (Das, 2002).

The activities of ALT and AST were significantly increased in tissues of *O.mossambicus* exposed to 40°C compared to control. Elevation in the levels of ALT and AST in different tissues of *O.mossambicus* can be considered as a response to stress induced by acute high temperature exposure. The high activities of aminotransferases results in the generation of ketoacids like α-ketoglutarate and oxalocetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand. Significant elevation in ALT and AST activities were recorded in *Labeo rohita* exposed to high temperature (Das *et al*., 2006).

The activity of ALT has been significantly elevated in muscle tissue of fish exposed to 15°C. But the exposure to 15°C significantly decreased the activity of ALT in liver and gill tissues of *O.mossambicus*. Alanine amino transferase and aspartate aminotransferase in different organs of teleosts may

be affected differently by the ambient temperature (Jurss, 1981).The low temperature exposure caused an increased ALT activity in the muscle tissue of *Idus idus and Carassius auratus* (Lehmann, 1970 a,b). The increase in the aminotransferase activity in the muscle may signify that increasing amounts of free amino acid (alanine) are being liberated for the liver (Jurss, 1979, 1981). The aspartate aminotransferase activity in the different tissues of *O. mossambicus* exposed to 15°C increased significantly compared to control. Elevations in liver AST activity were noticed in Rainbow trout exposed to cold temperature (Jurss, 1981). The significance of the increase in AST activity may be related to gluconeogenesis. Seibert (1985) has reported that as the temperature decreases, gluconeogenesis becomes of increasing importance in the hepatocytes of *Salmo gairdneri*. A strategy for temperature acclimation is to produce different enzyme isoforms that function better at the new temperature (Ballantyne, 2001). Some evidence for thermal isoforms of ALT was provided in a study of the pond loach (Mester *et al.,* 1973).

Glutamate dehydrogenase activity was found to be elevated in all the tissues exposed to 40°C compared to control. The increased activity may have helped more energy generation by funneling more α- ketoglutarate in to citric acid cycle. Whereas in fishes exposed to 15°C, a decreased activity was observed in all the tissues compared to control. This suggests accumulation of glutamate in the tissues during cold acclimation. The accumulated glutamate may aid in meeting the energy demands by being a gluconeogenic substrate. It has been demonstrated in Nile tilapia (*Oreochromis niloticus*) that exposure to 15°C caused significant reduction in ammonia excretion (Alsop *et al*., 1999). In teleost fish ammonia is mainly produced by the catabolism of protein. Glutamate dehydrogenase is an important enzyme involved in the ammonia production. Maetz (1972) interpreted that the majority of the acute temperature

sensitivity was in the metabolic production mechanism of ammonia, rather than in the branchial excretion mechanism. Thus the reduction in ammonia excretion may be due to the decreased activity of glutamate dehydrogenase at low temperature.

The arginase activity was significantly increased in liver, gills and kidney of fishes exposed to 40°C. But arginase activity in the liver, gills and kidney of animals exposed to 15°C showed significant decrease compared to control. Vellas *et al*. (1982) studied the effect of increasing temperature on the activity of hepatic arginase in Rainbow trout (*Salmo gairdneri*). They observed an increase in the activity of arginase during first few days of high temperature acclimation but after 17 days of acclimation to high temperature arginase displayed complete thermal compensation. Jurss *et al.* (1987) reported that arginase activity was reduced at a low water temperature in the liver of Rainbow trout (*Salmo gairdneri*).

Oxygen consumption was found to be increased in *O. mossambicus* exposed to 40°C compared to control. Similar results have been reported by Krishnamoorthy *et al*. (2008), who observed a significant increase in the oxygen consumption of fish fingerlings of *Alepes djidaba* exposed to high temperature. The oxygen consumption rate of *O. mossambicus* at 15°C decreased significantly compared to control. Similar results were observed in Nile tilapia exposed to low temperature (Alsop *et al*., 1999).

The plasma ammonia content was significantly increased in *O.mossambicus* exposed to 40°C. The increase in plasma ammonia may be due to the increased catabolism of amino acids and due to the elevated activity of AMP deaminase and glutamate dehydrogenase. But the exposure to 15° C caused decreased plasma ammonia content compared to control in

O.mossambicus. The reason for this may be the decreased activity glutamate dehydrogenase which in turn decreases the ammonia production.

In the present study a significant increase in ammonia excretion was observed in fish exposed to 40°C compared to control. The increase in the ammonia excretion rate indicates that degradation of protein was more at higher temperature. Krishnamoorthy *et al*. (2008) reported that the ammonia excretion of fish fingerlings of *Alepes djidaba* was enhanced with increasing temperature. But a significant decrease was observed in the ammonia excretion of *Oreochromis mossambicus* exposed to 15°C compared to control. Similar results were observed in Nile tilapia exposed to low temperature (Alsop *et al*., 1999). Alsop *et al*. (1999) reported that decreasing acclimation temperature from 30°C to 15°C resulted in a decrease in total fuel use by approximately 55% in the Nile tilapia and the relative protein and lipid use decreased, while relative carbohydrate use increased greatly. This indicates that the protein catabolism is lower in cold temperatures.

A significant increase in the ammonia quotient was observed in *O.mossambicus* when exposed to 40°C, which indicate increased protein degradation. But fish exposed to 15°C showed a decreased ammonia quotient compared to control. Similar results were observed by Alsop *et al*. (1999), who stated that nitrogen quotients (NQ) decreased with decreased acclimation temperature. So at low temperature protein catabolism is reduced.

In conclusion, temperature affects the protein catabolism significantly. The organism is trying to attain homeostasis during acute temperature change. At high temperature the protein catabolism was elevated and this may be because of the increased metabolic rate. The low temperature exposure produces a reduction in the metabolic activity. The glutamate dehydrogenase activity was

decreased with a concomitant reduction in the ammonia excretion. The ammonia quotient was decreased at low temperature. The increased tissue free amino acids may be utilized for gluconeogenesis through aminotransferase reaction.

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Chapter 8

Effect of Acute Changes in Temperature on the Purine Catabolism of *Oreochromis mossambicus*

8.1 Introduction

Water temperature strongly influences the abundance and distribution of organisms. Prokaryotic life exists over an impressively broad thermal range, but multicellular animals are limited to a much narrower range of temperature. (Guderley, 2002). Although most animals have been specialized for life within an even smaller portion of this range, they may still experience marked daily or seasonal temperature fluctuations. These thermal fluctuations have functional consequences at many levels of biological organization. Endotherms achieve considerable thermal independence from their habitat by conserving much of their metabolically generated heat, thus maintaining a thermal differential with their habitat. In contrast, the body temperature of ectotherms is determined primarily by external heat sources. Whereas the body temperature of terrestrial ectotherms can differ from that of their immediate habitat, the majority of fish and many aquatic ectotherms have body temperature identical to that of their habitat.

Temperature affects virtually all levels of biological organization, from the rates of molecular diffusion and of biochemical reactions, to membrane permeability, to cellular, tissue and organ function and to their integration in the whole organism (Guderley, 2002). Fish is a cold blooded animal. Thus fish is affected by the temperature of the surrounding water which influences the body temperature, growth rate, food consumption, feed conversion and other body functions (Houlihan *et al*., 1993; Britz *et al*., 1997; Azevedo *et al*., 1998). Therefore, water temperature is a driving force in the fish life because its effects are more than those of any other single factor. Growth in fish is optimum within a defined temperature range (Gadowaski and Caddell, 1991).

Fish often select a preferred environmental temperature to optimize physiological processes (Coutant, 1977a, 1985), but the characteristics of their surrounding environment can change frequently. Fish can experience rapid changes in temperature of natural or anthropogenic origin. The natural changes in temperature are usually only small deviations from ambient conditions and only occasionally become severe and result in mortality (Coutant, 1985). Changes in temperature due to anthropogenic activities are often rapid and may present challenges for fishes. Anthropogenic sources of temperature shock include thermal effluents of power plants and factories (Coutant, 1977b; Birtwell and Kruzynski, 1989), hypolimnetic release of water from upstream reservoirs (Clarkson and Childs, 2000) and commercial fishing when by-catch is put on ice prior to release (Hyvarinen *et al*., 2004). Temperature shock can have negative effects for fish by impairing swimming performance (Hocutt, 1973) and disrupting physiological homeostasis (Galloway and Kieffer, 2003; Suski *et al*., 2006).

Each fish species has an ideal temperature range within which it grows quickly. However, fish move into more favourable areas of a stream to regulate their body temperatures. In warmer environments fish have a longer growing season and faster growth rate but tend to have a shorter life span than

in cool water. High water temperatures increase the metabolic rates, resulting in increased food demand. Although, fish can generally function in a wide range of temperatures, they do have an optimum range, as well as lower and upper lethal temperatures, for various activities (Beschta, *et al*., 1987).

Classical studies of stress in fish have focused on the organismal stress response. The characteristic feature of this organismal stress response is the rapid release of stress hormones, including cortisol and catecholamines, resulting in the mobilization of energy reserves in an attempt to re-establish homeostasis (WendelaarBonga, 1997; Fabbri *et al*., 1998; Mommsen *et al*., 1999). Fish are ectothermic vertebrates that inhabit an aquatic environment with high temperature conductivity. As a result, temperature is an important factor influencing their bio geographic distribution over evolutionary time. In addition, daily and seasonal temperature fluctuations have an important impact during the lifetime of individual fish. Therefore, fish are a convenient model to study the effects of thermal stress in the intact organism on both short and long time scales (Basu *et al*., 2002). Among the natural stressors fish can experience throughout their life cycle are thermal changes. Fluctuations in water temperature can result from a transient (daily change) or a seasonal change. In either case, to deal with the environmental change, fish respond by altering physiological functions including those associated with the stress response (Barton and Iwama, 1991). The physiological stress response in fish is mediated by the neuro-endocrine system and includes the release of hormones such as cortisol and adrenaline (Barton and Iwama, 1991). In response to most stressors fish will exhibit an increase in plasma cortisol levels, which is generally followed by an elevation in plasma glucose levels. A physiological stress response also occurs at the cellular level, which is characterized by the induction of a conserved group of proteins named the heat shock proteins (HSP). The HSPs have been measured in almost all organisms including fish (Iwama *et al.*, 2004).

Temperature shifts may alter the equilibrium between synthesis and degradation of biological structures, change metabolic requirements, favour certain functions over others and alter trophic interactions. Given the extent of these thermal effects, it is not surprising that animals show a variety of strategies, from biochemical to behavioural, to cope with thermal change. The increasingly obvious problem of global warming has given greater urgency to the understanding of biological responses to temperature, particularly in the ectothermal organisms that have limited independence from changes in environmental temperature (Guderley, 2002).

The studies on the effects of temperature on purine catabolism of fish are scanty. Thus in the present study enzymes and metabolic parameters related to purine catabolism of *O. mossambicus* exposed to different temperatures for 72 hours were experimented. The parameters studied included enzymes such as AMP deaminase, xanthine oxidase, uricase and allantoinase. Also the parameters such as serum urea and serum uric acid were estimated.

8.2 Materials and Methods

Collection, maintenance, acclimation, experimental design, preparation of tissue and serum samples were the same as explained in detail in chapter3, section 3.2. Methods used for the biochemical analysis and statistical analysis were the same as explained in chapter 4, section 4.2.1 and 4.2.2. Experimental design for the study was the same as explained in chapter 7, section 7.2.1.

8.3 Results

8.3.1 Activity of AMP deaminase

The activity of AMP deaminase in different tissues of *O. mossambicus* exposed to 40°C showed significant increase ($P<0.05$) compared with control group (Fig 8.1 and Table 8.1) (One-way ANOVA followed by Tukey's test).

No significant variation in the activity of AMP deaminase was observed in fish exposed to 15°C compared to control.

Figure 8.1 AMP deaminase activity in the various tissues exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

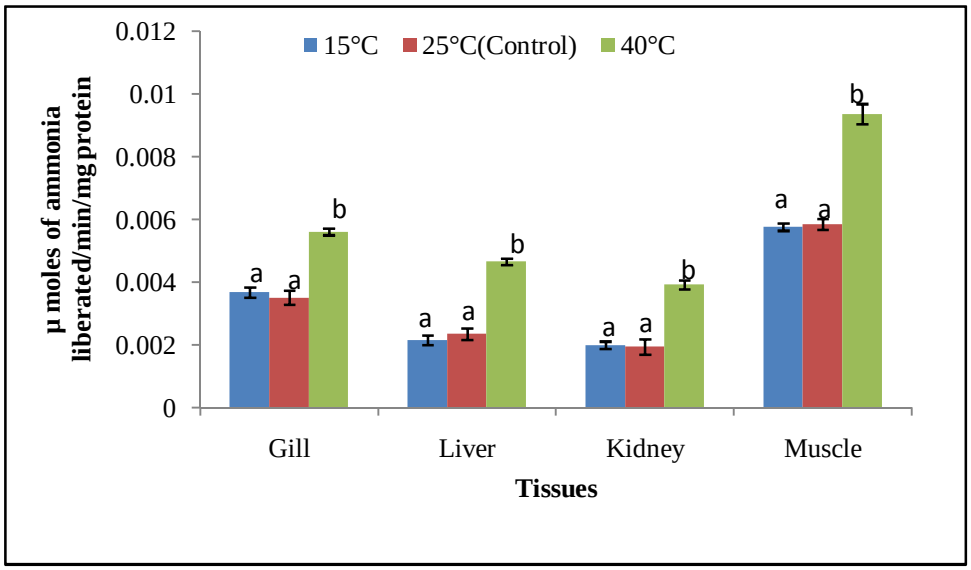

Table 8.1 Effects of exposure to acute changes in temperature on AMP deaminase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Tissues	AMP deaminaseactivity		
	Groups		
	15°C	25°C (Control)	40°C
Gills	0.00369±0.000163 [a]	0.0035±0.00023 [a]	0.00562±0.00011 [b]
Liver	0.00216±0.00015 [a]	0.0024±0.00018 [a]	0.00467±0.000102 [b]
Kidney	0.00201±0.000114 [a]	0.0019±0.00025 [a]	0.00393±0.000141 [b]
Muscle	0.00577±0.000116 [a]	0.0059±0.00018 [a]	0.00937±0.000320 [b]

Values are expressed as µ moles of ammonia liberated / min / mg protein.

Two factor ANOVA (Table 8.2) revealed that there was a significant variation (P<0.05) in AMP deaminase activity in all the experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 8.5) revealed that there was significant difference (P<0.05)

between temperatures. Between 15°C and 40°C exposed groups there was a significant difference at P<005. There was no significant difference between 15°C and control group. Multiple comparison tests also (Table 8.6) revealed that there was a significant difference between tissues. The highest activities were seen in muscle tissue followed by gills.

Table 8.2 Two-factor ANOVA for tissue AMP deaminase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	9.87E-005	2	4.93E-005	1483.365	0.000
Between Tissues	0.000	3	6.95E-005	2089.714	0.000
concentration * Tissue	6.91E-006	6	1.15E-006	34.632	0.000
Error	2.00E-006	60	3.33E-008		
Total	0.002	72			

df- degrees of freedom

8.3.2 Activity of xanthine oxidase

In the present study the activity of xanthine oxidase in the different tissues of *O. mossambicus* exposed to 40°C showed significant increase (P<0.05) compared with control group (Fig 8.2 and Table 8.3) (One-way ANOVA followed by Tukey's test).A statistically significant decrease in xanthine oxidase activity was observed in the tissues of the 15° C exposed fishes compared to control (Fig 8.2 and Table 8.3) (One-way ANOVA followed by Tukey's test).

Figure 8.2 Xanthine oxidase activity in the various tissues exposed to acute temperture change. Each bar diagram represents mean ± S.D. On each set of bars values with different lower case letters vary significantly (P<0.05) in each tissue on different treatments (One-way ANOVA)

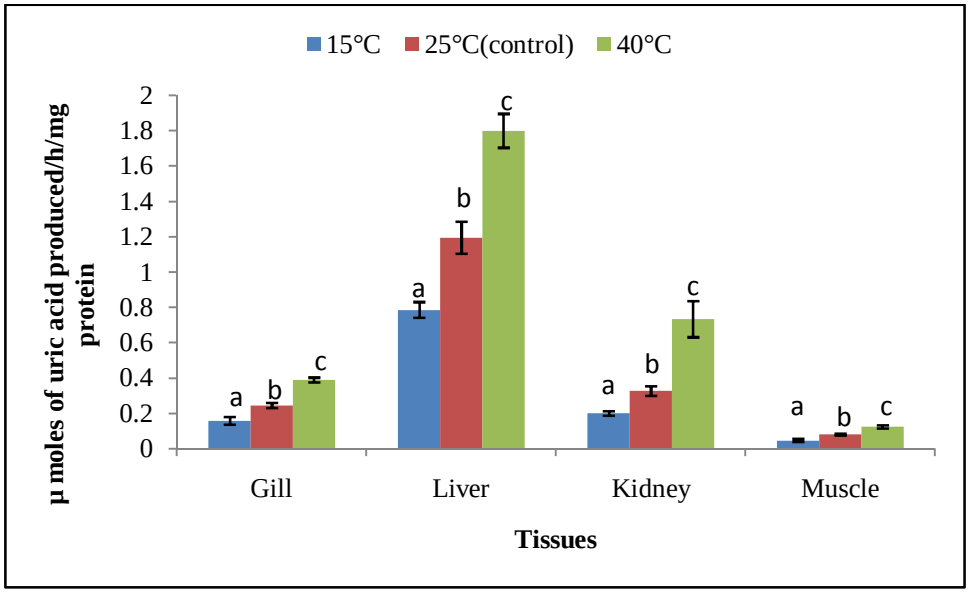

Table 8.3 Effects of exposure to acute changes in temperature on xanthine oxidase activity (mean ± S.D) present in different tissues of *O.mossambicus.* Values in the same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Tissues	Xanthine Oxidase activity		
	Groups		
	15°C	25°C (Control)	40°C
Gills	0.157±0.021[a]	0.243±0.015[b]	0.388±0.014[c]
Liver	0.785±0.044[a]	1.194±0.091[b]	1.799±0.096[c]
Kidney	0.199±0.0119[a]	0.326±0.027[b]	0.732±0.102[c]
Muscle	0.045±0.0087[a]	0.0782±0.0046[b]	0.12±0.01[c]

Values are expressed as µmoles of uric acid produced/mg protein/hr

Two factor ANOVA (Table 8.4) revealed that there was a significant variation (P<0.05) in xanthine oxidase activity in acute temperature exposed experimental groups when compared to control group. Subsequent analysis by multiple comparison test (Table 8.5) revealed that there was significant difference (P<0.05) between control and 15°C and 40°C exposed groups and between 15°C and 40°C exposed groups. Multiple comparison tests also

(Table 8.6) revealed that there was a significant difference between tissues. The highest activities were seen in liver tissue.

Table 8.4 Two-factor ANOVA for tissuexXanthine oxidase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	2.653	2	1.327	500.213	0.000
Between tissues	14.661	3	4.887	1842.661	0.000
concentration * tissue	1.580	6	0.263	99.301	0.000
Error	0.159	60	0.003		
Total	37.480	72			

df- degrees of freedom

Table 8.5 Results of Multiple comparison using TUKEY'S test (Concentrations)

Groups	Parameters	
	AMP deaminase	Xanthine Oxidase
25°C (control) Vs 15°C	0.968[d]	0.000[a]
25°C (control) Vs 40°C	0.000[a]	0.000[a]
15°C Vs 40°C	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

Table 8.6 Results of Multiple comparison using TUKEY'S test (Tissues)

Groups	Parameters	
	AMP deaminase	Xanthine Oxidase
Gills Vs Liver	0.000[a]	0.000[a]
Gills Vs Kidney	0.000[a]	0.000[a]
Gills Vs Muscle	0.000[a]	0.000[a]
Liver Vs Kidney	0.000[a]	0.000[a]
Liver Vs Muscle	0.000[a]	0.000[a]
Kidney Vs Muscle	0.000[a]	0.000[a]

The values are significant at a=P<0.05 and not significant at d

8.3.3 Activity of uricase

Uricase activity in the liver of *O. mossambicus* exposed to 40°C showed significant increase (P<0.05) compared to control (One-way ANOVA

followed by Tukey's test) (Fig 8.3 and Table 8.7). One-way ANOVA followed by Tukey's test also revealed a significant decrease at ($P<0.05$) in uricase activity in the liver of *O. mossambicus* exposed to 15°C.

Fig 8.3 Effects of acute temperature change on uricase activity in the liver of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

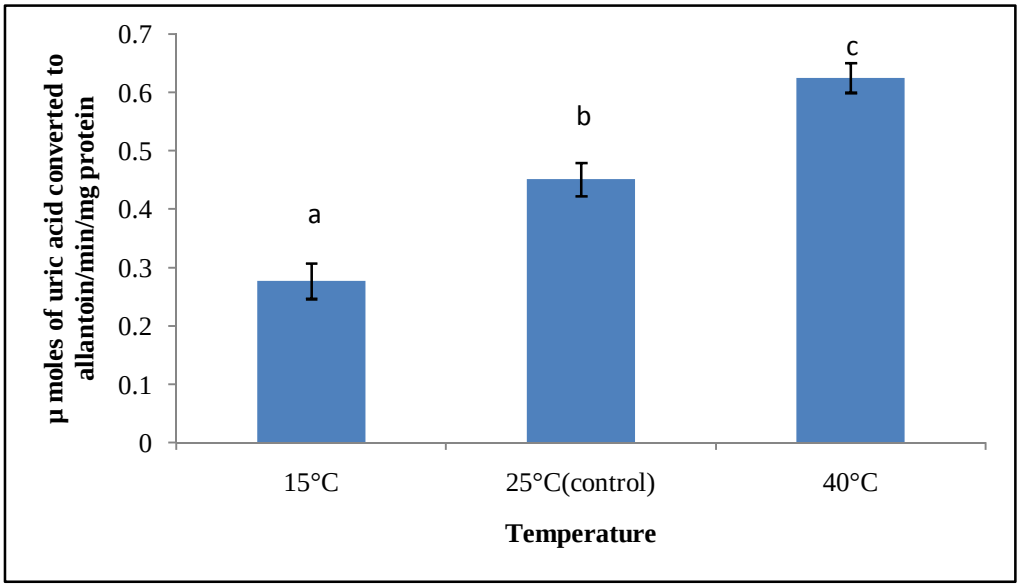

Table 8.7 Effects of exposure to acute changes in temperature on uricase activity (mean ± S.D) present in the liver of *O.mossambicus.* Values with different lower case letters vary significantly ($P<0.05$) between treatment groups (One-way ANOVA)

Group	Temperature		
	15°C	25°C (Control)	40°C
uricase activity	0.277 ± 0.03^{a}	0.451 ± 0.029^{b}	0.626 ± 0.025^{c}

Values are expressed as micromoles of uric acid converted to allantoin / minute / mg protein.

Table 8.8 One way ANOVA for uricase activity

Source of Variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.364	2	0.182	191.249	0.000
Within concentration	0.014	15	0.001		
Total	0.378	17			

df- degrees of freedom

8.3.4 Activity of allantoinase

In the present study the activity of allantoinase in the liver of *O. mossambicus* exposed to 15°C showed significant decrease (P<0.05) compared to control. Significant increase (P<0.05) in allantoinase activity was observed in the liver of 40°C exposed fish compared to control group (Fig 8.4 and Table 8.9) (One-way ANOVA followed by Tukey's test).

Fig 8.4 Effects of acute temperature change on allantoinase activity in the liver of *O. mossambicus.* Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly (P<0.05) (One-way ANOVA)

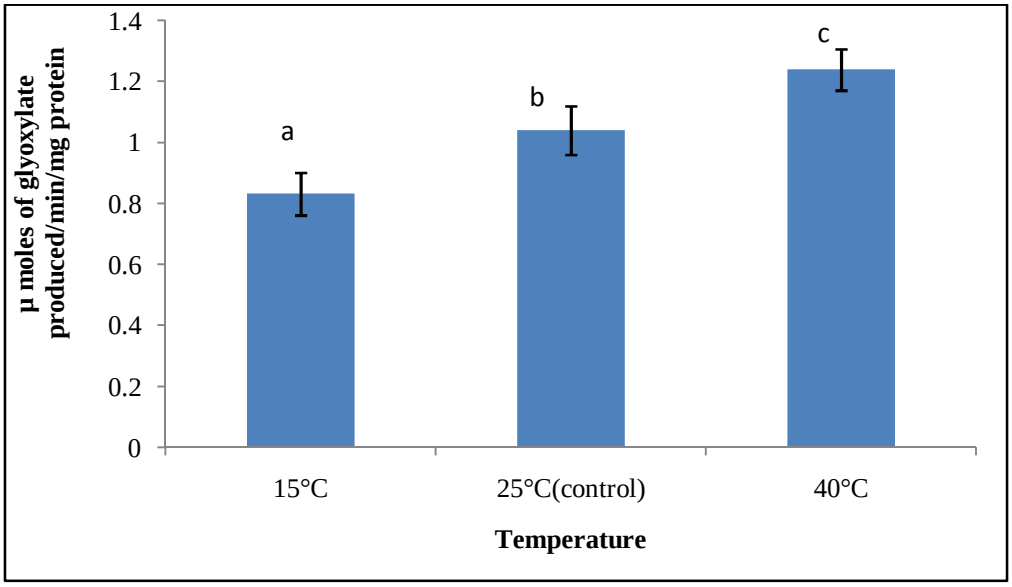

Table 8.9 Effects of exposure to acute changes in temperature on allantoinase activity (mean ± S.D) present in the liver of *O.mossambicus.* Values with different lower case letters vary significantly (P<0.05) (One-way ANOVA).

Group	Temperature		
	15°C	25°C (control)	40°C
Allantoinase activity	0.832±0.07[a]	1.04±0.08[b]	1.24±0.067[c]

Values are expressed as micromoles of glyoxylate produced / minute / mg protein.

Table 8.10 One way ANOVA for allantoinase activity

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	0.495	2	0.248	40.916	0.000
Within concentration	0.091	15	0.006		
Total	0.586	17			

df- degrees of freedom

8.3.5 Serum urea

Urea content in serum of *O. mossambicus* exposed to 40°C showed significant increase ($P<0.05$) compared to control group. There was significant decrease observed in serum urea content of 15°C exposed fish compared to control group. (Fig 8.5 and Table 8.13) (One-way ANOVA followed by Tukey's test). The ANOVA table is shown below (Table 8.11).

Figure 8.5 Levels of urea in the serum of *O.mossambicus* exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

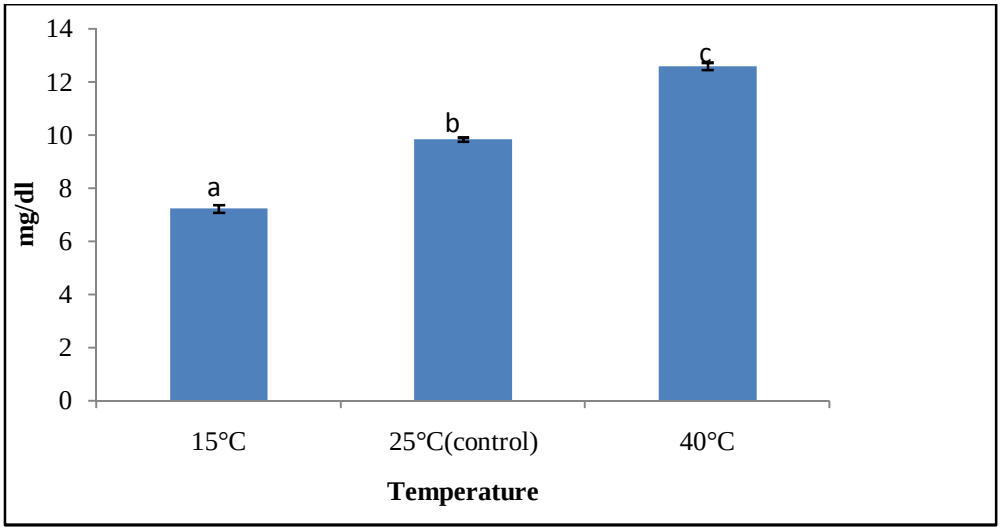

Table 8.11 One way ANOVA for serum urea

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	85.995	2	42.998	2783.813	0.000
Within concentration	0.232	15	0.015		
Total	86.227	17			

df-degrees of freedom

8.3.6 Serum uric acid

One-way ANOVA followed by Tukey's test showed that there was significant ($P<0.05$) increase in the serum uric acid content in 40°C exposed fish compared to control. There was significant decrease observed in the serum uric acid content of fish exposed to 15°C compared to control (Fig 8.7 and Table 8.13). The ANOVA table is shown below (Table 8.12).

Figure 8.6 Levels of uric acid in the serum of *O.mossambicus* exposed to acute temperature change. Each bar diagram represents mean ± S.D. On each bar, values with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA)

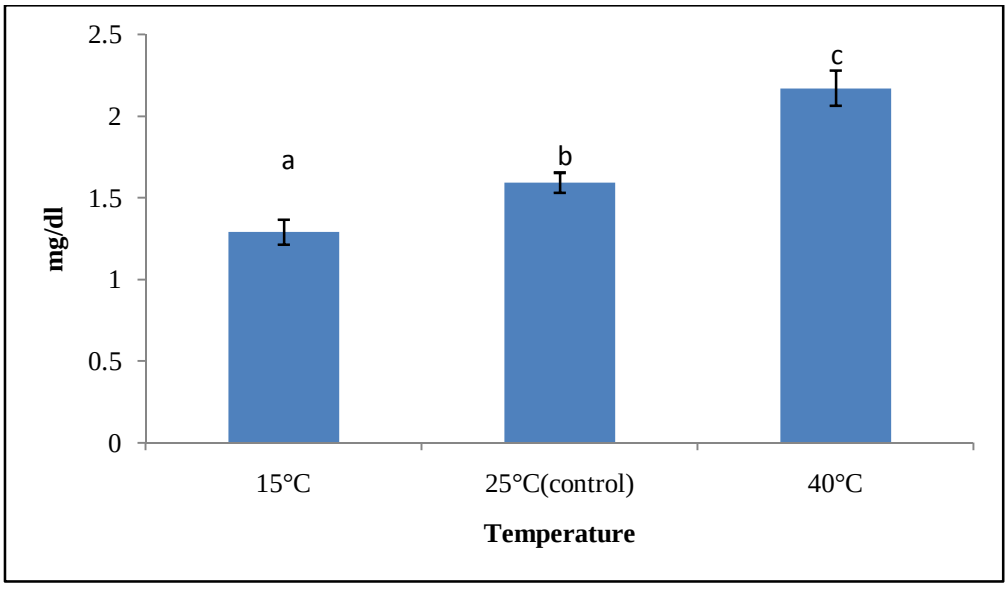

Table 8.12 One way ANOVA for serum uric acid

Source of variation	Sum of Squares	df	Mean Square	F	Sig.
Between concentrations	2.400	2	1.200	168.996	0.000
Within concentration	0.107	15	0.007		
Total	2.506	17			

df-degrees of freedom

Table 8.13 Effects of exposure to acute changes in temperature on serum parameters of *O.mossambicus.* Values in the same raw with different lower case letters vary significantly ($P<0.05$) (One-way ANOVA).

Serum parameters	Temperature		
	15°C	25°C (control)	40°C
Urea	7.22 ± 0.142^{a}	$9.8\ 3\pm0.075^{b}$	12.57 ± 0.143^{c}
Uric acid	1.3 ± 0.08^{a}	1.6 ± 0.06^{b}	2.2 ± 0.11^{c}

Values are expressed as mg/dl

8.4 Discussion

Fish, probably the only true obligate vertebrate poikilotherms, occupy a remarkable range of thermal niches. Adaptation to these niches has taken place over thousands of years and explains the wide diversity of environments now occupied by fish species. However, many of these species will be subjected to seasonal and even more acute temperature changes. Changes in temperature potentially cause greater consequences in fish. Physiological and biochemical parameters studied in the present investigation serve as sensitive indicators of the impacts of environmental changes and provide insight into how stressors affect fish in ways other than immediate mortality.

In the present study an increased activity of AMP deaminase was observed in the gills, liver, kidney and muscle of *O.mossambicus* exposed to 40°C for 72 hours compared to control group. But the AMP deaminase activity in the tissues of the fish exposed to 15°C did not show any significant variation compared to control. Walsh and Somero (1981) observed that acute

temperature decreases had no effect on adenylate concentration and adenylate energy charge (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al*. (1971) the value of energy charge $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$). In contrast acute temperature increases led to large changes in adenylate concentrations in *Metridium senile.* Sudden increase in temperature can impose a high demand for ATP that the adenylate energy charge is difficult to maintain at normal levels (Walsh and Somero, 1981).This indicates that higher temperature cause a fall in the adenylate energy charge. AMP deaminase may stabilize the adenylate energy charge (Chapman and Atkinson, 1973; Solano and Coffee, 1978). So the increase in the activity of AMP deminase may aid in the stabilization of adenylate energy charge.

Xanthine oxidase is an important enzyme participating in the nucleic acid metabolism of fish. The activity of xanthine oxidase was significantly increased in liver, kidney, muscle and gill tissues of fish exposed to 40°C compared to control. But the activity of xanthine oxidase decreased in fish exposed to 15°C compared to control. Xanthine oxidase activity of fish has been shown to change when fish are exposed to environmental pollutants (Hegazi *et al*., 2010, Basha and Rani, 2003). The activity of xanthine oxidase increased at high temperature in juvenile Chinese Sturgeon (*Acipenser Sinensis*) (Feng *et al*., 2011). The decreased activity of xanthine oxidase at 15°C indicates that the rates of enzyme catalyzed reactions are reduced by the low heat content of the cellular environment.

An increased uricase and allantoinase activity was observed in the liver of *O.mosambicus* exposed to 40°C. The elevated activity results in the

increased synthesis of urea from purines. Similar results were reported by Vellas (1965), who found that the activities of uricase, allantoicase and allantoinase in the liver of Carp increased with a rise in temperature. Vellas and Parent (1982) observed an increase in the activity of uricase with a rise in temperature in the liver of Rainbow trout. A decreased activity of hepatic uricase and allantoinase was observed in *O.mosambicus* exposed 15°C. The depressed activity of the enzymes may be the result of a reduction in general metabolism due to low temperature.

The serum urea and uric acid content were elevated in *O.mossambicus* exposed 40°C compared to control group. The increase may be due to the elevated activities of arginase and uricolytic enzymes such as uricase and allantoinase. Acute exposure to 15°C caused a decrease in the serum urea and uric acid content compared to control in *O.mossambicus.* Alsop *et al*. (1999) observed decreased urea excretion in *O.niloticus* exposed to 15°C. The reduced activity of xanthine dehydrogenase and uricolytic enzymes caused a reduction in the serum uric acid and urea content in *O.mosambicus.*

O.mossambicus exposed to acute rise in temperature showed significant increase in the activities of enzymes related to purine catabolism. This results in the elevation of urea and uric acid content in the serum. In conclusion the acute rise in temperature increased the urea production of *O.mossambicus* from purines. In contrary the exposure to low temperature decreased the activities of enzymes. As a result the purine catabolism was reduced and there was a decline in the urea synthesis.

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Chapter 9

Summary and Conclusions

Fish may encounter many situations that disrupt their homeostasis. Many physiological changes that occur in response to environmental disturbances are now used routinely for assessing stressed states in fish. The initial stress response is considered adaptive, one designed to help the fish to overcome the disturbances and regain its normal or homeostatic state. If the stressor is severe or long-lasting, however, the fish may no longer be able to cope with it and as a result, the fish enters a maladaptive or distressed state leading to decreased performance, which may lead to death. Many of the physiological and biochemical changes in fish during stress still remain within the realm of experimental research and require further study. The present investigation dealt with the changes in protein and purine catabolism on exposure to cadmium, salinity and temperature in the fresh water adapted euryhaline teleost *Oreochromis mossambicus.*

Important findings f the study can be summarized as:

- The LC_{50} value of cadmium chloride in *O. mossambicus* was found to be 9.233 mg/l as per probit analysis. 95% confidence limit ranged between 9.117 and 9.329 mg/l. Sub lethal concentrations of $CdCl_2$ which corresponds to $l/10^{th}$, $l/5^{th}$ and $1/3^{rd}$ of the lethal concentration value were selected for conducting experimental studies.

- Reduction in the protein content in the tissues of *O. mossambicus* suggests the activation of protein catabolism to counteract the cadmium ion (Cd^{++}) induced toxic stress. During stress conditions the balance between anabolism and catabolism will be impaired. The metabolism may shift towards more catabolic state and the tissue protein may undergo proteolysis.
- The increased level of free amino acids in tissues indicates that the amino acids may be fed in to citric acid cycle through aminotransferases to cope with the high energy demand posed by the cadmium ion (Cd^{++}) stress. The highest increase in free amino acid content in liver suggests the major role of liver in amino acid catabolism.
- The elevation in aminotransferase activities in different tissues of *O. mossambicus* on exposure to cadmium ion (Cd^{++}) indicates changes in energy metabolism in response to an enhanced energy demand to compensate the stress situation.
- Stimulation of GDH in the liver, kidney and muscle tissues of *O.mossambicus* on exposure to cadmium ion (Cd^{++}) may cause an increase in glutamate oxidation, resulting in increase in ammonia production and α- ketoglutarate formation at the expense of NAD. Cd^{++} induces the generation of glutamate through tissue transamination followed by their conversion to α– ketoglutarate through oxidative deamination to favour gluconeogenesis or energy production
- The increase in the activity of GDH was found to be most prevalent in liver and kidney. This indicates significant role of these organs in the deamination.

- Significant decrease in arginase activity may be due to inhibition of the enzyme by cadmium ions.
- Significant increase in the level of plasma ammonia in *O. mossambicus* on exposure to cadmium ion (Cd^{++}) seems to be a combined effect of elevated, stress induced, ammonia production and an unchanged excretion despite an elevated plasma-to-water gradient.
- The ammonia excretion rate was not altered in *O. mossambicus* on exposure to cadmium ion (Cd^{++}) because of the impaired ability to excrete ammonia across the gill which is a typical response to metal exposure.
- The decline in the oxygen consumption rate in *O. mossambicus* exposed to sub-lethal concentrations of cadmium ion (Cd^{++}) points to the alteration in the normal respiratory metabolism. This is due to the intimate contact with water contaminated with cadmium ions which decreases the oxygen diffusing capacity of the gills. Excessive secretion and coagulation of mucus may impair gas exchange across the secondary lamellae epithelium.
- The ammonia quotient in treated fish increased significantly which indicates a marked increase in the catabolism of proteins during cadmium ion (Cd^{++}) induced stress. Cadmium toxicity in the fish *O. mossambicus* enhances the catabolism of proteins to meet the extra energy demand.
- Elevation in the activity of AMP deaminase in the gill, liver, kidney and muscle tissue of *O.mossambicus* exposed to sub lethal cadmium ion (Cd^{++}) concentrations may be due to the activation of the enzyme by limited proteolysis during stress.

- The enhanced activity of xanthine oxidase in tissues of *O. mossambicus* suggests that the elevated catabolism of purines and increased synthesis of uric acid during cadmium induced stress.
- Depressed activity of uricase in the liver tissue of *O.mossambicus* may be due to its inhibition by cadmium (Cd^{++}).
- A significant decrease in the allantoinase activity was seen in *O.mossambicus* exposed to sub-lethal concentrations of cadmium (Cd^{++}). The larger molecular size of cadmium may result in steric hindrance of the active site of allantoinase so that the enzyme is inhibited by the metal.
- The increased serum uric acid content in *O.mossambicus* may be due to the elevated activity of xanthine oxidase and also because of the kidney dysfunction in response to metal exposure.
- The elevation of serum urea content may be due to kidney dysfunction of the fish *O.mossambicus* in response to cadmium (Cd^{++}) exposure.
- The significant decrease in the levels of protein in different tissues of *O.mossambicus* on exposure to 20 ppt and 30 ppt salinity for 48 hours suggests the possibility of increased protein hydrolysis due to increased energy needs during salinity stress.
- Elevation in the free amino acid content in different tissues of *O.mossambicus* exposed to 20 ppt and 30 ppt salinity was due to the increased protein hydrolysis. The amino acids may be utilized for ATP production.
- The enhanced activities of ALT and AST in all the tissues of fish acclimated to 20 ppt and 30 ppt suggest increased amino acid

catabolism during acute salinity change. The oxidation of aspartate and alanine by their respective aminotransferases can lead to the accumulation of glutamate, which may be deaminated via glutamate dehydrogenase for being fed in to the citric acid cycle.

- An elevation in glutamate dehydrogenase activity in the gills, liver and kidney of fish exposed to 20 ppt and 30 ppt may be due to activation by leucine or activation by ADP and AMP.
- The Significant increase in arginase activity in the liver and gills of *O.mossambicus* exposed to 20 ppt and 30 ppt salinity may be due to an elevation in the cortisol level during acute salinity exposure.
- Increased oxygen consumption at 20 ppt and 30 ppt could be attributed to the increased requirement of osmoregulation and changes in the metabolism.
- The elevated plasma ammonia and increase in the ammonia excretion rate in *O.mossambicus* exposed to 20 ppt and 30 ppt salinity may be due to the increased catabolism of amino acids and increased activity of AMP deaminase.
- The significant increase in the ammonia quotient in *O.mossambicus* exposed to 20 ppt and 30 ppt salinity indicates increased protein degradation. Thus it is clear from the present study that proteins are very important fuels for energy production during stress conditions in fish.
- The present study shows that the time immediately after salinity acclimation is critical. Large amount of energy is required for osmoregulation during this critical phase.

- The study also indicates that protein catabolism is minimum in isosmotic salinity.
- Enhanced activity of AMP deaminase in the gill, liver, kidney and muscle tissue of *O.mossambicus* exposed to 20 ppt and 30 ppt salinity for 48 hours may due to the activation by sodium or potassium ions. Another reason for the increased activity is the limited proteolysis of AMP deaminase in response to salinity stress.
- Elevated activity of xanthine oxidase in the liver tissue of fish exposed to 20 ppt and 30 ppt salinity suggests an enhanced degradation of purine nucleotides leading to uricogenesis.
- The liver tissue of *O.mossambicus* exposed to 20 ppt and 30 ppt salinity showed an increased uricase and allantoinase activity. This may be due to an elevation in the level of cortisol during acute salinity exposure.
- The rise in serum uric acid content in *O.mosambicus* exposed to 20 ppt and 30 ppt salinity may be due to the increased activity of xanthine oxidase. The increased serum urea content in *O.mosambicus* exposed to 20 ppt and 30 ppt salinity may be due to the increased activity of enzymes such as arginase, uricase and allantoinase.
- No significant difference in the activities of enzymes and the level of metabolites was observed in the fish exposed to 10 ppt. salinity. This indicates that the energetic cost of osmoregulation is least in isosmotic salinity.
- Depletion of tissue protein content in *O.mossambicus* exposed to 40°C suggests that this is due to increased energy needs during stress.

- Decreased protein content was observed in tissues of *O.mossambicus* exposed to 15 °C. High rate of protein degradation at low temperature is suggested as an adaptive response to keep the steady-state concentration of oxidatively modified proteins at an acceptable level.
- Free aminoacid content was found to be significantly elevated in all tissues of *O.mossambicus* exposed to acute temperature change. The increased free amino acid pool can be used for ATP production by transamination reactions or by gluconeogenic pathway. Increase in free amino acid levels may be the result of breakdown of protein for energy and impaired incorporation of amino acids required in protein synthesis.
- Elevation in the levels of ALT and AST in different tissues of *O.mossambicus* exposed to 40°C can be considered as a response to stress induced by acute high temperature exposure. The high activities of aminotransferases results in the generation of ketoacids like α-ketoglutarate and oxalocetate for contributing to gluconeogenesis and/or energy production necessary to meet the excess energy demand.
- The activity of ALT has been significantly elevated in muscle tissue of *O.mossambicus* exposed to 15°C. But the exposure to 15°C significantly decreased the activity of ALT in liver and gill tissues. The increase in the aminotransferase activity in the muscle may signify that increasing amounts of free amino acid (alanine) are being liberated for the liver.
- Enhanced aspartate aminotransferase activity in the different tissues of *Oreochromis mossambicus* exposed to 15°C signifies that gluconeogenesis becomes more important during cold acclimation.

- Elevated glutamate dehydrogenase activity in the tissues of *O.mossambicus* exposed to 40°C may have helped more energy generation by funneling more α- ketoglutarate in to citric acid cycle. In *O.mossambicus* exposed to 15°C, a decreased activity was observed in all the tissues. This suggests accumulation of glutamate in the tissues during cold acclimation. The accumulated glutamate may aid in meeting the energy demands by being a gluconeogenic substrate.
- Enhanced activity of arginase in liver, gills and kidney of fishes exposed to 40°C results in increased urea production. Activity of arginase was reduced in fish exposed to 15°C. This explains the reduction in the synthesis of urea at low temperature.
- Oxygen consumption was found to be increased in *O. mossambicus* exposed to 40°C. The oxygen consumption rate of *O. mossambicus* at 15°C decreased significantly. This reflects the changes in metabolism.
- Enhanced plasma ammonia content and increased rate of ammonia excretion in *O. mossambicus* exposed to 40°C indicates increased rate of amino acid catabolism at high temperature. The decreased rate of ammonia excretion and reduced plasma ammonia content in fish exposed to 15°C may be due to the decreased activity of Glutamate dehydrogenase.
- A significant increase in the ammonia quotient was observed in *O.mossambicus* exposed to 40°C, which indicates increased protein degradation during stress. But fish exposed to 15°C showed a decreased ammonia quotient. This may be due to the decrease in ammonia excretion and oxygen consumption.

- Elevated AMP deaminase activity in *O.mossambicus* exposed to 40°C points to the fact that higher temperature causes a fall in the adenylate energy charge (Adenylate energy charge= the mole fraction of ATP plus half the mole fraction of ADP. According to Chapman *et al.* (1971) the value of energy charge $EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$).

  AMP deaminase may stabilize the adenylate energy charge. So the increase in the activity of AMP deminase may aid in the stabilization of adenylate energy charge. The decreased activity of xanthine oxidase in *O.mossambicus* exposed to 15°C indicates that the rate of enzyme catalyzed reactions are reduced by the low heat content of the cellular environment.

- The increased activity of xanthine oxidase in liver, kidney, muscle and gill tissues of fish exposed to 40°C indicates that the degradation of purines are high at high temperature.

- An increased uricase and allantoinase activity was observed in the liver of *O.mosambicus* exposed to 40°C. This may lead to an enhanced urea synthesis from purines. The reduced activity of uricase and allantoinase in *O.mosambicus* exposed to 15°C may be due to reduced metabolic activity at low temperature.

- The elevated serum urea and uric acid content in *O.mosambicus* exposed to 40°C may be due to the elevated activities of arginase and uricolytic enzymes such as uricase and allantoinase.

- The reduced activity of xanthine dehydrogenase and uricolytic enzymes caused a reduction in the serum uric acid and urea content in *O.mosambicus* exposed to 15°C.

The results obtained signifies that *O. mossambicus* when exposed to different levels of cadmium ion, salinity and temperature show great variation in the catabolism of proteins and purines. The organism is trying to attain homeostasis in the presence of stressors by increasing or decreasing the activity of certain enzymes. Parameters employed in this study can be adapted for future investigations as biomarkers for assessing stressed states in fish. The present study revealed that the protein and purine catabolism in *O. mossambicus* is sensitive to environmental stressors.

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