Biochemical Effects of Thermal Stress in a Tropical Teleost Fish *Etroplus suratensis* (Bloch, 1790)

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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 Thermal Stress in a Tropical Teleost Fish *Etroplus suratensis* (Bloch, 1790)"** is an authentic record of the research work carried out by **Mrs. Susan Joy** 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 for the award of any other degree, diploma or associateship in any University. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee have been incorporated in the thesis.

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(Co-Guide)

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August 2018

Declaration

I hereby declare that the thesis entitled **"Biochemical Effects of Thermal Stress in a Tropical Teleost Fish *Etroplus suratensis* (Bloch, 1790)"** is a genuine record of research work done by me under the supervision and guidance of **Dr. Babu Philip**, Retired Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution earlier.

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Susan Joy

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List of Abbreviations

%	- 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
ANOVA	- Analysis of Variance
APHA	- American Public Health Association
CT Max	- Critical thermal maximum
CT Min	- Critical thermal minimum
df	- Degrees of freedom
Dist.H_2O	- Distilled water
dL	- Decilitre
DO	- Dissolved oxygen
EDTA	- Ethylene diamine tetra acetic acid
FAO	- Food and Agricultural Organization
g	- Gram
g/ L	- Gram per litre
h	- Hour
H_2	- Hydrogen
H_2O_2	- Hydrogen peroxide
H_2O	- Water
O_2	- Oxygen
HSPs	- Heat shock proteins
HSE	- Heat shock element
HSF	- Heat shock factor
IARC	- International Agency for Research on Cancer
L	- Litre
M	- Molar

mg	- Milligram
mg/ dL	- Milligram per decilitre
mg/ g	- Milligram per gram
mg/ L	- Milligram per litre
min	- Minute
ml	- Milli litre
mM	- Millimolar
mm/ L	- Millimole/ litre
mRNA	- Messenger Ribo nucleic acid
N	- Normal
NAD+	- Nicotinamide adenine dinuclcotide (oxidised)
NADH	- Reduced nicotinamide adenine dinucleotide
NaOH	- Sodium hydroxide
nm	- Nano metre
OD	- Optical density
$O_2^{\cdot -}$	- Super oxide radical
ppm	- Parts per million
ppt	- Parts per thousand
ROS	- Reactive oxygen species
RNS	- Reactive nitrogen species
rpm	- Revolutions per minute
SD	- Standard deviation
SPSS	- Statistical Package for Social Sciences
TCA	- Tri chloro aceticacid
UILT	- Upper incipient lethal temperture
USEPA	- United States Environmental Protection Agency
WHO	- World Health Organization
Wt	- Weight
SOD	- Superoxide Dismutase
Cat	- Catalase
LPO	- Lipid Peroxidation
GST	- Glutathione-S-Transferase
GR	- Glutathione Reductase

GPx	- Glutathione Peroxidase
GSH	- Reduced Glutathione
GSSH	- Oxidised Glutathione
HDL	- High Density Lipoprotein
LDL	- Low density Lipoprotein
VLDL	- Very Low Density Lipoprotein
SDS PAGE	- Sodium Dodecyl Polyacrylamide Gel Electrophoresis
PCR	- Polymerase Chain reaction
TBARS	- Thiobarbituric acid reactive substances
W	- Watts
TCA-TBA-HCl	- Trichloroacetic acid – Thiobarbituric acid – hydrochloride
MDA	- Malondialdehyde
U	- International units
PUFA	- Polyunsaturated fatty acids
LOOH	- Lipidhydroperoxide
AP-1	- Activator Protein 1
NFkB	- Nuclear Factor kappa B
TNF	- Tumor Necrosis Factor
TBS	- Tris Buffered Saline
RIPA	- Radioimmunoprecipitation Assay Buffer
ORF	- Open Reading Frame
BLAST	- Basic Local Alignment Search Tool
PCR	- Polymerase Chain Reaction
CR	- Cortisol Receptor
bp	- Base Pair
ORF	- Open Reading Frame
OH	- Hydroxyl radical
CDNB	-1-chloro 2,4 dinitrobenzene
DTNB	- 5,5-dithio-bis-2-nitrobenzoic acid

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Chapter 1

General Introduction

Contents

Most of the organisms on Earth are ectotherms which have to survive and adapt to temperature fluctuations (Hochachka and Somero, 2002; Guschina and Harwood 2006; Somero 2010). Temperature affects all aspects of physiology by influencing the reactive rates as well as the physical properties of biological molecules (Hochachka and Somero, 2002; Crockett and Londraville, 2006). For marine ectotherms including fish, environmental temperature has the pervasive effects on physiological and biochemical functions at all levels of biological organization, from molecule to organism (Hochachka and Somero, 2002; Hofmann *et al*., 2002; Donaldson *et al.,* 2008). According to the tolerance range of temperature, the fishes can be classified into two groups, eurythermal and stenothermal species. Eurythermal fish can maintain their metabolic activity at extreme temperatures, where as in stenothermal fishes, changes in environmental temperature may lead to the poor maintenance of

physiological homeostasis resulting in temperature stress (Hochachka and Somero, 2002; Somero, 2010; Long *et al.,* 2012). The rise of water temperature due to climate change puts additional stress on the freshwater ecosystems. Temperature, salinity and dissolved oxygen are the major factors influencing the aquatic environment. The rates of many physiological processes are influenced by the water temperature which directly affects biological performance in aquatic ectothermic animals. Due to the role of water temperature as a major metabolic modifier, the performance of an aquatic animal could be affected by any changes of the environment (Mjoun *et al.,* 2010). Oxygen solubility will be affected by water temperature and this in turn affects the metabolism of aquatic organisms (Boyde, 2002). This condition will lead to a spatial shift in animal distribution as water temperature limits their physiological functions (Diaz and Breitburg, 2009). Thermal changes would pose threats to aquaculture yield and productivity for economically important freshwater fishes. Water temperature changes can induce either detrimental or adaptive alteration in the performances of aquatic organisms.

Due to environmental disturbances many physiological changes occur which are now used routinely for assessing stressed states in fish. Responses to stress are mediated through neuronal and endocrine pathways, known as the primary response, following initial perception of the stressor. These, in turn, can influence secondary physiological features and tertiary or whole animal (fish) performance which could result in stress-induced alterations in fish populations. The initial stress response is considered adaptive, one designed to help the fish to overcome the disturbance 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 it enters a maladaptive or distressed state leading to decreased performance, a pathological condition which may be lethal. In fish, typical primary responses which are used for evaluating stress include determining circulating levels of cortisol and, to a lesser extent, catecholamines (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). Secondary responses include measurable changes in blood glucose, lactate, major ions (e.g., chloride, sodium) and osmolality, tissue levels of glycogen and lactate, and heat-shock proteins at the cellular level. To evaluate responses of fish to acute stressors, physiological measurements provide a useful approach but they may not necessarily be so for monitoring fish experiencing sub lethal chronic stress. The stressors are severe enough to challenge the fish's homeostatic mechanisms beyond their capacity to adjust; physiological mechanisms will generally adapt to compensate for the stress.

Endocrine and cellular stress responses are likely mechanisms by which fish cope with stressfully elevated temperatures and may serve as strong bioindicators for measuring these sub lethal effects. As it is in other vertebrates, cortisol is the major corticosteroid stress hormone in fish (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999). Cortisol plays a critical role in the stress response because, among other functions it is responsible for adjusting metabolic pathways and mobilizing energy stores in the liver through gluconeogenesis pathway (Vanderboon *et al*., 1991; Wendelaar Bonga, 1997). In response to a real or perceived stressor, the hypothalamic–pituitary–interrenal axis is activated and releases cortisol in fish (Mommsen *et al*., 1999). In addition to the

endocrine stress response, there is increasing attention on the use of heat shock proteins (HSPs), as potential biomarkers for thermal stress (Iwama *et al.,* 1999; Wikelski and Cooke, 2006). Inducible isoforms of HSPs are up regulated in the presence of denatured proteins, which can result from a variety of environmental stressors, including elevated temperature (Tomanek, 2010; Deane and Woo, 2011). Other factors, including social interaction may influence HSP concentrations (Currie *et al*., 2010) and there is a complex interaction between HSPs and the endocrine stress response in fish (Boone *et al*., 2002).

In the present study, three important aspects such as effect of thermal stress on antioxidant activity, hematological parameters and Heat shock proteins in *Etroplus suratensis* were investigated.

1.1 Antioxidants

Several types of antioxidants are found in all fish species to protect their lipids against damage caused by reactive oxygen species (ROS). These compounds belong to various chemical groups and make use of their antioxidative effects via different modes of action. These include antioxidant enzymes, amino acids, peptides, ascorbic acid, carotenoids and phenolic compounds such as tocopherols and ubiquinones. Stress which is induced by changes in temperature is associated with enhanced generation of reactive oxygen species, which may seriously affect immune function and lead to oxidative stress (Fisher and Newell, 1986; Shin *et al*., 2010a). Overproduction of ROS in response to any stress can lead to oxidative damage (Halliwell and Gutteridge, 1989) and increased lipid peroxidation, and may affect cell viability by causing membrane

damage and enzyme inactivity (Kim and Phyllis, 1998; Pandey *et al*., 2003). Complex antioxidant defense systems maintain homeostasis in changing environments and protect aerobic organisms against ROS and subsequent oxidative stress-induced damage (Bagnyukova *et al*., 2007). Antioxidants are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST), and glutathione reductase (GR) or compounds such as metallothionein, ascorbic acid, and vitamin E (alpha-tocopherol) (McFarland *et al.,* 1999). Antioxidant defense systems are found in the liver, gills and kidneys of marine organisms (Basha and Rani, 2003) and have the following functions. SOD, CAT and GPX directly scavenge ROS and inactivate it. SOD breaks down superoxide through the process of dismutation to O_2 or H_2O_2 (Kashiwagi *et al*., 1997). H_2O_2 produced by SOD is sequentially reduced to H_2O and O_2 by CAT (Kashiwagi *et al.,* 1997). Glutathione peroxidase uses reduced glutathione to reduce H_2O_2 to H_2O thereby counteracting the toxicity of H_2O_2 (Kashiwagi *et al.,* 1997). ROS are the most powerful oxidants formed in biological systems which can readily attack any biological molecule and it also attack polyunsaturated fatty acids which can lead to lipid peroxidation (Gutteridge, 1995). Lipid peroxidation proceeds through a free radical chain mechanism involving initiation, propagation and termination steps. In fish lipid oxidation is influenced by several catalytic systems for oxygen activation. To overcome the spin restriction between ground state oxygen and lipids, the reaction requires initiation which may be the activation of ground state oxygen ($^3O_2$) into singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HOO•), peroxides (LOOH), or transformation of unsaturated

lipids into lipid radicals (L•). Under most circumstances, autooxidation starts in the presence of initiators with an extraction of hydrogen atom from a fatty acid to produce the free radical. Subsequently the reaction proceeds through propagation reactions, which produce further free radicals. In the terminating reaction two free radicals combine to produce non-radical products. Lipid hydroperoxides (LOOH) are formed in the propagation phase when lipid radicals react with oxygen. They are relatively stable, but transition metal ions (Me), such as iron and copper, as well as heam compounds catalyze their decomposition both by oxidation and by reduction;

$$LOOH + Me^{n+} \rightarrow LO\bullet + OH^{-} + Me^{n+1}$$

$$LOOH + Me^{n+1} \rightarrow LOO\bullet + H^{+} + Me^{n+}$$

Transition metal ions are therefore important prooxidants for the initiation of lipid oxidation. In fish, other components such as proteins, amino acids, vitamin D and ascorbate can interact with these free radicals to terminate the reaction. When components other than lipids terminate the reaction, they are often referred to as antioxidants. Antioxidants reduce free radicals and are themselves oxidized. After that they may be reduced to their active forms by other reducing systems. Glutathione-S- transferase (GST) is a chain breaking antioxidant. It interferes with the propagation step of lipid oxidation by reacting with the lipid derived radicals. Chain breaking antioxidants can be divided into hydrogen or electron donors to peroxyl or hydroxyl radicals and hydrogen or electron acceptors from carbon-centered radicals (Scott, 1997). Hydrogen or electron donors comprise phenol antioxidants and the hydrogen or

electron acceptors include the stable phenoxyl radicals and quinonoid compounds (Scott, 1997).

The action of chain breaking antioxidants is as follows

$LOO\bullet + AH\bullet \longrightarrow LOOH + A\bullet$ (A – antioxidant)

$LO\bullet + AH \longrightarrow LOH + A\bullet$

$A\bullet + LOO\bullet \longrightarrow$ Non-radical products

$A\bullet + A\bullet \longrightarrow$ Non-radical products

The ability of an antioxidant to reduce another antioxidant or a lipid derived radical is based on their reduction potentials (Buettner and Jurkiewicz, 1996). Antioxidants can act at different stages in the oxidation process and most of them have more than one mechanism of action. Their mode of action can be divided into the following categories (Symons and Gutteridge, 1998).

1) Removing oxygen or decreasing local O_2 concentrations.
2) Removing catalytic metal ions.
3) Removing reactive oxygen species such as $O_2\bullet^-$ and H_2O_2.
4) Scavenging initiating radicals such as •OH, LO• and LOO•.
5) Breaking the chain of an initiated sequence.
6) Quenching or scavenging singlet oxygen.

1.2 Reliable indicators of thermal stress

The magnitude of the stress response in fish may vary according to ontogeny, genetic, and environmental factors (Schreck, 1981). Temperature is an environmental factor which can modify the stress response when fish encounter conditions such as abrupt thermal changes or exposure to cold or warm water. In fish the physiological stress response can depend on the temperature to which the fish are acclimated. Temperature variations in environment affect many properties and functions of biomolecules. It also affects the structural components of the cell, such as folding, assembly, activity and stability of proteins, structure and rigidity of lipids, and fluidity and permeability of cell membrane. In fish, determination of blood parameters may be important in establishing the effect of heat stress. In fish, elevation of circulating levels of glucose (hyperglycemia) following stressful disturbances is a major metabolic response to stress (Love, 1980; Wedemeyer *et al.*, 1984). An increase in plasma glucose indicates mobilization of energy reserves such as tissue glycogen through glycogenolysis and may reflect the degree of metabolic activity (Umminger 1977; Love, 1980). Stress-induced increase in blood glucose is an adaptive response to provide an energy source for the fish during stressful conditions (Love, 1980). In addition to plasma glucose, other secondary responses to stress include changes in plasma lactate, liver glycogen, hydromineral balance and hematological features such as hematocrit and circulating lymphocytes (Mazeaud *et al.*,1977; Wedemeyer and McLeay 1981; Wedemeyer *et al.*, 1984).When placed under stressful condition, plasma cortisol and glucose levels tend to respond more quickly and mortality rates are often higher in fish acclimated to warm

water, than cold water (Strange, 1980; Barton and Schreck, 1987). In fishes blood glucose and total serum cholesterol levels are physiological adaptation mechanisms that can be affected by high ambient temperatures. Blood glucose and lipid parameters show greater differences in hot conditions than in the comfort zone. The marked decrease in total serum cholesterol levels may have a relation with the increase in total body water or the decrease in acetate concentration which is the primary precursor for the synthesis of cholesterol. Protein parameters decreased during thermal stress and this may be due to increase in plasma volume as a result of heat shock which causes results in decreases plasma protein concentration. Prolonged exposure of solar radiations increased plasma total protein, albumin, and globulin. This might be due to vasoconstriction and decreased plasma volume during heat stress (Helal *et al*., 2010).

1.3 Heat Shock Proteins

Heat shock proteins (Hsps), also known as stress proteins are a suite of highly conserved proteins of varying molecular weight (16-100 kDa) produced in all cellular organisms when they are exposed to various kinds of stress. They are a family of highly conserved cellular proteins present in all organisms including fish. The transcription of HSP is mediated by the interaction of heat shock factors (HSF) with heat shock elements (HSE) in gene promoter regions. They play a pivotal role in protein homeostasis and cellular stress response within the cell (Feder and Hofmann, 1999; Iwama *et al*., 2004; Mao *et al.*, 2005; Multhoff, 2007; Keller *et al.*, 2008a). Hsps play an important role as helper molecules or chaperones, and it is now studied that the up-regulation in response to

stress is universal and not restricted to heat stress. Stressors such as anoxia, ischemia, toxins, protein degradation, hypoxia, acidosis and microbial damage will also lead to up-regulation of Hsps. In the regulation of normal protein synthesis within the cell Hsps has a major role. Among the Hsp families, Hsp90 and Hsp70 are critical to the folding and assembly of other cellular proteins and are also involved in regulation of kinetic partitioning between folding, translocation and aggregation within the cell. Hsps also have a wider role in relation to the function of the immune system, apoptosis and various aspects of the inflammatory process. In aquatic animals, Hsps have been shown to play an important role in health, in relation to the host response to environmental pollutants and food toxins and in particular in the development of inflammation and the specific and non-specific immune responses to bacterial and viral infections particularly in fishes.

Hsp induction can increase tolerance to subsequent stressors. Hsp28, Hsp70, and Hsp90 induction in the renal epithelium of the white flounder protects the cells against the damaging effects of severe heat and 2,4-dichlorophenoxyacetic acid on sulphate transport (Brown *et al*., 1992; Renfro *et al.*, 1993; Sussman-Turner and Renfro, 1995). Stress induced increases in Hsps in fish may occur in a threshold manner, rather than in a graded way dependent on the degree of the stressor. Heat stress can induce various heat shock proteins in cell lines (Kothary *et al.*, 1984), primary cell culture, and in tissues from whole animals (Koban *et al*., 1991). Hsp90 mRNA in Chinook salmon *(Oncorhynchus tshawytscha*), and Hsp54 and Hsp70 in Atlantic salmon (Smith *et al.*, 1999) are produced in response to osmotic stress. In tissues of fish exposed to

environmental contaminants, such as heavy metals (Heikkila *et al.*, 1982), industrial effluents (Janz *et al.*, 1997), pesticides (Sanders, 1993) and polycyclic aromatic hydrocarbons (Vijayan *et al.*, 1997) elevated levels of various heat shock proteins have been measured. One of the most important physiological functions associated with the stress induced accumulation of the inducible Hsp70 was acquired thermotolerance, which is defined as the ability of a cell or organism to become resistant to heat stress after a prior sub lethal heat exposure. Hsp70 was associated with the development of tolerance to a variety of stresses, including hypoxia, ischemia, acidosis, energy depletion, cytokines such as tumor necrosis factor-α (TNF-α) and ultraviolet radiation. The phenomenon of acquired thermotolerance is transient in nature and depends primarily on the severity of the initial thermal stress. The greater the initial heat dose, the greater the magnitude and duration of thermotolerance. The expression of thermotolerance following heating will occur within several hours and last 3–5 days in duration. The similar kinetics of thermotolerance demonstrated by cells, tissues, and animals suggest that the morbidity and mortality associated with whole body heating is due in part to the dysfunction of some critical target tissues. Development of thermotolerance results from the improved tolerance of the weakest organ and cell systems. Presumably, the tissues are both heat sensitive and vital to the animal. Cellular manipulations that either block Hsp70 accumulation or overexpress certain Hsps have been shown to either increase or decrease heat sensitivity. Hsps appear to play a role in protecting cells from damage generated by a variety of stressors. Their synthesis is associated with protection against light-induced damage to the retina and ischemia-

reperfusion injury to the heart, liver, and kidney. Studies of cardiac shock followed by resuscitation have revealed that hepatocytes synthesize members of the Hsp70 family early in the course of recovery. The fact that Hsp70 message is preferentially translated by a cell under stress to the exclusion of other messages may result in the inability of the cell to produce some proteins or respond to additional signals. In this model, the cell may "choose" self-preservation over tissue preservation to the detriment of the organ. This model may be particularly relevant in a situation where Hsp70 accumulation could be utilized as a biomarker of cellular injury. Subsequent resumption of translation resulted in Hsp mRNA being translated into Hsps before the synthesis of other proteins take place within the cell. The period of translational arrest in response to heat stress could be shortened if cells were first made thermotolerant. A primary function of Hsps during cellular stress is to maintain translation and protein integrity. Cells that were made thermotolerant also produced less Hsp during a second challenge compared with unheated cells, suggesting there is a regulation of Hsp synthesis that is dependent on the levels of these proteins existing within the cell. The increase in Hsp 70 synthesis in heat-stressed leukocytes was inversely proportional to the length of the initial "conditioning" exercise stress, suggesting that cells regulate the amount of these stress proteins in response to repeated challenges. An additional issue related to the development of thermotolerance deals with the possibility that Hsps, through their interaction with cellular proteins during translational arrest, play a role in preventing protein denaturation and processing

There are many possible applications of measuring the stress response in fishes, and other aquatic organisms. They range from resolution of the generalized stress response, to the monitoring of the quality of the aquatic environment through the stressed states of the organisms that live there. However, these applications can only be developed if there is unequivocal evidence for a relationship between the stressed state of the animal and the cellular stress response. The fact that stressors cause the induction of specific proteins offers the possibility of developing diagnostic probes for monitoring the condition of fish and their environment. The evidence showing that increased levels of Hsps induce tolerance of cells, tissues, and whole fish to subsequent stressors suggests that it may be possible to develop strategies to enhance tolerance to stressors by inducing the cellular stress response. A non-lethal heat shock (NLHS) of 37°C for 30 min followed by 6 hour recovery maximally induced endogenous Hsp70 and optimally enhanced the resistance of Artemia (*Artemia parthenogenetica*) larvae against *Vibrio campbellii* and *Vibrio proteolyticus*, two Vibrio species known to infect brine shrimp (*Artemia salina*). The two-fold increase in larval survival, in association with stress protein synthesis, suggests a protective role for Hsp70. Exposure of Artemia larvae to a combined hypothermic and hyperthermic shock enhanced the amount of a 70 kDa polypeptide which reacted with antibody to Hsp70. Protection against infection by *V. campbellii* was significantly enhanced in the larvae, with the result again supporting a causal link between Hsp70 accumulation induced by heat stress and enhanced resistance to infection. In shrimp other than Artemia where Hsp70 build-up after a 24 hour hyperthermic stress from 29°C to 37°C correlates with attenuation of gill-associated

virus (GAV) replication in the black tiger prawn. Supplying exogenous Hsps, either by feeding with Hsps encapsulated in bacteria or injecting recombinant Hsp70, represents another way to limit Vibrio infection in aquatic organisms. Feeding with *E. coli* YS2 over-producing DnaK, the prokaryotic equivalent of Hsp70, enhances Artemia larvae survival approximately two- to three-fold upon challenge with pathogenic *V. campbellii*. Larvae were fed with heated bacterial strains LVS 2 (*Bacillus* species), LVS 3 (*Aeromonas hydrophila*), LVS 8 (Vibrio sp), GR 8 (*Cytophaga* species*)* and GR 10 (*Roseobacter* species*)*, all of which produce increased amounts of DnaK when compared to non-heated bacteria. Improvement in larval resistance to *V. campbellii* infection correlates with escalating amounts of DnaK, suggesting a protective role for this protein, either via chaperoning or by immune enhancement. In fish, intra-coelomal injection with DnaK and GroEL, proteins equivalent to mammalian Hsp70 and Hsp60, combined with a non-lethal heat shock, safeguards *Xiphophorus maculates* from death caused by *Yersinia ruckeri*. The resistance of aquatic organism to stress is enhanced by endogenous DnaK / Hsp70. Hsp70 may stabilize cells against injury due to thermal stress, assist the proper folding of cell proteins synthesized in response to bacterial pathogens and facilitate the storage and re-folding of partially denatured proteins. Hsps are thought to influence the production of cell surface peptides which are presented to the immune system, facilitating recognition of diseased cells and they are involved with Toll- like receptors, a major element of the innate immune system.

Among the three indigenous cichlids, *Etroplus suratensis* is the largest and native of peninsular India, occurring primarily in Kerala and

Southern Karnataka; the other species being *Etroplus maculatus* and *Etroplus canarensis*. The family Cichlidae that occur in freshwater as well as brackishwater habitats comprises over 700 species of fishes. Cichlid fishes speciation in tropical lakes has been reported to be miraculous and they appear conservative structurally, occupy a prodigious range of ecological niche. Cichlids have a most important role in tropical freshwater fisheries and aquaculture. From Egyptian frescos, Cichlid specimen has been recognized. *Etroplus* is the only genus endemic to India among the Cichlid group. *E. suratensis*, popularly known as Karimeen is widely distributed in almost all the brackish and freshwaters, along the coastal tracts from south Canara to Malabar on the west coast to Chilka lake on the east coast. It is essentially a brackish water fish that has become naturally acclimatized to freshwaters. *E. suratensis* seeds are widely seen in backwaters of Kerala. It is an economically important food fish and it fetches a very high price locally due to its delicacy. The quality of the flesh and its palatability has been determined by the environmental features in which they grow. The fish was reported to constitute almost 10 percent of the total fish landings from the backwaters of Kerala. Due to this it is the first Indian food fish that has been transplanted to any foreign country. It is much suited to aquaculture owing to good palatability, herbivorous feeding habits, and its hardy and nonpredaceous habit. They are also valued as ornamental fishes because of their unique coloration and remarkable patterns. The colouration and markings are highly susceptible to change in relation to the emotional condition and life-phase of this fish. It is ideally compatible for polyculture with both freshwater and brackish water fish and it breeds naturally in confined conditions. The

unique parental and custodial care, monogamously mating habits, small clutch size and their exclusive substrate breeding nature, hamper the production of these fishes in disturbed habitats. Information on the biological features is indispensable for devising valid programs for conservation of this fishes. Life history traits and intra specific variations are acquired over evolutionary history and because of this each species aid to buffer against various natural and anthropogenic stresses. Biological characterization will also be of immense use for identifying the characteristic of the species that qualify them as suitable candidates for aquaculture.

Systematic position of the experimental animal *Etroplus suratensis* (common name -Karimeen) employed in this study is as follows (Fig 1.1)

Phylum	:	Vertebrata
Subphylum	:	Craniata
Super class	:	Gnathostomata
Series	:	Pisces
Class	:	Teleostei
Subclass	:	Actinoptergii
Super order	:	Acanthoptergii
Order	:	Perciformes
Suborder	:	Labroidei
Family	:	Cichlidae
Genus	:	*Etroplus*
Species	:	*suratensis*

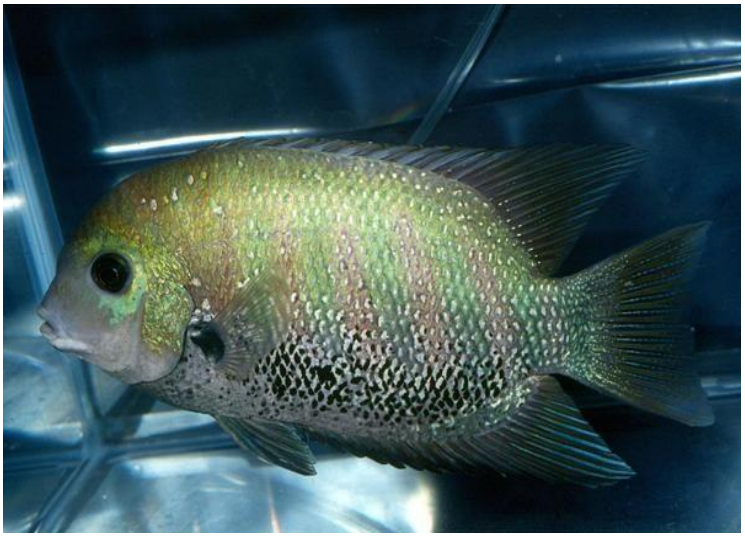


Fig. 1.1 *Etroplus suratensis* (Bloch, 1790)

1.4 Objectives of the study

The specific objectives of the study are:

1) To investigate the effect of thermal stress on the antioxidant defense response in *Etroplus suratensis*

2) To evaluate the extent of oxidative stress at the time of thermal stress in *E. suratensis* by lipid peroxidation

3) To study the biochemical effects of thermal stress on carbohydrate, lipid and protein profile of *E. suratensis*

4) To study the expression profile of Heat Shock Proteins (Hsps) in *E. suratensis* and its molecular characterization.

1.5 Outline of the thesis

The thesis is presented in six chapters. Chapter 1 is the general introduction. Chapter 2 deals with the glutathione independent antioxidant activity during thermal stress. Chapter 3 deals with the glutathione dependent antioxidants activity. Chapter 4 deals with the changes in carbohydrate, lipid and protein profile during thermal stress. Chapter 5 give emphasis on Heat shock proteins (Hsps), particularily Hsp 70, its identification, PCR amplification and molecular characterization. The whole study is summarized in Chapter 6 with special emphasis on salient findings of the study. This is followed by a list of References, GenBank Submission and Publications.

Chapter 2

Effect of Acute Temperature Fluctuations on Glutathione Independent Antioxidants of *Etroplus suratensis*

Contents

2.1 Introduction

Aquatic organisms are exposed to local and global environmental stressors. Exposure of organisms to various stressors may results in various physiological and biochemical changes. At the organismal level these changes are mediated by the neuroendocrine system (Nakano *et al.*, 2014). In addition to the neuroendocrine stress response, there is cellular stress response when organisms are exposed to stressful situations. These stress responses affect the general health, disease resistance, reproduction and growth of the organism. The physiological state of organism is affected by the environmental temperature. As a result, their biological geographic distribution can be affected by temperature. In ectotherms intensification of respiration at higher temperatures would result in enhanced reactive oxygen species (ROS). To quickly dispose ROS and

maintain their intracellular concentration at physiological levels, all aerobic cells possess antioxidant defenses including superoxide dismutase (SOD), catalase (CAT), glutathione dependent (GSH) antioxidant enzymes.

2.2 Review of Literature

Temperature is considered as the major abiotic factor which influences the life in the aquatic environment (Brett, 1971). It was reported that temperature fluctuations can induce stress in fishes (Wedemeyer *et al*., 1990). Fishes offer an ideal and convenient model to study the effects of thermal and other complex stressors for short and long duration of time. This is because fishes are typical ectothermic vertebrates. The metabolic rate and oxygen consumption of ectothermic animals change with changes in environmental temperature (Hochachka and Somero, 2007). Such variations in the metabolism induce the generation of reactive oxygen species (ROS) in the mitochondria (Boveris and Chance, 1973; Boveris, 1977; Halliwell and Gutteridge, 2007; Nelson and Cox, 2008). Reactive oxygen species are chemically reactive molecules containing oxygen. They are formed as natural byproducts of the normal metabolism of oxygen and have important roles in cell signalling and homeostasis (Cadenas, 1989). During the time of environmental stress such as heat exposure, the level of ROS can increase dramatically (Gerschman *et al*., 1954). When the production and accumulation of ROS is beyond the organism's capacity to handle, there is oxidative stress (Vingare *et al*., 2012). Some reactive oxygen species can initiate lipid peroxidation, a self – propagating process in which a peroxy radical is formed when the ROS has the ability to abstract a

hydrogen atom from an intact lipid. The reaction of ROS with lipids is one of the most prevalent mechanisms of cell damage (Halliwell and Gutteridge, 1989). An increase in concentration of ROS leads to free radical chain reactions which in turn damage cellular proteins (Stadtman and Levine, 2000), lipids (Rubbo *et al.*, 1994), polysaccharides (Kaur and Halliwell, 1994) and DNA (Richter *et al*., 1988). The rate of generation of ROS such as superoxide anion ($O_{2.}^{-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) is related to consumption of oxygen. ROS are molecules produced physiologically and continuously at the mitochondrial cristae mainly as by-products of oxygen consumption (Boveris and Chance, 1973). The intensification of respiration at higher temperatures results in higher ROS formation and the adaptive responses by the antioxidant defenses occur when the organism is shifted from low to high temperatures. Cellular stress biomarkers have been widely used for the development of ecological indices and in the assessment of oxidative stress on exposure to environmental contaminants. Extent of lipid peroxidation and the activities of catalase and superoxide dismutase are the commonly used biomarkers for habitat quality assessment. Environmental variables are strongly related to seasonality and extreme natural events such as temperature and salinity. These factors have significant effect on oxidative stress biomarkers. So these are the confounding factors which may result in difficulties in interpretation of patterns of biomarkers. It is assumed that the frequency, intensity and duration of extreme natural events may increase in the future. As a result of this, heat waves will be more common in many parts of the world. However environmental assessment must take into account the fact that

temperature fluctuations may hinder the interpretation and comparison of biomarkers such as oxidative stress biomarkers. The cellular stress response of different tissues may be different due to difference in their distribution and function. It has been reported that antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) work to nullify ROS and maintain their intracellular concentration at physiological levels (Jeevitha *et al.,* 2014). Some co-adjuvant enzymes such as glutathione reductase (GR) and glutathione-S-transferase (GST) (Halliwell and Gutteridge, 2007) also play the same role. Antioxidant enzymes usually act in a coordinated manner in order to ensure the optimal protection against oxidative stress (Morales-Medina *et al*., 2017). Nevertheless, when organisms are exposed to environmental stress, ROS can increase extensively causing injury to cells. For the survival of critical temperature stress the interaction between oxidative stress, antioxidants and Heat shock proteins (Hsps) (Heise *et al.,* 2006) is very crucial.

Superoxide dismutase catalyzes the dismutation of superoxide ion into oxygen and hydrogen peroxide. Hydrogen peroxide is decomposed into water and oxygen (Halliwell and Gutteridge 2007). Univalently reduced $O_2^{\cdot -}$ are reduced to uncharged hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD) activity. H_2O_2 thus produced diffuses freely through mitochondrial and cellular membranes. If H_2O_2 is not enzymatically decomposed, it can be converted to the very short-lived and highly aggressive $^{\cdot}OH$, via the transition metal catalyzed Fenton reaction (Halliwell and Gutteridge, 1985). The Fenton reaction is a driving force in tissue damage and apoptosis and is involved in ROS

signalling functions. In order to control ROS production, aerobic cells are endowed with an array of antioxidant enzymes which either convert O_2 to H_2O_2 by superoxide dismutase, convert H_2O_2 to water and oxygen by catalase, or use H_2O_2 to oxidize substrates (peroxidases, e.g. glutathione peroxidase) (Abele 2004). The enzymatic antioxidant system is supplemented by small molecule antioxidants such as glutathione, vitamins E, A, and C, urate and biliverdin (Di Giulio *et al*., 1989; Storey, 1996).

Lipid peroxidation (LPO) is a complex process where a cascade of free radical initiated reactions on unsaturated fatty acids leads to the formation of certain peroxide radicals that can cause damage to various tissues. Among lipids, polyunsaturated fatty acids are more sensitive to oxidative modifications by ROS. The intensity of lipid peroxidation is assessed by measuring the levels of primary products, conjugated dienes and lipid peroxides, and end products of lipid peroxidation such as malondialdehyde (MDA) and other aldehydes, which are assayed with thiobarbituric acid and expressed as thiobarbituric acid-reactive substances (TBARS) (Rice-Evans *et al*., 1991; Halliwell and Gutteridge, 1989). Most of the lipid peroxidation products are toxic and mutagenic. They may form DNA adducts, giving rise to mutations and altering gene expression (Marnett, 1999). Peroxidized membranes become rigid and this may cause changes in their permeability and integrity. LPO and oxidative stress are highly correlated and the products of LPO can be used as biomarkers for the evaluation of oxidative stress status (Lushchak and Bagnyukova, 2006a). Stress biomarkers have been widely used in the assessment of exposure of aquatic organisms to environmental contaminants (Haux and Forlin, 1988). However, the levels of these biomarkers may

depend on environmental variables other than chemical pollutants. It is thus relevant to evaluate the effect of other important variables on oxidative stress biomarker levels in relevant species of the ecosystem and in species important in aquaculture.

Pearl spot or *E. suratensis* is a tropical euryhaline culture fish widely cultured and distributed in Peninsular India and Sri Lanka. It is an oval-shaped cichlid with a short snout, small mouth not extending past the front margin of the eye with a greyish-green colouration on the flanks, with 6 to 8 dark bars and a dark spot at base of the pectoral fin. Most scales on the sides are with a pearly spot. Macrophytic fragments form the most important component of its diet along with molluscs, although detritus, diatoms and animal matter. Jayaprakash *et al.* (1990) studied its potential as a suitable culture fish in ponds and tanks and the captive breeding and hatchery rearing technologies were standardized. Culturing *E. suratensis* in open cage is done either as monoculture or as polyculture along with other brackish water fishes (Ignatius *et al*., 2010). It can be also farmed under extensive and intensive farming in freshwater, brackish water eco-systems and in homestead ponds in the backyard of houses. This can serve as an occupation for poverty alleviation and high valued fish production in southern states of India. *E. suratensis* was selected as the animal model for the study owing to its wide availability, ease of rearing, maintenance and sustainability in laboratory conditions. It is known as the State Fish of Kerala. It was reported that environmental contaminants such as nonylphenol induced oxidative stress in *E. maculatus* which leads to the alterations in the antioxidant enzyme defense (Asifa *et al*., 2016).

2.3 Objectives of the study

1) To investigate the effect of thermal stress on tropical culture fish *E. suratensis* by evaluating oxidative stress biomarkers such as superoxide dismutase, catalase and lipid peroxidation at tissue level.

2) An attempt is made to trace the pattern of antioxidant defense response of selected organs of *E. suratensis* in the event of a thermal stress.

3) To evaluate the extent of oxidative stress at the time of thermal stress in *E. suratensis* by lipid peroxidation.

4) To identify the difference in antioxidant enzyme activity during the selected temperatures.

2.4 Materials and methods

2.4.1 Biological Model

The tropical brackish water cichlid fish *E. suratensis* (Bloch, 1790) commonly known as 'pearl spot' was selected as animal model for the study owing to its aquaculture potential, ease of rearing, maintenance and sustainability in laboratory conditions.

2.4.2 Experimental design

2.4.2.1 Collection and maintenance of fish

Apparently healthy adult *E. suratensis* (35± 3g) were collected from culture ponds of fisheries station of Kerala University of Fisheries and Ocean studies, located at Puthuvyppu, Kochi, Kerala, India. The fishes

were acclimatized to laboratory condition for one month in recirculating aquaculture tanks. The water quality parameters were estimated daily according to the procedure of APHA (1998). The dissolved oxygen content, pH, temperature and salinity of water in the experimental tank were 7.8 ppm, 7.0 ± 0.32, 26 ± 2^{o}C and 0 ppt respectively in natural light and dark cycle. Hardness of the water was below detectable amounts. Fishes were fed on a commercial pellet (Godrej Grower, Godrej Agrovet Pvt Ltd, Maharashtra, India) *ad libitum.* The laboratory acclimatized fishes were sorted into batches of seven animals each and were kept in 60 L tubs for the experiment. Water in the tub 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 is likely to affect the organism.

2.4.2.2 Determination of CT Max (Critical Thermal Maximum) and CT Min (Critical Thermal Minimum)

The maximum temperature in which fish can survive is designated as CT Max (Mora and Ospina, 2001). CT Max was estimated by increasing the temperature by one degree in every one hour starting from the ambient temperature (27^{o}C). Similarly CT Min, minimum temperature in which fish can survive is estimated by decreasing temperature by one degree in every one hour from ambient temperature (27^{o}C). CT Max and CT Min were determined based on the loss of equilibrium of 50% of the total fishes (20 fishes for each temperature) with respect to increase or decrease of temperature. CT Max and CT Min were observed as 40^{o}C and 14^{o}C respectively.

2.4.2.3 Experimental design for the study of the effects of temperature

After acclimatization, fishes were divided into three groups to be maintained at 16^{o}C, 27^{o}C (ambient temperature) and 38^{o}C respectively. These three temperatures represent sub CT Min, ambient, sub CT Max respectively. These three temperature regimes were selected based on CT Max and CT Min temperature exhibited by the experimental organism as described above. Twenty eight fishes were maintained for the study at each temperature. For the study, each group was transferred into thermostatized tanks of 500 L set at the selected temperatures and maintained for 72 hours. Fishes were fed *ad libitum* throughout the experimental period to avoid the effect of starvation on normal physiological processes. The water was changed daily and the test solutions were renewed every 24 hours to maintain the dissolved oxygen concentrations at optimum level (USEPA, 1975). Any other factor likely to influence temperature was nullified by maintaining a suitable control (27^{o}C).

2.4.2.4 Preparation of tissue samples for the biochemical study

At 0th and at every 24th hour of treatment, seven fishes were randomly selected from each group and were sacrificed after placing them in a sedative solution (clove oil, 1 mL/ L) for five minutes. The animal was dissected and tissues such as gills, liver, muscle and brain were collected and were wiped thoroughly using blotting paper to remove blood and other body fluids. Then they are washed in ice cold 0.33 M sucrose solution and again blotted dry and the desired amount of the tissues were weighed. The tissues were homogenized in a homogenizer (KEMI Model No. KHH1) in ice cold Tris - HCl buffer (0.1 M, pH 7.5)

to get 5% homogenate and the supernatant fractions were collected after centrifugation at 11,200 x g for 15 minutes at 4ºC and used for biochemical analysis.

2.4.3 Methods used for the biochemical analysis

Estimation of Superoxide Dismutase (SOD; E.C 1.15.1.1)

Superoxide dismutase activity was assayed by the method of Das *et al*. (2000).

Principle

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound which subsequently reacts with naphthylethylenediamine to produce a red azo compound whose absorbance is measured at 543 nm.

Reagents

1) 50 mM Phosphate buffer, pH 7.4
2) 20 mM L-Methionine
3) l% (v/v) Triton X-100
4) 10 mM Hydroxylamine hydrochloride
5) 50 µM EDTA
6) 50 µM Riboflavin
7) Griess reagent: 1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride. These are mixed in equal proportion just before the use.

Procedure

The tissue homogenate of gills, muscle, liver and brain tissues were prepared in Tris HCl buffer of pH 7.5. Pipetted 1.5 mL aliquot of the reaction mixture in a test tube which consists of 0.3 mL each of phosphate buffer, methionine, Triton X-100, hydroxylamine hydrochloride and EDTA. To this 100 µL of the sample was added followed by pre incubation at 37°C for 5 min. Into this 80 µL of riboflavin was added and the tubes were exposed for 10 min to 200W Philips fluorescent lamp. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time (10 minutes), 1.0 mL of Griess reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm against buffer taken as blank. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

Estimation of Catalase (CAT; E.C 1.11.1.6)

Catalase was assayed by the method of Mahely and Chance, (1954).

Principle

Catalase is an extraneous enzyme present in most of the cells and acts by catalyzing decomposition of hydrogen peroxide to water and oxygen.

Reagents

1) 0.01 M phosphate buffer, pH 7
2) 30 mM H_2O_2

Procedure

Enzyme extract were prepared by homogenizing tissues in Tris HCl buffer of pH 7. Reaction mixture consists of 2.5 mL of phosphate buffer, 0.5 mL of H_2O_2 and 0.2 mL of enzyme extract. Estimation was done spectrophotometrically by following the decrease in absorbance at 230 nm. The specific activity was expressed in terms of change in absorbance/ min/ extinction coefficient.

Estimation of Lipid Peroxidation

Product of lipid peroxidation malondialdehyde was estimated by Niehaus and Samuelson, (1968).

Principle

Malondialdehyde reacts with thiobarbituric acid (TBA) under acidic condition to form a pink coloured chromophore which was read spectrophotometrically at 535 nm.

Reagents

1) TCA – TBA- HCl reagent
   15% w/v TCA, 0.375% w/v thiobarbituric acid (TBA) in 0.25 N HCl.

Procedure

Homogenate of gills, liver, muscle and brain were prepared in Tris HCl buffer of pH 7.5. One mL of tissue homogenate was mixed with equal amount of TCA – TBA – HCl reagent. The contents were mixed thoroughly and heated for 15 minutes in boiling water bath. After cooling

the tubes were centrifuged for 10 minutes at 1100 x g. The supernatant was taken and the absorbance was read at 535 nm. Extinction coefficient of malondialdehyde is 1.56×10^{-5} M^{-1}/ cm^{-1} and the values were expressed as millimoles of malondialdehyde/ mg protein.

Estimation of Protein

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

Principle

The CO-NH group (peptide bond) present in the protein molecule reacts with copper sulphate in alkaline medium to give a blue colour, which was read at 660 nm.

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 reagent A and 0.1 mL of reagent 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)
5) Stock standard: 50 mg of bovine serum albumin as 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

The tissue homogenate of gills, muscle, liver and brain tissues were prepared in Tris HCI buffer of pH 7 and supernatant fractions were collected after centrifugation at 11,200 x g for 15 minutes. To the supernatant equal volume of 10% trichloroacetic acid was added to precipitate the proteins. The contents were allowed to stand for 30 minutes at room temperature and centrifuged at 1000 x g for 15 minutes. The sediment of protein was dissolved in 1 mL of 0.1N NaOH. After suitable dilution a known volume of the solution was used as samples. A set of working standard solutions ranging from 0.2 to 1.0 mL were pipetted out in to the test tubes. The volume in all the tubes was made up to 1.0 mL with distilled water. 5. 0 mL of alkaline copper reagent was added to all the test tubes. The contents in the tube 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.

Statistical Analysis

The statistical analysis was carried out using the software SPSS 22.0 package. Three- way analysis of variance (ANOVA) was carried out to compare between different temperature, tissues and hours of exposure. If significant difference was revealed by the ANOVA test, Tukey's test

was used to further elucidate which temperature, tissues and hours of exposure were significantly different. One-way ANOVA followed by Tukey's test was carried out for the comparison between different temperatures in each hours of exposure. Significance level was set at $P< 0.05$ in all the tests.

2.5 Results

In *E. suratensis* Critical Thermal Maximum (CT Max) was found to be 40^{o}C. Similarly Critical Thermal Minimum (CT Min) was found to be 14^{o}C.

Superoxide Dismutase

Superoxide dismutase activity was significantly affected by the time course of exposure, temperature and type of tissues investigated. SOD activity was in the range of 3.35 ± 0.09 to 3.92 ± 0.012 U/ mg protein in the control (27^{o}C) tissues, muscle and brain. Whereas, in the experimental group exposed to 38^{o}C, SOD activity was increased to 13.23 ± 0.60 U/ mg protein and 25.71 ± 0.01 U/ mg protein (3-6 fold) in the muscle and brain respectively (Fig. 2.1.C and D) at the end of 48th hour of experiment compared to control. At 16^{o}C, the activity was 13.34 ± 0.53 U/ mg protein and 14.01 ± 0.52 U/mg protein (3-3.2 fold) in the muscle and brain respectively (Fig. 2.1.C and D). In gills (Fig. 2.1.A) the SOD activity was 7.827 ± 0.185 U/ mg protein at 38^{o}C wherein, at 16^{o}C it was 8.55 ± 0.24 U/ mg protein compared to control. In liver (Fig. 2.1.B) maximum activity was at 16^{o}C during 72th hour of exposure. During the course of study the SOD level remained steady in the case of group reared at ambient temperature and a profound change in the SOD activity was observed in all the tissues of experimental groups selected for the study overtime.

Figure 2.1 A.B.C.D. Activity of superoxide dismutase (SOD) in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n =7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

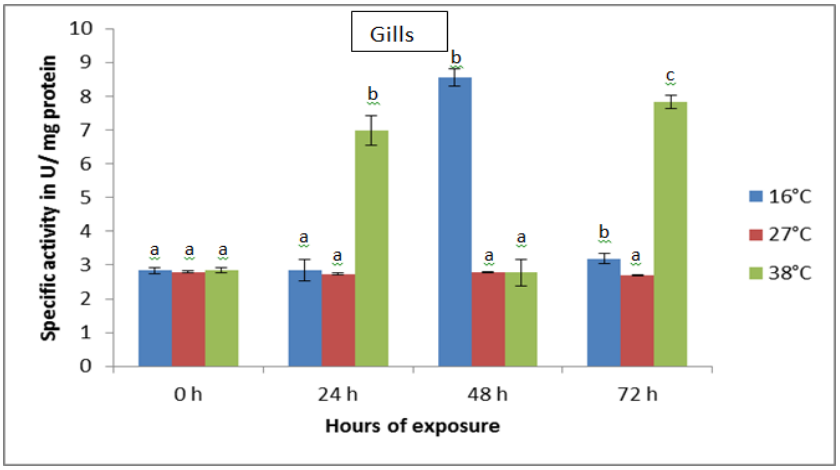
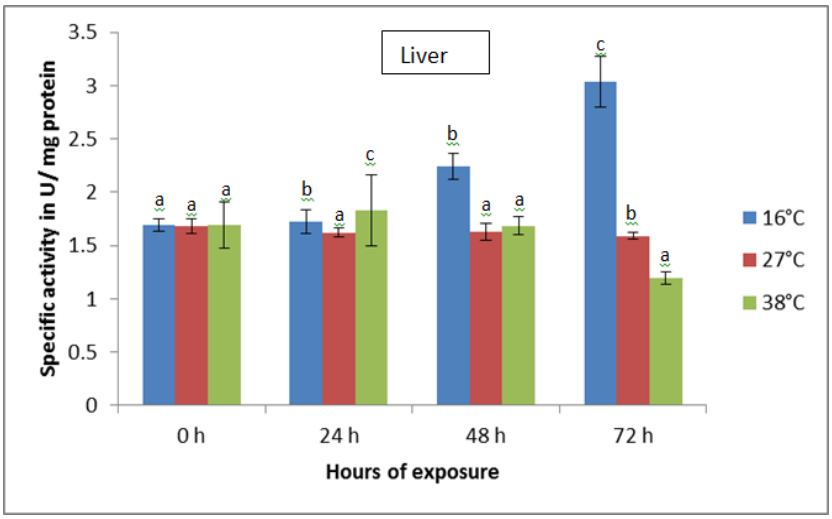


Figure 2.1 (A)



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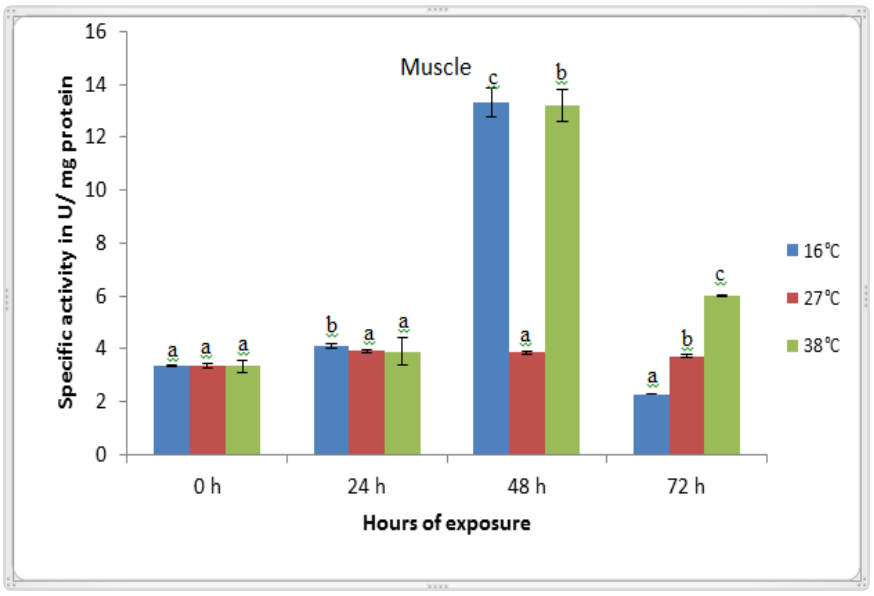

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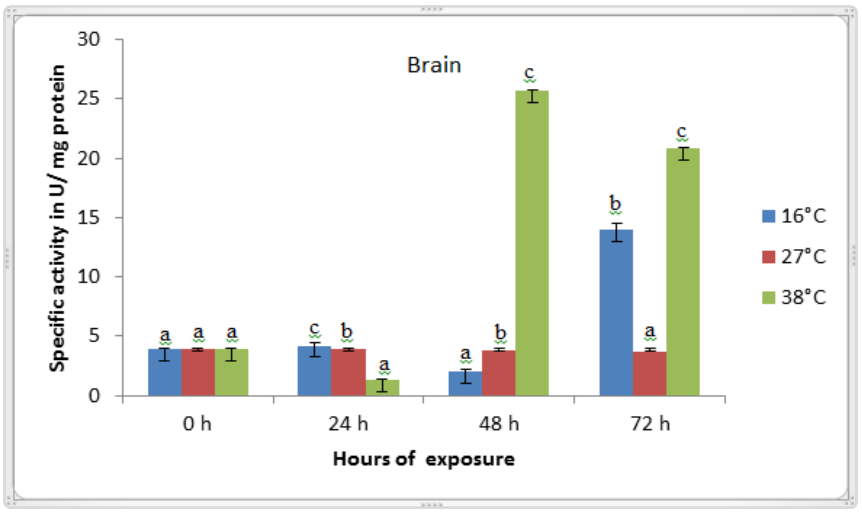

Figure 2.1 (D)

Table 2.1 Effect of different temperature on superoxide dismutase activity (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

Superoxide Dismutase activity				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16ºC	27ºC	38ºC
	0 h	2.840 ± 0.093[a]	2.8 ± 0.042[a]	2.840 ± 0.078[a]
	24 h	2.848 ± 0.32[a]	2.734 ± 0.053[a]	6.989 ± 0.44[b]
	48 h	8.554 ± 0.242[b]	2.783 ± 0.046[a]	2.783 ± 0.390[a]
	72 h	3.186 ± 0.151[b]	2.698 ± 0.045[a]	7.827 ± 0.185[c]
Liver				
	0 h	1.689 ± 0.056[a]	1.68± 0.067[a]	1.689 ± 0.218[a]
	24 h	1.72 ± 0.109[b]	1.62 ± 0.043[a]	1.828 ± 0.335[c]
	48 h	2.242 ± 0.125[b]	1.63 ± 0.078[a]	1.685 ± 0.081[a]
	72 h	3.036 ± 0.235[c]	1.59 ± 0.032[b]	1.197 ± 0.056[a]
Muscle				
	0 h	3.341 ± 0.034[a]	3.35 ± 0.098[a]	3.35 ± 0.224[a]
	24 h	4.12 ± 0.083[b]	3.91 ± 0.045[a]	3.903 ± 0.511[a]
	48 h	13.339±0.537[c]	3.860 ± 0.062[a]	13.221 ± 0.602[b]
	72 h	2.275 ± 0.023[a]	3.723 ± 0.056[b]	6.01 ± 0.045[c]
Brain				
	0 h	3.9 ± 0.0765[a]	3.96 ± 0.023[a]	3.9 ± 0.076[a]
	24 h	4.215 ± 0.197[c]	3.923 ± 0.012[b]	1.364 ± 0.057[a]
	48 h	2.035 ± 0.164[a]	3.860 ± 0.023[b]	25.717 ± 0.012[c]
	72 h	14.009 ± 0.527[b]	3.723 ± 0.074[a]	20.867 ± 0.068[c]

Values are expressed as specific activity in U/ mg protein

Three factor ANOVA (Table 2.2) revealed that between temperatures, tissues and hours of exposure significant difference were present (P<0.05). Subsequent analysis by multiple comparison tests using

Tukey's test revealed that between gills, liver, muscle and brain significant difference ($P<0.05$) was present. In between temperatures such as 16ºC, 27ºC and 38ºC also significant difference was present . In between 0 and 24 hours there was no significant variation .

Table 2.2 Three – factor ANOVA for Superoxide Dismutase

Source	**Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
Tissue	917.566	3	305.855	6406.379	0.000
Hours	962.641	3	320.880	6721.084	0.000 0.000
Temperature	180.607	2	90.304	1891.481	0.000
Tissue * Hours	815.328	9	90.592	1897.520	0.000
Tissue * Temperature	218.395	6	36.399	762.408	0.000
Hours * Temperature	185.966	6	30.994	649.201	0.000
Tissue * Hours * Temperature	825.825	18	45.879	960.975	0.000
Error	4.583	96	0.048		
Total	4110.912	143			

df – degrees of freedom

Catalase

Maximum catalase activity observed in muscle was 2.87 ± 0.045 U/ mg protein at 27ºC, 11.34 ± 0.345 U/ mg protein at 38ºC and 14.13 ± 1.02 U/ mg protein at 16ºC. It is clear from the result that antioxidant defense response offered by CAT was significantly high (6.5 fold) in muscle at 48th hour of experiment exposed to 38ºC (Fig. 2.2.C). In the experimental group reared at ambient temperature CAT activity remained steady whereas, a 5.1 and 4.71 fold increase in CAT activity (Fig. 2.2.C and 2.2.D) was observed in brain and muscle tissues respectively at 38ºC. In gills and liver (Fig. 2.2.A and 2.2.B) maximum activity was at 24th hour of exposure in the experimental group reared at 38ºC.

Figure 2.2 A.B.C.D. Activity of catalase (CAT) in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

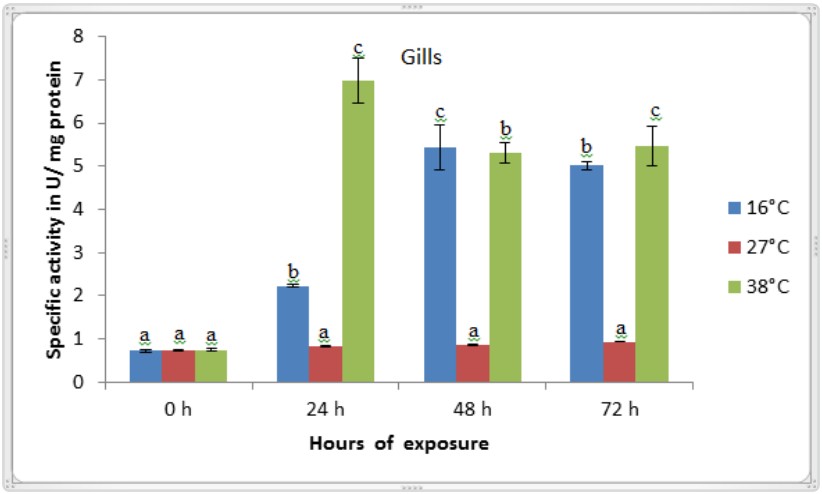

Figure 2.2 (A)

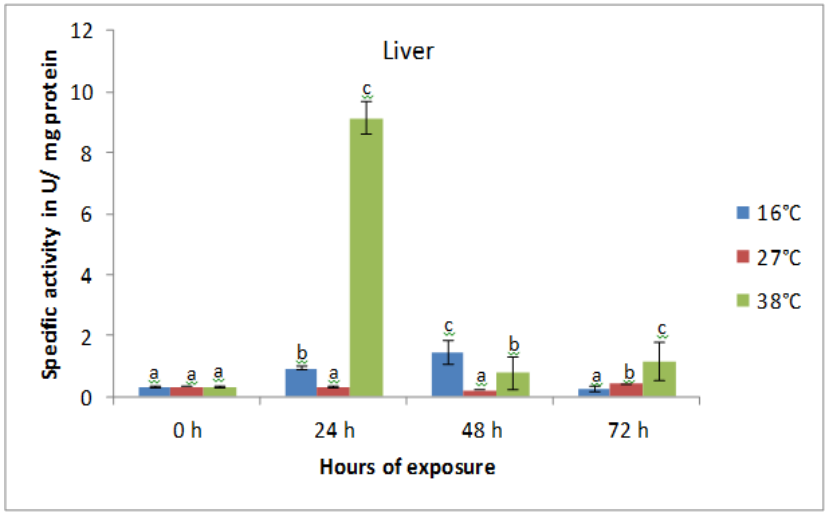

Figure 2.2 (B)

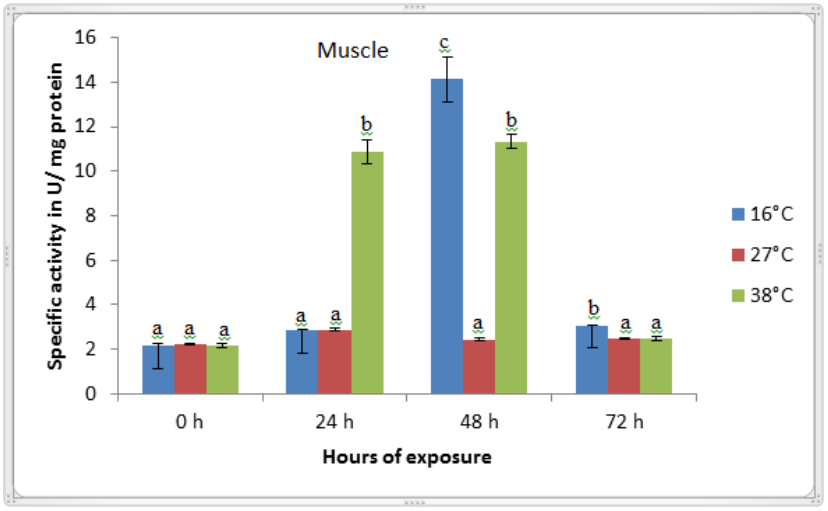

Figure 2.2 (C)

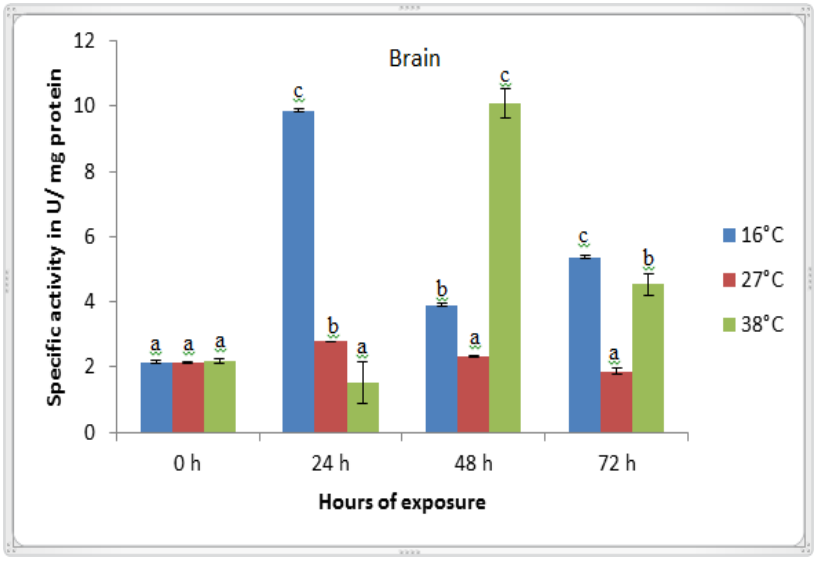

Figure 2.2 (D)

Table 2.3 Effect of different temperature on catalase activity (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

Catalase activity				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16°C	27°C	38°C
	0 h	0.724 ± 0.034[a]	0.73 ± 0.005[a]	0.74 ± 0.021[a]
	24 h	2.227 ±0.033[b]	0.821 ± 0 .006[a]	6.976 ± 0.527[c]
	48 h	5.425 ± 0.52[c]	0.852 ± 0.003[a]	5.32 ± 0.236[b]
	72 h	5.019 ± 0.093[b]	0.932 ± 0.007[a]	5.456 ± 0.463[c]
Liver				
	0 h	0.31± 0.024[a]	0.324 ± 0.004[a]	0.3 ± 0.038[a]
	24 h	0.930 ± 0.0499[b]	0.313 ± 0.005[a]	9.122 ± 0.543[c]
	48 h	1.475 ± 0.383[c]	0.214 ± 0.002[a]	0.778 ± 0.546[b]
	72 h	0.259 ± 0.074[a]	0.423 ± 0.002[b]	1.131 ± 0.63[c]
Muscle				
	0 h	2.134 ± 0.083[a]	2.19 ± 0.034[a]	2.15 ± 0.083[a]
	24 h	2.83 ± 0.073[a]	2.873 ± 0.045[a]	10.861 ± 0.516[b]
	48 h	14.138 ± 1.02[c]	2.435 ± 0.045[a]	11.341 ± 0.345[b]
	72 h	3.030 ± 0.045[b]	2.456 ± 0.032[a]	2.457 ± 0.092[a]
Brain				
	0 h	2.146 ± 0.042[a]	2.15 ± 0.023[a]	2.18 ± 0.065[a]
	24 h	9.874 ± 0.034[c]	2.786 ± 0.012[b]	1.514 ± 0.654[a]
	48 h	3.896 ± 0.045[b]	2.345 ± 0.023[a]	10.098 ± 0.435[c]
	72 h	5.370 ± 0.054[c]	1.875 ± 0.074[a]	4.528 ± 0.345[b]

Values are expressed as specific activity in U/ mg protein

Three factor ANOVA (Table 2.4) revealed that between temperature, tissues and hours of exposure significant difference were present (P<0.05). Subsequent analysis by multiple comparison tests using Tukey's test revealed that between gills, liver, muscle and brain

significant difference ($P<0.05$) was present. Significant difference were noticed between the three temperatures regimes (16°C, 27°C, 38°C) tested. In between hours of exposure there was significant variation.

Table 2.4 Three-factor ANOVA for Catalase

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	278.170	3	92.723	213.288	0.000
Temperature	306.550	2	153.275	352.574	0.000
Tissue	240.464	3	80.155	184.377	0.000
Hours * Temperature	172.638	6	28.773	66.186	0.000
Hours * Tissue	193.416	9	21.491	49.434	0.000
Temperature * Tissue	52.168	6	8.695	20.000	0.000
Hours * Temperature * Tissue	395.279	18	21.960	50.514	0.000
Error	41.734	96	0.435		
Total	1680.418	143			

df – degrees of freedom

Lipid peroxidation

Fig 2.3.A,B,C and D shows the levels of oxidative stress marker MDA of lipid peroxidation in various tissues during experiment. In gills (Fig. 2.3.A) MDA level was 1.96 ± 0.08 mM/ mg of protein at 16°C and in liver (Fig. 2.3.B) it was 0.315 ± 0.006 mM/ mg of protein at 16°C. In case of muscle (Fig. 2.3.C) MDA level was high in organism reared at 16°C where as in brain (Fig. 2.3.D) maximum activity was at 38°C. Among the four tissues LPO rate was maximum in gills during the 48th hour of exposure at 16°C. Group exposed to ambient temperature followed a steady response pattern.

Figure 2.3 A.B.C.D. Lipid Peroxidation in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each tissue on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

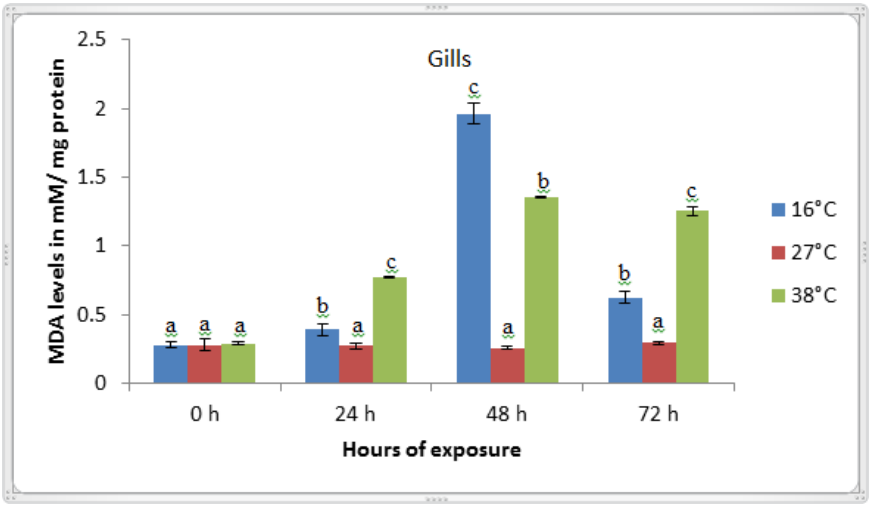

Figure 2.3 (A)

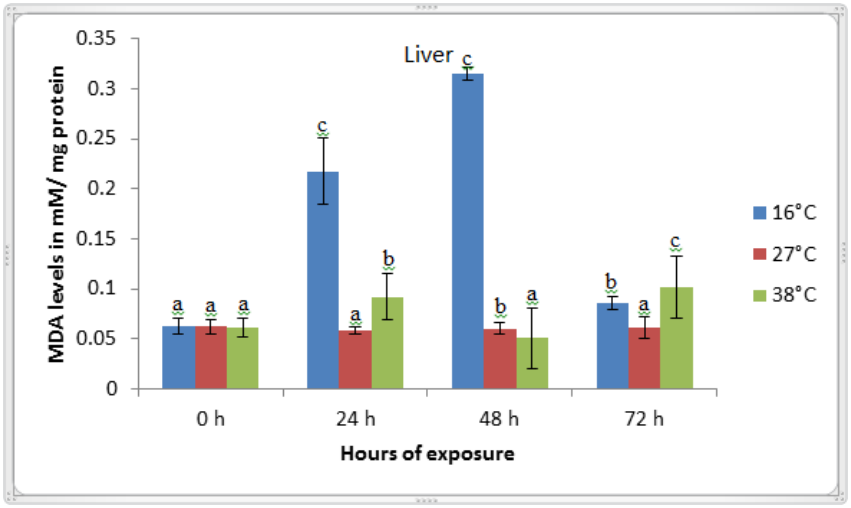

Figure 2.3 (B)

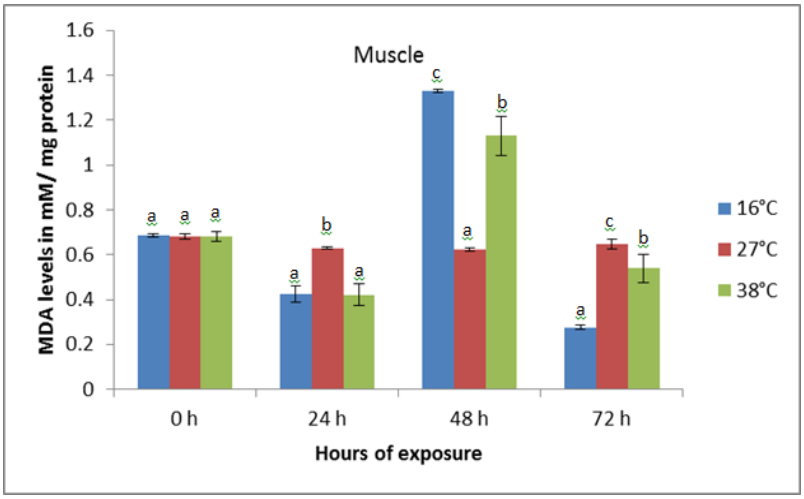

Figure 2.3 (C)

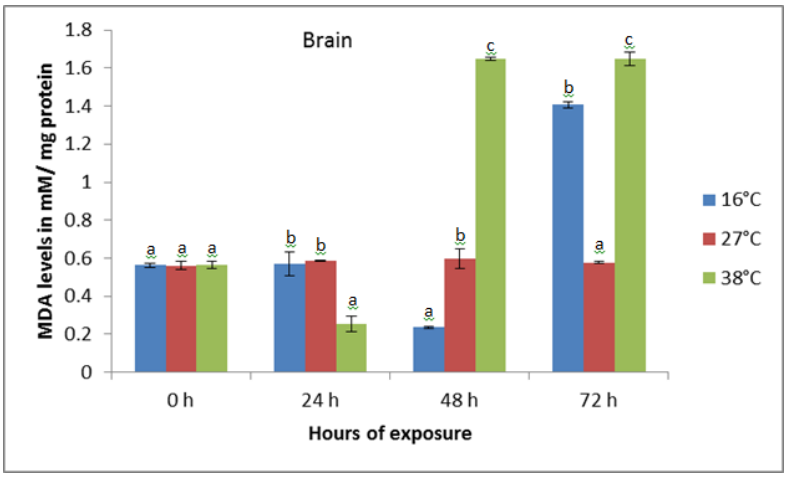

Figure 2.3 (D)

Table 2.5 Effect of different temperature on lipid peroxidation (mean ± SD, n=7) present in different tissues of *E. suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups(One-way ANOVA)

Lipid Peroxidation				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16ºC	27ºC	38ºC
	0 h	0.282 ± 0.02^{a}	0.28 ± 0.043^{a}	0.29 ± 0.013^{a}
	24 h	0.389 ± 0.04^{b}	0.27 ± 0.023^{a}	0.768 ± 0.005^{c}
	48 h	1.963 ± 0.08^{c}	0.258 ± 0.008^{a}	1.354 ± 0.007^{b}
	72 h	0.625 ± 0.04^{b}	0.259 ± 0.01^{a}	1.255 ± 0.031^{c}
Liver				
	0 h	0.062 ± 0.008^{a}	0.062 ± 0.007^{a}	0.061 ± 0.01^{a}
	24 h	0.217 ± 0.033^{c}	0.058 ± 0.003^{a}	0.091 ± 0.023^{b}
	48 h	0.315 ± 0.006^{c}	0.060 ± 0.006^{b}	0.05 ± 0.03^{a}
	72 h	0.086 ± 0.006^{b}	0.061 ± 0.011^{a}	0.1 ± 0.031^{c}
Muscle				
	0 h	0.685 ± 0.005^{a}	0.68 ± 0.012^{a}	0.6817 ± 0.023^{a}
	24 h	0.425 ± 0.036^{a}	0.628 ± 0.004^{b}	0.4218 ± 0.05^{a}
	48 h	1.328 ± 0.008^{c}	0.623 ± 0.006^{a}	1.13 ± 0.087^{b}
	72 h	0.276 ± 0.009^{a}	0.649 ± 0.022^{c}	0.538 ± 0.064^{b}
Brain				
	0 h	0.562 ± 0.009^{a}	0.56 ± 0.021^{a}	0.565 ± 0.017^{a}
	24 h	0.569 ± 0.062^{b}	0.587 ± 0.004^{b}	0.254 ± 0.042^{a}
	48 h	0.236 ± 0.005^{a}	0.598 ± 0.052^{b}	1.647 ± 0.008^{c}
	72 h	1.407 ± 0.015^{b}	0.578 ± 0.006^{a}	1.647 ± 0.035^{c}

Values are expressed as MDA levels in mM/ mg protein

Three factor ANOVA (Table 2.6) revealed that between temperatures, tissues and hours of exposure significant difference were present ($P<0.05$). Subsequent analysis by multiple comparison test using Tukey's test revealed that between gills and muscle no significant difference ($P<0.05$) was present. In between temperatures such as 16°C, 27°C and 38°C significant difference was present . In between 0 and 24 hours of exposure there was no significant variation .

Table 2.6 Three-factor ANOVA table for Lipid Peroxidation

Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Hours	4.425	3	1.475	2504.287	0.000
Temperature	2.140	2	1.070	1816.124	0.000
Tissue	10.091	3	3.364	5710.134	0.000
Hours * Temperature	2.463	6	0.410	696.850	0.000
Hours * Tissue	4.674	9	0.519	881.685	0.000
Temperature * Tissue	2.088	6	0.348	590.664	0.000
Hours * Temperature * Tissue	6.163	18	0.342	581.242	0.000
Error	0.057	96	0.001		
Total	32.099	143			

df- degrees of freedom

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2.6 Discussion

Temperature transitions either from higher to lower or from lower to higher temperatures result in alterations of enzyme activities. Some changes were registered in antioxidant enzyme activities under temperature transitions. The recovery of frog, *Rana ridibunda*, from winter hibernation and following exposure to 20°C increased activities of superoxide dismutase, catalase and glucose-6-phosphate dehydrogenase (Bagnyukova *et al*., 2003). Hence, even relatively mild thermal stress can cause an enhancement of antioxidant potential including primary and associated antioxidant enzyme activities (Lushchak and Bagnyukova 2005, Lushchak *et al.*, 2005a). In the present study we tested the effect of three temperatures, 38^{o}C, 27^{o}C and 16^{o}C respectively on the activities of antioxidant enzymes involved in oxidative metabolism in the organs such as gills, liver, muscle and brain of *E. suratensis*. Occurrence of oxidative stress along with enhanced respiration, oxygen consumption and metabolic rate in ectotherms as a result of increase in temperature from ambient condition have been already reported by many researchers (Watters and Smith, 1973; Parihar and Dubey, 1995; Bagnyukova *et al*., 2003; Heise *et al*., 2006). At low temperatures, the rate of poly unsaturation in the mitochondrial membranes and thereby mitochondrial respiration rate increases in fishes (Sladjan *et al*., 2010). This could enhance the formation of ROS and proton leakage which in turn favors peroxidation of the membrane lipids. The augmented level of stress markers particularly ROS and LPO levels in the tissues may be due to the disruption of the mitochondrial electron transfer and the accumulation of ROS in tissues during cold stress (Sahoo and Kara 2014, Kammer, 2011

Johnston and Maitland, 1980; Egginton and Sidell, 1989) relative to warm-acclimatized animals. A change in temperature can crucially affect many physiological and metabolic aspects of the organism and the effects often depend on gradients and directions of alterations. Antioxidant activity and its expression had been used to evaluate the response of organisms under thermal stress in gold fish (Bagnyukova *et al*., 2007). Induction of antioxidant enzymes is an important line of defense against oxidative stress in biological systems (Parihar and Dubey, 1995). The response of antioxidant and associated enzyme systems may vary differently in different organs as the stress progresses (Nakano *et al.,* 2014). It is obvious from the present study that the change in temperature (from ambient condition) induced oxidative stress in *E. suratensis* and hence we analysed the antioxidant profile of four different organs - gills, liver, muscle and brain of *E. suratensis*.

Increase in superoxide dismutase (SOD) activity clearly shows that the temperatures selected for the study induced oxidative stress in *E. suratensis*. The stress was prominent in the group exposed to high temperature. The maximum SOD activity was observed in the brain of experimental group at 38^{o}C and this result is in accordance with the findings of Lushchak and Bagnyukova (2005). The generation of superoxide radicals during progressive temperature stress may induce the upregulation of SOD activity. It has been reported that SOD activity was maximum in liver and minimum in muscle of *Rana ridibunda* when transferred from 5^{o}C to 20^{o}C for 24 hour (Bagnyukova *et al*., 2003). Bagnyukova *et al*. (2003) reported that prolonged exposure to high temperature upregulated the expression of antioxidant enzymes such as

superoxide dismutase in liver and muscle whereas in our study, activity was maximum in brain and muscle. The stress marker levels were comparatively low in liver tissues of fishes reared at both experimental temperatures where as in gold fish the activity was maximum in liver (Bagnyukova *et al*., 2007, 2006). The trend was almost similar in tissues treated at 16^{o}C (Abele and Puntarulo 2004). The maximum contribution to the defense mechanism against thermal stress was offered by the brain during the experiment which was in agreement with the findings of Lushchak and Bagnyukova (2006). SOD activity was high in low temperature (16^{o}C) as compared to high temperature (38^{o}C) in all tissues except in brain which was in accordance with findings of Bagnyukova *et al*. (2007). The increases in brain SOD activity with exposure to high and low temperature stress in the present study possibly indicate its role in scavenging superoxide radicals. Among antioxidants, superoxide dismutase activity demonstrated a strong and uniform increase in all tissues indicating possibly its key role in protection against reactive oxygen species produced at high temperature. SOD activity is induced by lipid peroxidation products and probably by a shift in redox status of a tissue (Lushchak and Bagnyukova, 2005). This up-regulation might be realized via redox sensitive transcriptional factors like AP-1 and NFκB (Droge, 2002).

Catalase (CAT) is an important antioxidant enzyme barrier of oxidative stress like SOD. It mainly participates in the neutralization of toxic radical generated as part of hydrogen peroxide catabolism at the time of stress. The highest levels of catalase activity were observed in fish exposed to temperatures outside their thermal optimum, which means that thermal stress affects the cellular responses (Vinagre *et al*., 2012).

Though the catalase levels were increased in all the tissues evaluated, a maximum activity was found in muscle during 48th hour of exposure to both the tested temperatures which clearly indicate that the muscles were more stressed compared to other organs. This may be due to its maximum exposure to environment compared to other organs (Lushchak and Bagnyukova, 2006). Next to muscle, maximum activity was observed in brain followed by other organs. It was already reported that the activity of catalase was increased in brain of gold fish over the intermediate times such as 6, 12 and 24 hour after transfer to 23^{o}C and catalase response in liver showed decreased activity over time (Bagnyukova *et al*., 2007). Wherein, low temperature stress the pattern of SOD and catalase responses was similar to that of high temperature effects (Abele *et al.*, 2002). Roche and Boge (1996) tested the effects of temperature on oxidative stress biomarkers in *Dicentrarchus labrax* and reported that catalase activity was increased by thermal stress after 12 hour of exposure of temperature stress. The antioxidant enzymes especially SOD and catalase play an important role in antioxidant defenses in gold fish subjected to various kinds of stress (Lushchak *et al*., 2005a). During the experimental course, the enzymes under study demonstrated a coordinated response to stressful conditions thereby offering possible protection against O^{2-} radicals.

In most tissues the progressive lipid peroxidation (LPO) is evidenced by increased levels of lipid peroxidation byproducts such as MDA and LOOH as biomarkers. LPO in stressed fish is considered to be the results of metabolites derived from damaged tissues. The temperature rise increased levels of lipid peroxides and TBARS, but the response was

highly tissue specific. Hermes-Lima (2004); Rau *et al*. (2004) reported that lipid peroxidation has been shown to be a sensitive marker under different stressful condition. Indian catfish, *Heteropneustes fossils*, subjected to temperature stress exhibited significant rise of TBARS levels at tissue level (Parihar and Dubey, 1995). LPO marker levels were found increased in gills when exposed to low temperature selected suggesting their sensitiveness to the thermal stress (Martinez, 2005) (Parihar and Dubey, 1995). Among the tissue selected, lipid peroxidation was high in gills, followed by brain (Lushchak and Bagnyukova, 2006). The organ displaying the lowest level of peroxidation in the current study was liver where as in gold fish it was muscle (Bagnyukova *et al.,* 2003). However the levels of LPO markers in brain changed overtime in experimental groups treated at 38ºC clearly indicating that high temperature significantly caused oxidative damage to tissues (Sahoo and Kara 2014). Lushchak and Bagnyukova (2006a) reported that lipid peroxidation products are not only markers of oxidative stress but they are also involved in triggering the upregulation of antioxidant enzymes. In *Cyprinus carpio*, seven days exposure to warmer temperature brought a decrease in percentage of PUFAs as reported by Raddy *et al.* (1990) and a similar rapid modification of lipid composition might occur during the experimental period and hence the organism may disturbed by both low and high temperatures. Oxidative stress affects cellular integrity only when antioxidants are no longer capable of coping with ROS or other superoxide radicals. ROS reacts with the unsaturated fatty acid of cellular or subcellular membranes. In the study it was found that oxidative stress response was directly related to temperature, it is lowest at the optimal temperature and it increases at the lower and upper

thermal limits. Thus in the present study, an acute exposure to thermal stress leads to the exhaustion of antioxidants and thereby to a decline in the defense offered by them resulting in the loss of cellular integrity. At some point, vital functions are so impaired that, according to Portner's (2001) model of thermal tolerance, animals enter a state of temporary survival, from which they cannot return to their normal activity. As temperature stress progress, antioxidant enzymes denature, while heat shock proteins (HSP) may come into play as suggested by Portner (2001) in a final effort to prolong passive survival. Thus, antioxidant enzymes seem to be promising biomarkers for immediate early and acute heat stress in marine ectotherms (Abele and Puntarulo, 2004).

In conclusion, the present results regarding the expression patterns of multiple redox – related biomarkers, such as SOD, CAT and LPO in response to thermal stressors suggest that severe thermal stress due to heat shock induced extensive oxidative stress in *E. suratensis*, which may lead to the oxidation of biomolecules resulting in damage to various tissues. The elevated activities of the antioxidant enzymes and other stress markers studied in the present work were probably due to the inflated primary defense of the organism against the oxidative stress imposed by the temperature variation. But a sustained thermal stress inactivated their functionality overtime and as a result, the fish failed to survive under stressed environment or habitat. In the current global warming scenario the study has its significance because temperature induced mortality is a threat to aquaculture and affects the livelihood of fishermen all over the world.

Chapter 3

Effect of Acute Temperature Fluctuations on Glutathione Dependent Antioxidants of *Etroplus suratensis*

Contents

3.1 Introduction

Although most animals rely heavily on aerobic metabolism to sustain life, reliance on oxidative metabolism for energy can be detrimental. Oxidative stress is an important component of the stress response in aquatic organisms which are exposed to thermal stress. Ectotherms, including fish are consistently subjected to varying ambient temperature and they must cope with its fluctuations. An increase of environmental temperature leads to metabolic activation combined with an increase in oxygen consumption results in oxidative stress. This oxidative stress has serious deleterious effects which affect the cellular functions and eventually result in death. Organism quickly raises

antioxidant potential in response to oxidative stress. Glutathione dependent antioxidants play an important physiological role in fishes during oxidative stress. Glutathione-dependent enzymes such as glutathione peroxidase (GPx) which detoxifies both H_2O_2 and organic hydroperoxides, glutathione-S-transferase (GST) which detoxifies various compounds by conjugating them with glutathione, glutathione reductase (GR) which reduces oxidized glutathione using NADPH, and associated enzymes like glucose-6-phosphate dehydrogenase (G6PDH) which supplies reduced equivalents for GR (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004).

3.2 Review of Literature

The metabolic rate of ectothermic animals and their oxygen consumption are proportional to environmental temperature (Hochachka and Somero, 1984). Temperature is an abiotic factor that influences many biological processes (Windisch *et al.,* 2011). Wedemeyer *et al.* (1990) reported that temperature can function as a stressing agent in fish and the metabolic rate of an ectothermic organism is strongly related to environmental temperature. The thermal tolerance of an organism is closely related to its aerobic ability because both heating and cooling may alter oxygen balance in the tissues, which result in generation of reactive oxygen species (ROS). These ROS react with lipids and nucleic acids causing oxidative stress and damage to the cells (Abele *et al.,* 1998; Stadtman and Levine, 2000; Martinez-Alvarez *et al.,* 2005; Halliwell and Gutteridge, 2007). The mitochondria are thought to consume over 90% of the cellular oxygen in unstressed cells and are considered the major sites

of aerobic cellular ROS production (Boveris and Chance, 1973; Staniek and Nohl, 1999; Lenaz, 1998; Han *et al.*, 2001). It was reported that mitochondrial electron transport chains *in vitro* convert around 2% of the oxygen consumed to univalently reduced superoxide anions, the extent to which this happens *in vivo* and the rate of escape of radicals to the cytoplasm is still under debate (Guidot and McCord, 1999; Gnaiger *et al.*, 2000; St.-Pierre *et al*., 2002). Moreover, ROS are also produced by the microsomal systems of the endoplasmic reticulum (Chu and La Peyre, 1993; Winston *et al*., 1996) and by various enzymatic oxidase reactions. The rate of ROS generation, including superoxide anion ($O_{2.}^{-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (.OH) is related to oxygen consumption (Halliwell and Gutteridge, 1989). So the intensification of respiration at a higher temperature would result in enhanced ROS production, and therefore the adaptive responses by antioxidant defenses would be needed for fishes when shifting from low to high ambient temperatures. An increase or decrease in temperature causes oxidative stress as well as up-regulation of genes of two functional groups "oxygen and reactive oxygen species metabolism" and "response to oxidative stress" (Malek *et al.*, 2004). During thermal stress more oxygen is consumed in peripheral tissues and therefore limitation of oxygen supply to central tissue can occur and produce a state of thermally induced transient hypoxia. The onset of heat-induced hypoxia in body fluids and tissues has been found when the animals were warmed beyond their pejus temperatures (Tp), indicating the limits of the species-specific thermal optimum range (Heise *et al.*, 2006). In fish, capacity limits of cardiac performance account for a progressive mismatch between oxygen supply

and demand in the whole animal during warming (Mark *et al.*, 2002; Farrell, 2002; Lannig *et al.*, 2004). At high and low temperatures, oxygen extraction by working skeletal muscle during exercise would further increase oxygen demand. When a critical temperature (Tc) is reached, an aerobic scope is limited and mitochondria progressively switch to anaerobic energy production, which allows only time limited survival (Zielinski and Portner, 1996; Sommer *et al*., 1997; Portner, 2002). Hypoxic signaling, heat shock response and oxidative stress during warming are interactive cellular processes (Heise *et al.*, 2006). Studies using graded short-term exposure to temperatures mildly and severely elevated over the control temperature (27 ± 1^{o}C) make it possible to distinguish between the effects of hyper and hypothermia and the combination of heat and functional hypoxia beyond the critical temperature limit. Antioxidant defenses may be enzymatic or non-enzymatic (Sies, 1985; Martinez-Alvarez *et al.*, 2005; Yilmaz *et al*., 2006). The accurate way to examine antioxidant responses to temperature changes is to use experimental induction of temperature shock that is short-term transfer to significantly higher or lower temperature. This action can disturb antioxidant potential and cause oxidative stress (Parihar and Dubey, 1995; Heise *et al.*, 2006; Lushchak and Bagnyukova, 2006, 2006a).

The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) is a low-molecular weight antioxidant involved in a number of crucial cellular functions, such as ROS scavenging, detoxification of electrophiles, maintenance of thiol-disulfide status and signal transduction (Droge *et al.*, 1994; Halliwell and Gutteridge, 1999). The ratio of reduced to oxidized glutathione (GSH/GSSG) is a good indicator of oxidative stress intensity.

Usually, a relatively high ratio is maintained intracellularly, but under oxidative conditions its level can be lowered. Anderson (1996) reported that glutathione is present in cells in millimolar concentrations, up to 10 mM. The GSH antioxidant system is comprised of GSH and its associated enzymes, such as glutathione peroxidase (GPx) and glutathione reductase (GR). GSH, along with the GPx enzymes, reduces various oxidized products within a cell, forming oxidized GSH (GSSG). The antioxidant activity of GSH relies on GSSG being readily reduced back to GSH by GR. The total cellular pool of GSH is the result of a balance between its synthesis within the cell (via glutamylcysteine synthetase (GCS) and glutathione synthetase enzymes), and its transport from the cell (Meister, 1995; Kidd, 1997; Anderson, 1998; Hayes and McLellan, 1999). Although some free GSH may be lost by reversible interactions with protein thiols (Lind *et al*., 2002), excretion of GSH and GSSG from cells can account for intracellular GSH turnover of hepatocytes, kidney cells and erythrocytes in mammals (Lunn *et al*., 1979; Lauterburg *et al.*, 1984; Scott *et al.,* 1993). In mammals, GSH levels are highly relative to other antioxidant and thiol molecules (Kidd, 1997; Hayes and McLellan, 1999). Wallace (1989) reported that in fish GSH levels and the functional activity of GSH-associated enzymes are relatively low. GSH turnover appears to be lower in fish than mammals (Gallagher *et al.,* 1992). The basis for lower GSH levels, turnover and activity of associated enzymes in fish relative to mammals is not fully understood, but may be due to one of two factors. Fish are able to excrete hydrogen peroxide from their gills (Wilhelm-Filho *et al.,* 1994), and thereby decreasing the need for GSH antioxidant system. Secondly, lower metabolic rate in fish, from either or

both lower body temperature and reduced metabolic cost may decrease the necessity for GSH in fish. Davidson and Schiestl (2001) reported that increased metabolism results in increased oxygen uptake by mitochondria, and consequently increased production of reactive oxygen species such as hydrogen peroxide. The lower metabolic rates of fish should result in decreased demand for GSH, and this may account for the observed low GSH levels, turnover and activity of associated enzymes compared to mammals. The effect of temperature on the GSH antioxidant system has been examined to some extent in fish and mammals. In mammals, hyper and hypothermia results in a transient decrease followed by an increase in GSH levels, an increase in excretion of hepatic GSH, and an increase in lipid peroxidation (Skibba *et al.,* 1991; Ohtsuka *et al.,* 1994). Short-term thermal stress was found to reduce GSH levels and increase lipid peroxidation in catfish, *Heteropneustes fossilis* (Parihar *et al.*, 1996; Parihar *et al.*, 1997), and snakeheads, *Channa punctata* (Kaur *et al.*, 2005). Although fewer studies have directly addressed the effects of long-term temperature changes on GSH, seasonal changes in GSH levels have been associated in part with changes in temperature in various ectothermic animals (Dziubek, 1987; Perez-Pinzon and Rice, 1995; Gorbi *et al*., 2005). GSH turnover, levels and activity of associated enzymes in fish would increase with increasing temperature, and that temperature has a role in GSH antioxidant system in fish. In mammals GSH antioxidant enzymes activity was maximum in liver where as in fish the activity was maximum in gills and kidney (Gallagher and Di Giulio, 1992: Lushchak *et al*., 2001).

3.3 Objectives of the study

1) The present study was aimed to investigate the effect of thermal stress on tropical culture fish *E. suratensis* by evaluating oxidative stress biomarkers such as glutathione peroxidase, glutathione reductase, glutathione-S-transferase and total reduced glutathione at the tissue level.

2) An attempt was made to trace the pattern of antioxidant defense response of selected organs of *E. suratensis* in the event of a thermal stress and discussed the adaptability of this species under an elevated temperature environment.

3) To identify the difference in antioxidant activity at selected extreme temperatures with respect to control.

3.4 Materials and methods

Collection, maintenance, acclimation, determination of Critical Thermal Maximum (CT Max), Critical Thermal Minimum (CT Min), experimental design, preparation of tissue samples were same as explained in detail in chapter 2

3.4.1 Methods used for the biochemical analysis

Estimation of Glutathione-S-Transferase (GST; E.C 2.5.1.18)

Glutathione-S-Transferase was assayed by the method of Habig *et al*. (1973).

Principle

Glutathione-S-Transferase catalyses the reaction of l-chloro 2, 4 dinitrobenzene (CDNB) with the sulphhydryl group of glutathione.

$$\text{CDNB} + \text{GSH} \xrightarrow{\text{GST}} \text{CDNB-S-glutathione}$$

The conjugate, CDNB-glutathione, absorbs light at 340 nm and the activity of the enzyme can therefore be estimated by measuring the increase in absorbance.

Reagents

1) 0.5 M phosphate buffer, pH 6.5
2) 30 mM CDNB in 95% ethanol (30 mg of CDNB was dissolved in 5 mL of distilled water)
3) 30 mM reduced glutathione (14 mg of reduced glutathione was dissolved in 1.5 mL of distilled water

Procedure

The tissue homogenate of gills, muscle, liver, and brain tissues prepared in Tris HCI buffer of pH 7.5 was taken for the assay. To 1.0 mL of buffer, 0.1 mL of sample, 1.7 mL of water and 0.1 mL of CDNB were added. The tubes were then incubated at 37^{o}C for 5 minutes. After suitable period of incubation, 0.1 mL of reduced glutathione was added. The increase in absorbance of the reaction system was measured against that of the blank at 340 nm. The enzyme activity was calculated in terms of µmoles of CDNB conjugate formed/ min/ mg protein using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$

Estimation of Glutathione Reductase (GR; E.C 1.6.4.2)

Glutathione reductase activity was estimated according to the method of Beutler (1984).

Principle

Glutathione reductase catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and the activity is assayed by measuring the decrease in absorbance at 340 nm.

$$\text{NADPH (NADH)} + H^+ + \text{GSSH} \xrightarrow{\text{GR}} \text{NADP}^+ (\text{NAD}^+) + 2\text{GSH}$$

Reagents

1) 0.3 M phosphate buffer, pH 6.8
2) 25 mM EDTA (93 mg of EDTA was dissolved in 10 mL of distilled water)
3) 12.5 mM oxidized glutathione (11.5 mg of oxidized glutathione (GSSG) was dissolved in 1.5 mL of distilled water)
4) 3 mM NADPH (2.5 mg of NADPH was dissolved in l mL of distilled water)

Procedure

The tissue homogenate of gills, muscle, liver, and brain tissues were prepared in 0.1M Tris HCl buffer of pH 7.5 and was taken for the assay. To 0.2 mL of tissue sample, 1.5 mL of buffer, 0.5 mL EDTA, 0.2 mL GSSG and 0.1 mL of NADPH was added. The decrease in optical density

of the enzyme was measured against that of the blank at 340 nm. The enzyme activity was calculated in terms of µmoles of NADPH oxidized/ min/ mg protein.

Estimation of Glutathione Peroxidase (GPX; E.C 1.11.1.9)

Glutathione peroxidase activity was estimated according to the method of Rotruck *et al.* (1973).

Principle

A known amount of enzyme preparation was allowed to react with hydrogen peroxide in the presence of reduced form of glutathione (GSH) for a specified time period. Then the remaining GSH was measured by the method of Ellman.

$$2GSH + H_2O_2 \xrightarrow{\text{Se-GPx}} GSSG + 2H_2O$$

Reagents

1) 0.4 M Sodium phosphate buffer pH 7.0
2) 10 mM Sodium azide
3) 2.5 mM Hydrogen peroxide
4) 4 mM Reduced glutathione
5) 10% TCA
6) 25 mM EDTA
7) DTNB reagent – 40 mg of DTNB in 1% sodium citrate

8) Reduced glutathione standard: 20 mg reduced glutathione was dissolved in 100 mL of water.

Procedure

Homogenate of gills, muscle, liver, and brain tissues prepared in Tris HCl buffer of pH 7.5 were taken for the assay. To 0.4 mL of buffer, 0.1 mL of sodium azide, 0.2 mL of reduced glutathione, 0.1 mL of H_2O_2, 0.2 mL of enzyme were added. Tubes were incubated at 37ºC for 10 minutes. The reaction was then terminated by the addition of 0.5 mL of 10% TCA. To determine the glutathione content, 1.0 mL of the supernatant was removed by centrifugation and to this 3.0 mL of sodium phosphate buffer and l.0 mL of DNTB reagent was added. The colour developed was read at 412 nm against buffer taken as blank. Standards in the range of 40 - 200 µg were taken and treated in a similar manner. The activity was expressed in terms of µg of glutathione consumed/ min/ mg protein.

Estimation of Total reduced glutathione

Total reduced glutathione (GSH) was estimated according to the method of Moron *et al.* (1979).

Principle

Glutathione (GSH) was measured by its reaction with DTNB to give a compound that absorbs at 412 nm.

Reagents

1) 0.2 M sodium phosphate buffer, pH – 8
2) DTNB (5,5-dithio-bis-2-nitrobenzoic acid) reagent: 40 mg DTNB was dissolved in 100 mL of l% trisodium citrate.
3) 5% TCA
4) Standard glutathione: 20 mg reduced glutathione was dissolved in 100 mL of distilled water.

Procedure

The tissue homogenate of gills, muscle, liver, and brain tissues prepared in 0.1M Tris HCl buffer of pH 7.5 was taken for the assay. 0.5 mL of tissue homogenate was precipitated with 2 mL of 5% TCA. To 1.0 mL of the supernatant, 3.0 mL of sodium phosphate buffer and 1.0 mL of DTNB reagent were added. The absorbance was read within 2 minutes at 412 nm against a reagent blank. A series of standards was also treated as in glutathione peroxidase assay. The amount of glutathione was expressed as µg of glutathione obtained/ mg protein.

Estimation of Protein

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

Principle

The CO-NH group (peptide bond) present in the protein molecule reacts with copper sulphate in alkaline medium to give a blue colour, which was read at 660 nm.

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 reagent A and 0.1 mL of reagent 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)
5) Stock standard: 50 mg of bovine serum albumin as 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

The tissue homogenate of gills, muscle, liver and brain tissues were prepared in Tris HCl buffer of pH 7 and supernatant fractions were collected after centrifugation at 11,200 x g for 15 minutes. To the supernatant equal volume of 10% trichloroacetic acid was added to precipitate the proteins. The contents were allowed to stand for 30 minutes at room temperature and centrifuged at 1000 x g for 15 minutes. The sediment of protein was dissolved in 1 mL of 0.1N NaOH. After suitable dilution a known volume of the solution was used as samples. Different

volumes of the working standard solution ranging from 0.2 to 1.0 mL were pipetted out in to the test tubes. The volume in all the tubes was made up to 1.0 mL with distilled water. 5.0 mL of alkaline copper reagent was added to all the test tubes. The contents in the tube 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 absorbance of the blue colour developed was measured at 660 nm in a 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.

Statistical Analysis

The statistical analysis was carried out using the software SPSS 22.0 package. Three-way analysis of variance (ANOVA) was carried out to compare between different temperature, tissues and hours of exposure. If significant differences were revealed by the ANOVA test, Tukey's test was used to further elucidate which temperature, tissues and hours of exposure were significantly different. One-way ANOVA followed by Tukey's test was carried out for the comparison between different temperatures in each hour of exposure. Significance level was set at $P < 0.05$ in all the tests.

3.5 Results

Glutathione-S-Transferase (GST)

GST activity was significantly affected by the time course of exposure, temperature and type of tissues investigated. GST activity was in the range of 0.182 ± 0.008 to 0.6512 ± 0.021 µM of GSH – CDNB conjugate formed/ min/ mg protein in the control (27^{o}C) tissues. Whereas, in the experimental group exposed to 38^{o}C, GST activity was 0.3494 ± 0.006 µM of GSH – CDNB conjugate formed/ min/ mg protein and 0.330316 ± 0.0052 µM of GSH – CDNB conjugate formed/ min/ mg protein in the muscle and brain respectively (Fig. 3.1.C and D) at the end of 48th hour of experiment compared to control. At 16^{o}C, the activity was 0.189 ± 0.0013 µM of GSH – CDNB conjugate formed/ min/ mg protein and 0.2010 ± 0.0012 µM of GSH – CDNB conjugate formed/ min/ mg protein in the muscle and brain respectively (Fig. 3.1.C and D). In gills (Fig. 3.1.A) the GST activity was 0.622 ± 0.0239 µM of GSH – CDNB conjugate formed/ min/ mg protein at 38^{o}C wherein, at 16^{o}C it was 0.62143 ± 0.0053 µM of GSH – CDNB conjugate formed/ min/ mg protein compared to control. In liver (Fig. 3.1.B) maximum activity was at 27^{o}C during 48th hour of exposure. During the course of study the GST level remained steady in the case of group reared at ambient temperature and a profound change in the GST activity was observed in all the tissues of experimental groups selected for the study overtime (Table 3.1)

Figure 3.1 A.B.C.D Activity of glutathione–S-transferase (GST) in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different

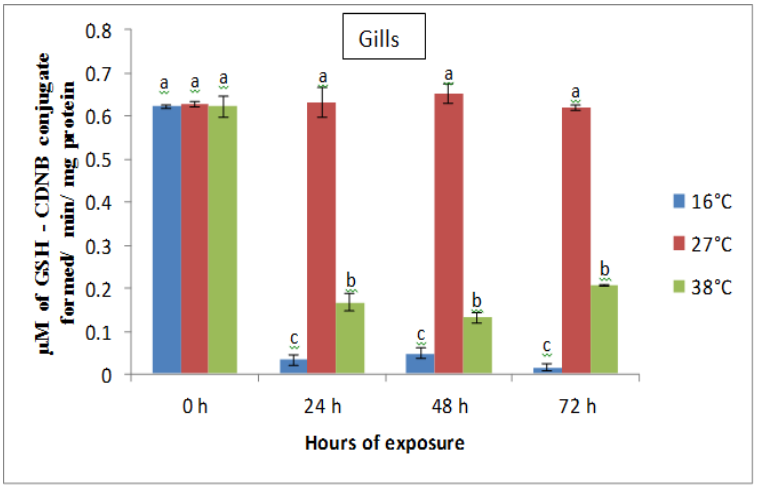

Figure 3.1 (A)

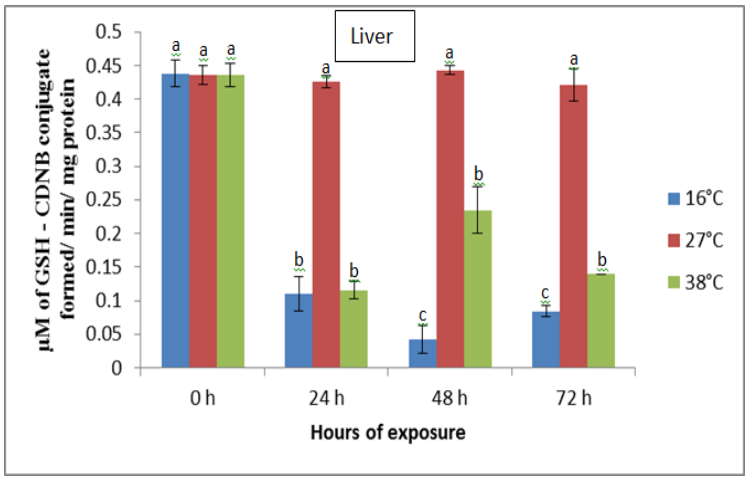

Figure 3.1 (B)

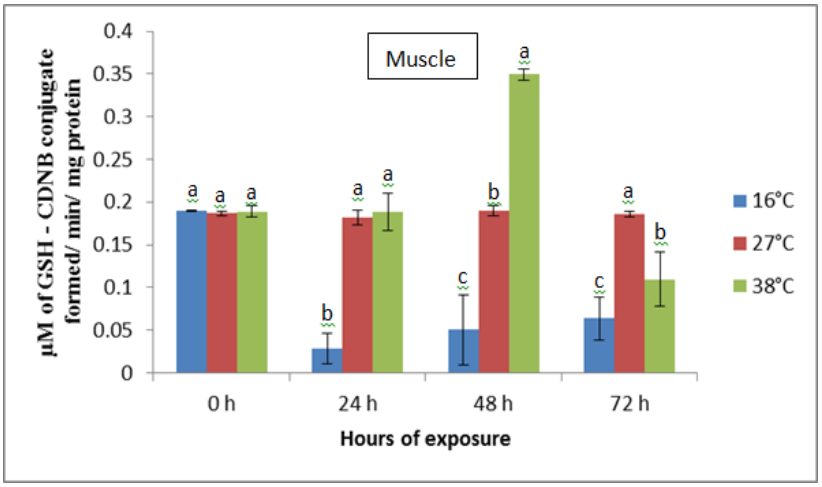

Figure 3.1 (C)

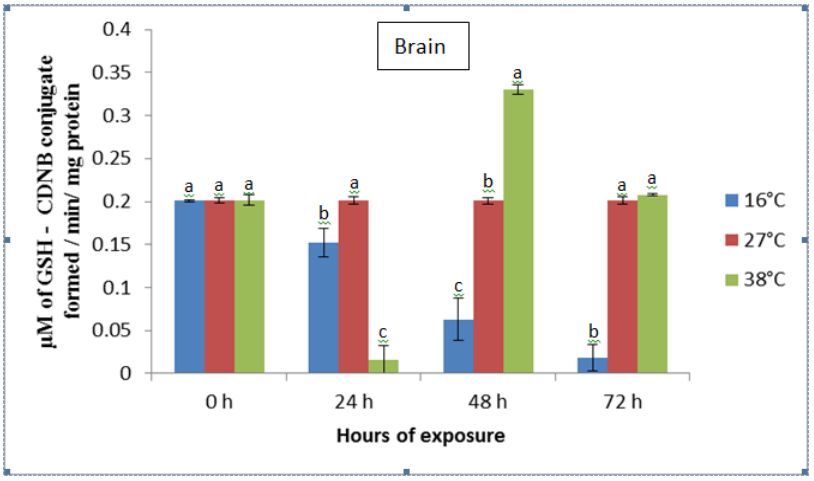

Figure 3.1 (D)

Table 3.1 Effect of different temperatures on GST activity (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

Glutathione-S-transferase activity				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16ºC	27ºC	38ºC
	0 h	0.621 ± 0.0053[a]	0.627 ± 0.0062[a]	0.622 ± 0.023[a]
	24 h	0.033 ±0.0136[c]	0.632 ± 0.034[a]	0.16747±0.019[b]
	48 h	0.04837±0.011[c]	0.6512 ± 0.021[a]	0.1316 ± 0.012[b]
	72 h	0.0159±0.0072[c]	0.6183 ±0.0056[a]	0.2057± 0.009[b]
Liver				
	0 h	0.43791±0.019[a]	0.4362 ±0.0138[a]	0.435± 0.017[a]
	24 h	0.1098± 0.025[b]	0.426 ± 0.0087[a]	0.11535±0.013[b]
	48 h	0.0420±0.0209[c]	0.4429± 0.0063[a]	0.2346±0.0353[b]
	72 h	0.0835±0.0083[c]	0.421± 0.0238[a]	0.1389±0.0005[b]
Muscle				
	0 h	0.1898±0.0013[a]	0.187± 0.0025[a]	0.189± 0.0064[a]
	24 h	0.02905±0.017[b]	0.182± 0.0089[a]	0.1883±0.0213[a]
	48 h	0.0506±0.0409[c]	0.1898±0.0063[b]	0.3494±0.006[a]
	72 h	0.0637±0.0253[c]	0.186±0.0037[a]	0.1097±0.0314[b]
Brain				
	0 h	0.2010±0.0012[a]	0.2013± 0.0034[a]	0.2018± 0.0062[a]
	24 h	0.1518±0.0164[b]	0.2010±0.0043[a]	0.0157±0.0173[c]
	48 h	0.0628±0.0248[c]	0.2010±0.0038[b]	0.3303± 0.0052[a]
	72 h	0.01813±0.015[b]	0.2010±0.0046[a]	0.2078 ± 0.008[a]

Values are expressed as µM of GSH – CDNB conjugate formed/ min/ mg protein

Three – factor ANOVA table (Table 3.2) revealed that between temperature, tissue and hours of exposure significant differences were present (P<0.05). Subsequent analysis by multiple comparison tests using Tukey's test revealed that between gills, liver, muscle and brain significant difference (P<0.05) was present. In between

temperatures such as 16ºC, 27ºC and 38ºC also significant difference was present . In between 0 and 24, 48, 72 hours of exposure there was a significant difference but between 24, 48, 72 hours of exposure, there was no significant variation.

Table 3.2 Three- factor ANOVA for Glutathione–S-Transferase

Source	Sum of Squares	df	Mean Square	F	Sig.
Days	0.921	3	0.307	533.457	0.000
Temperature	1.424	2	0.712	1237.176	0.000
Tissue	1.079	3	0.360	624.735	0.000
Days * Temperature	0.488	6	0.081	141.338	0.000
Days * Tissue	0.473	9	0.053	91.231	0.000
Temperature * Tissue	0.725	6	0.121	210.036	0.000
Days * Temperature * Tissue	0.367	18	0.020	35.395	0.000
Error	0.055	96	0.001		
Total	5.531	143			

df –degrees of freedom

Glutathione Reductase (GR)

Maximum GR activity observed in muscle was 13.49117 ± 0.44068 µM of NADPH oxidized/ min/ mg protein at 16ºC, 0.725 ± 0.0681 µM of NADPH oxidized/ min/ mg protein at 27ºC and 7.6379 ± 0.0391 µM of NADPH oxidized/ min/ mg protein at 38ºC. It is clear from the result that antioxidant defense response offered by GR was significantly high in muscle at 48th hour of experiment exposed to 16ºC. In the experimental group reared at ambient temperature GR activity remained steady. In gills at 38ºC the activity was 4.054 ± 0.02317 µM of NADPH oxidized/ min/ mg protein (Fig 3.2.A). In the liver, the maximum activity was during 72 hours of exposure in the experimental group reared at 38ºC. (Fig 3.2.B) where as in muscle and brain the activity was 13.49117± 0.44068 µM of NADPH oxidized/ min/ mg

protein and 7.195747± 0.17422 µM of NADPH oxidized/ min/ mg protein (Fig 3.2.C and D) (Table 3.3).

Figure 3.2 A.B.C.D. Activity of glutathione reductase (GR) in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

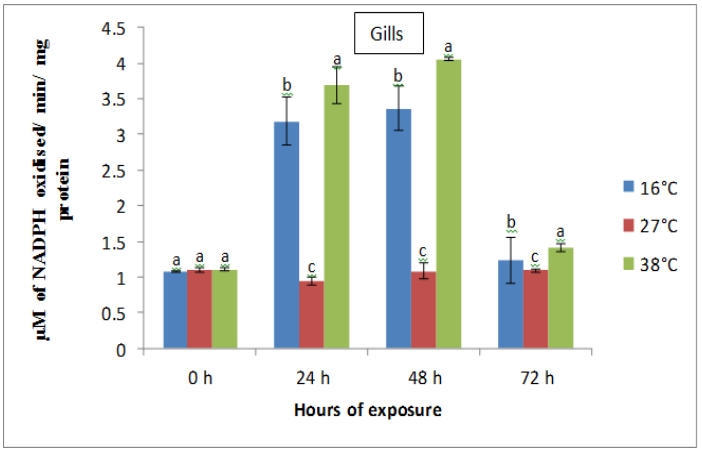

Figure 3.2 (A)

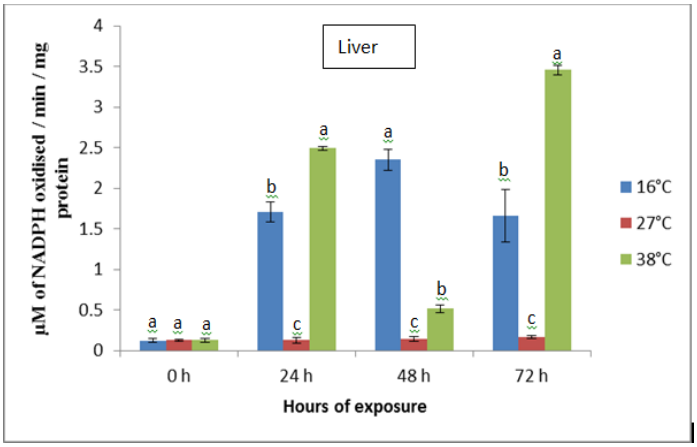

Figure 3.2 (B)

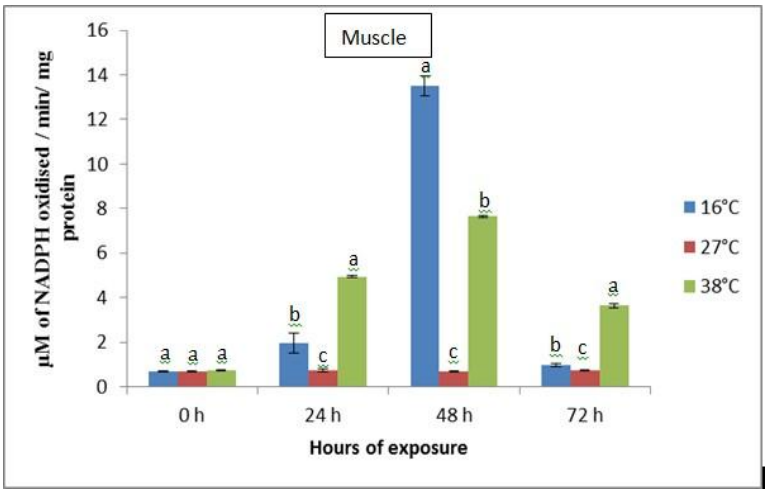

Figure 3.2 (C)

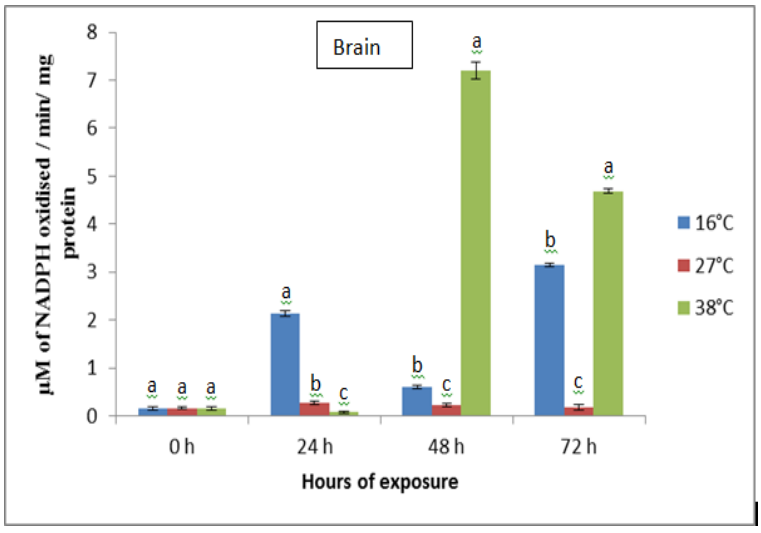

Figure 3.2 (D)

Table 3.3 Effect of different temperatures on GR activity (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Glutathione Reductase activity				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16°C	27°C	38°C
	0 h	1.0822±0.0153[a]	1.093± 0.0321[a]	1.11± 0.023[a]
	24 h	3.183± 0.3305[b]	0.937±0.061[c]	3.6956±0.261[a]
	48 h	3.3629± 0.318[b]	1.0822± 0.109[c]	4.054± 0.023[a]
	72 h	1.2269±0.3219[a]	1.09± 0.026[c]	1.408±0.057[b]
Liver				
	0 h	0.121±0.0223[a]	0.125±0.0124[a]	0.128± 0.025[a]
	24 h	1.70832±0.126[b]	0.1249±0.0342[c]	2.4920± 0.0234[a]
	48 h	2.3527±0.131[a]	0.142± 0.0290[c]	0.5170±0.0459[b]
	72 h	1.6606± 0.321[b]	0.163± 0.0189[c]	3.454± 0.0621[a]
Muscle				
	0 h	0.6972± 0.032[a]	0.6932± 0.0248[a]	0.712± 0.039[a]
	24 h	1.958±0.4396[b]	0.725± 0.0681[c]	4.9473±0.0543[a]
	48 h	13.491± 0.440[a]	0.693± 0.0248[c]	7.6379±0.039[b]
	72 h	0.9557± 0.083[b]	0.7132±0.0239[c]	3.642± 0.1052[a]
Brain				
	0 h	0.1453± 0.032[a]	0.1614± 0.0237[a]	0.1532± 0.0421[a]
	24 h	2.1255± 0.057[a]	0.276±0.032[b]	0.0758±0.017[c]
	48 h	0.596± 0.0290[b]	0.231± 0.034[c]	7.1957± 0.174[a]
	72 h	3.1393±0.032[b]	0.1723±0.057[c]	4.690± 0.0432[a]

Values are expressed as µM of NADPH oxidized/ min/ mg protein

Three – factor ANOVA table (Table 3.4) revealed that between temperature, tissues and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests using Tukey's test revealed that between gills, liver, muscle and brain

significant difference ($P<0.05$) was present . In between temperatures, such as 16ºC, 27ºC and 38ºC also significant difference was present. In between 24 and 48 hours of exposure, there was no significant variation.

Table 3.4 Three- factor ANOVA for Glutathione Reductase

Source	Sum of Squares	df	Mean Square	F	Sig.
Days	156.323	3	52.108	9698.233	0.000
Temperature	140.755	2	70.377	13098.539	0.000
Tissue	72.560	3	24.187	4501.612	0.000
Days * Temperature	83.923	6	13.987	2603.285	0.000
Days * Tissue	151.195	9	16.799	3126.695	0.000
Temperature * Tissue	33.738	6	5.623	1046.542	0.000
Days * Temperature * Tissue	211.656	18	11.759	2188.505	0.000
Error	0.516	96	0.005		
Total	850.667	143			

df – degrees of freedom

Glutathione Peroxidase (GPx)

In the case of fish treated at 38ºC, highest GPx activity was observed in gills (51.05 ± 0.52 µg/ min/ mg protein) at 48th hours of the experiment (Fig. 3.3.A). Whereas, highest Glutathione peroxidase activity was observed in muscle (78.865 ± 0.786 µg/ min/ mg protein) at 16ºC after 48th hours of exposure but brought back to near baseline during 72th hours (Fig. 3.3.C). In liver and brain highest activity (12.10 ± 0.139 µg/ min/ mg protein and 29.92 ± 0.790 µg/ min/ mg protein) was observed at 48th hours of exposure in experimental group reared at 16ºC (Fig. 3.3.B and D). The results clearly show that GPx activity was significantly high in tissues stressed at low temperature compared to those stressed at high

temperature. At ambient temperature GPx profile was steady overtime (Table 3.5).

Figure 3.3 A.B.C.D. Activity of glutathione peroxidase (GPx) in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

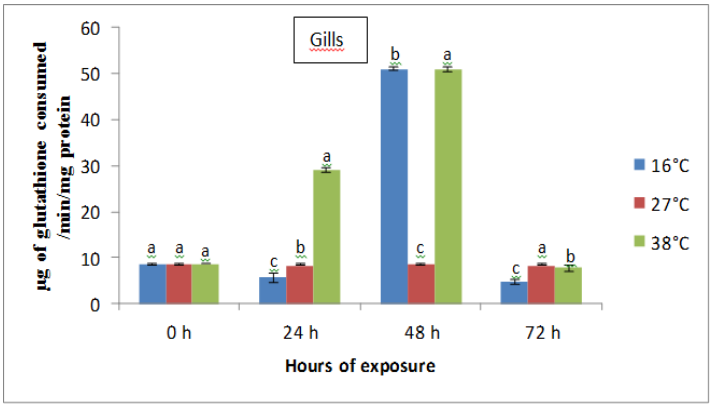

Figure 3.3 (A)

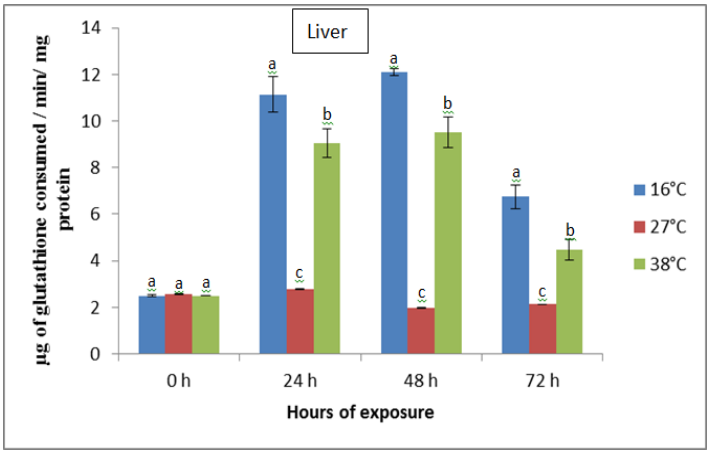

Figure 3.3 (B)

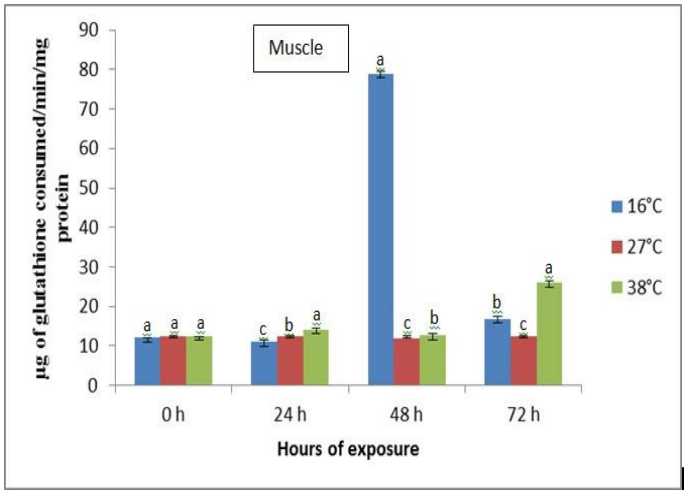

Figure 3.3 (C)

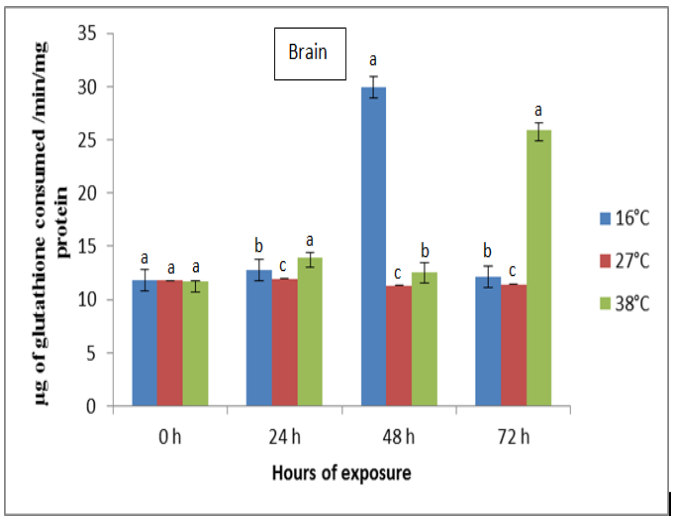

Figure 3.3 (D)

Table 3.5 Effect of different temperatures on GPx activity (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Glutathione Peroxidase activity				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16°C	27°C	38°C
	0 h	8.532± 0.021[a]	8.53± 0.034[a]	8.63± 0.057[a]
	24 h	5.6098± 0.971[c]	8.32± 0.0045[b]	29.1346±0.505[a]
	48 h	50.975±0.3543[b]	8.53± 0.0056[c]	51.053±0.5058[a]
	72 h	4.826± 0.588[c]	8.17± 0.0078[a]	7.734± 0.7177[b]
Liver				
	0 h	2.49± 0.043[a]	2.56± 0.021[a]	2.5± 0.012[a]
	24 h	11.143± 0.746[a]	2.787± 0.012[c]	9.049 ± 0.624[b]
	48 h	12.101±0.139[a]	1.98±0.014[c]	9.529± 0.657[b]
	72 h	6.7458± 0.504[a]	2.132± 0.012[c]	4.482± 0.4338[b]
Muscle				
	0 h	12.021± 0.042[a]	12.3± 0.016[a]	12.4± 0.009[a]
	24 h	10.9688±0.595[c]	12.56± 0.0193[b]	13.988±0.368[a]
	48 h	78.865± 0.786[a]	11.98±0.031[c]	12.602±0.564[b]
	72 h	16.755± 0.603[b]	12.42±0.023[c]	25.941± 0.435[a]
Brain				
	0 h	11.8± 0.0583[a]	11.8±0.016[a]	11.7± 0.0325[a]
	24 h	12.7671±0.581[b]	11.98±0.023[c]	13.988±0.451[a]
	48 h	29.9281±0.790[a]	11.32± 0.0143[c]	12.602± 0.813[b]
	72 h	12.121±0.117[b]	11.43±0.0153[c]	25.941± 0.682[a]

Values are expressed as µg of glutathione consumed/ min/ mg protein

Three – factor ANOVA (Table 3.6) revealed that between temperature, tissues and hours of exposure significant difference were present (P<0.05). Subsequent analysis by multiple comparison tests using Tukey's test revealed that between gills, liver, muscle and brain significant difference

($P<0.05$) was present. In between temperatures such as 16ºC, 27ºC and 38ºC also significant difference was present. In between 0, 24, 48 and 72 hours of exposure also there was significant variation .

Table 3.6 Three- factor ANOVA for Glutathione Peroxidase

Source	Sum of Squares	df	Mean Square	F	Sig.
Days	6311.943	3	2103.981	12677.748	0.000
Temperature	3564.883	2	1782.441	10740.279	0.000
Tissue	6085.971	3	2028.657	12223.875	0.000
Days * Temperature	6042.421	6	1007.070	6068.202	0.000
Days * Tissue	3889.883	9	432.209	2604.320	0.000
Temperature * Tissue	1628.502	6	271.417	1635.450	0.000
Days * Temperature * Tissue	5077.836	18	282.102	1699.834	0.000
Error	15.932	96	0.166		
Total	32617.371	143			

df- degrees of freedom

Total Reduced Glutathione

Fig 3.4. A,B,C and D shows the levels of oxidative stress marker total reduced glutathione in various tissues during experiment. In gills (Fig. 4A) total reduced glutathione level was 27.752 ± 2.913 µg/ mg of protein at 38ºC and in liver (Fig. 4B) it was 10.5793 ± 0.745 µg/ mg of protein at 16ºC. In case of muscle (Fig. 4C) levels was high in organism reared at 38ºC where as in brain (Fig. 4D) maximum activity was at 16ºC. Among the four tissues total reduced glutathione was maximum during the 48th hour of exposure in muscle at 38ºC. The group exposed to ambient temperature followed a steady response pattern.

Figure 3.4 A.B.C.D. Total reduced glutathione levels in gills (A), liver (B), muscle (C) and brain (D) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

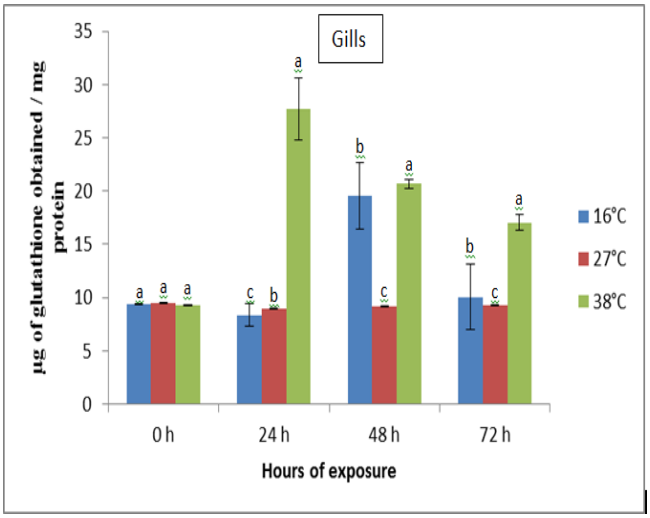

Figure 3.3 (A)

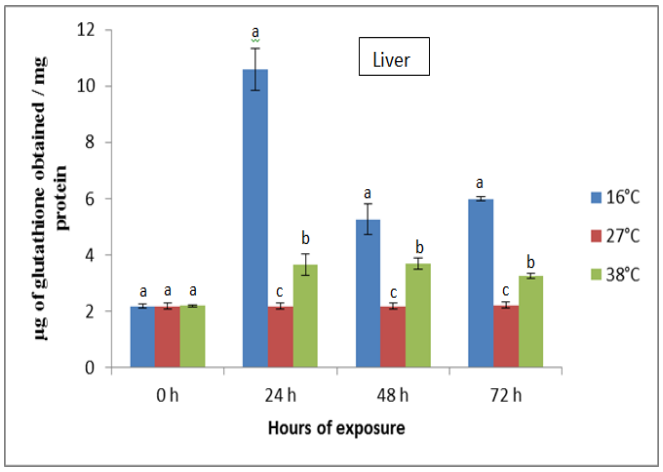

Figure 3.3 (B)

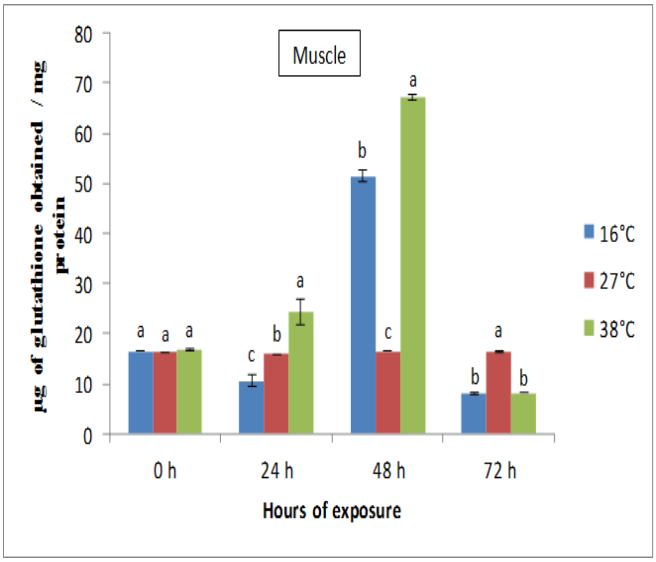

Figure 3.3 (C)

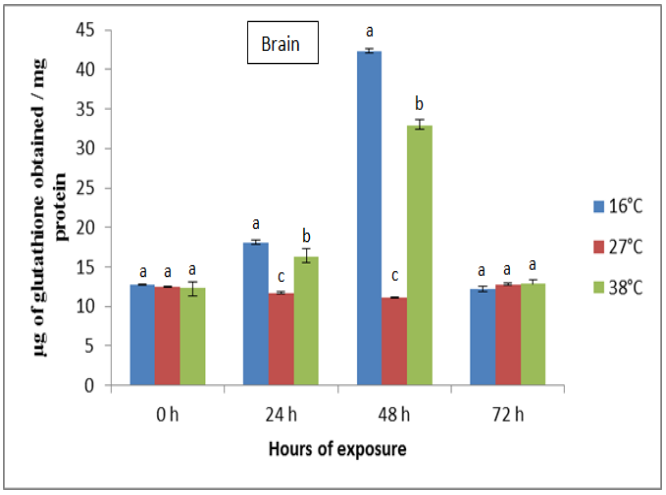

Figure 3.3 (D)

Table 3.7 Effect of different temperatures on total reduced glutathione (mean ± SD, n=7) present in different tissues of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Total Reduced glutathione				
Tissues	**Hours of exposure**	**Temperature**		
Gills				
		16°C	27°C	38°C
	0 h	9.400± 0.023[a]	9.523± 0.054[a]	9.321± 0.063[a]
	24 h	8.1439± 1.070[c]	8.9254± 0.037[b]	27.752± 2.913[a]
	48 h	19.543± 3.150[b]	9.138± 0.046[c]	20.680±0.388[a]
	72 h	10.040± 3.076[b]	9.263± 0.042[c]	17.031±0.7516[a]
Liver				
	0 h	2.182± 0.0638[a]	2.173±0.0129[a]	2.1883± 0.036[a]
	24 h	10.579± 0.745[a]	2.1718± 0.0234[c]	3.663±0.384[b]
	48 h	5.2657±0.552[a]	2.1820±0.062[c]	3.7003± 0.202[b]
	72 h	5.9951± 0.087[a]	2.21±0.0178[c]	3.2552± 0.093[b]
Muscle				
	0 h	16.5390±0.053[a]	16.238± 0.092[a]	16.832± 0.086[a]
	24 h	10.499±1.102[c]	15.8734±0.043[b]	24.3413±2.546[a]
	48 h	51.4543±1.085[b]	16.5390±0.024[c]	67.169±0.577[a]
	72 h	8.0323± 0.095[b]	16.321±0.0419[a]	8.1157± 0.023[b]
Brain				
	0 h	12.774± 0.095[a]	12.523±0.085[a]	12.319± 0.832[a]
	24 h	18.117±0.323[a]	11.7182±0.092[c]	16.32±0.994[b]
	48 h	42.324±0.287[a]	11.1145 ±0.031[c]	32.953± 0.732[b]
	72 h	12.22±0.3219[a]	12.774±0.1342[a]	12.836±0.5632[a]

Values are expressed as µg of glutathione obtained/ mg protein

Three – factor ANOVA (Table 3.8) revealed that between temperature, tissues and hours of exposure significant difference were present ($P<0.05$). Subsequent analysis by multiple comparison tests using Tukey's test revealed that between gills, liver, muscle and brain significant difference ($P<0.05$) was present. In between temperatures such as 16°C, 27°C and 38°C also significant difference was present. In between 0, 24, 48 and 72 hours of exposure also there was significant
variation .

Table 3.8 Three- factor ANOVA for Total Reduced Glutathione

Source	Sum of Squares	df	Mean Square	F	Sig.
Temperature	2335.212	2	1167.606	846.032	0.000
Days	2911.690	3	970.563	703.258	0.000
Tissue	8081.326	3	2693.775	1951.876	0.000
Temperature * Days	1835.351	6	305.892	221.645	0.000
Temperature * Tissue	915.463	6	152.577	110.556	0.000
Days * Tissue	8293.814	9	921.535	667.733	0.000
Temperature * Days * Tissue	5851.412	18	325.078	235.548	0.000
Error	132.489	96	1.380		
Total	30356.758	143			

df- degrees of freedom

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3.6 Discussion

The activity and expression of antioxidant enzymes have been used to evaluate the response of organisms under thermal stress (Park *et al.* 2008a, b; Mueller *et al*., 2012). Abele *et al*. (1998) reported that environmental temperature increases the consumption of oxygen, which in turn promotes the generation of reactive oxygen species (ROS). Increase in environmental temperature result in an increase of oxygen consumption (Hochachka and Somero, 2002). Lushchak and Bagnyukova (2006) reported that increase in temperature and enhanced oxygen consumption may promote the ROS generation, oxidation of cellular constituents and response of antioxidant and associated enzyme systems. Between the tolerance to temperature and resistance to oxidative stress there is a clear link present. An increase or decrease in environmental temperature reorganizes intermediary metabolism, changing the chemical composition particularly lipids levels (Cossins, 1981; Hochachka and Somero, 2002;). It modifies the physical and chemical state of membranes and fish behavior (Cossins *et al*., 1977). The GSH dependent antioxidant enzymes such as GPx, GR and GST are used as markers to distinguish between oxidative and thermal stress in fishes (Heise *et al.*, 2006; Bagnyukova *et al*., 2007; Leggatt *et al*., 2007; Grim *et al*., 2013). GPx acts to consume H_2O_2 and then transforms reduced glutathione (GSH) into oxidized glutathione (GSSG) (Wendel, 1981). GR acts by regenerating reduced glutathione (Halliwell and Gutteridge, 2007). The general function of the GST enzymes is the detoxification of electrophile compounds by glutathione conjugation. GSH and the enzymes associated with its metabolism provide a major defense against ROS induced cellular

damage. GSH interacts with ROS and acts as a chain breaking antioxidant (Cadenas, 1989).

Thermal stress induced significant changes in the glutathione metabolism. Lushchak *et al.* (2005a) and Nakano *et al*. (2014) reported that GPx activity increased during stress conditions. Though GPx activity increased in both the temperatures tested, the stress was prominent in the experimental group exposed to a low temperature which was in accordance with the findings of Storey (1996). Lushchak and Bagnyukova (2006) reported that GPx activity increased by two-fold in muscle after 24 hours. The maximum GPx activity was observed on the 48th hour of exposure in both the cases. The GPx activity was maximum in gills and low in muscle and brain, whereas, the activity was minimum in the liver at 38^{o}C. At 16^{o}C muscles were found more stressed compared to other tissues with a low response in gills and a minimum in brain and liver. It has been reported that, in eelpout, GPx activity does not show much variation between the control and experimental group (Heise *et al*., 2005) which is different from present study in which significant variation in GPx activity is noticed between the control and experimental group. There are reports suggesting that GPx activity changed overtime in response to the increase in temperature in liver (Machado *et al.,* 2014). It is clear from the present results that a 7- 10 fold increase in GPx activity has occurred in *E. suratensis* during the exposure to the thermal stress (16^{o}C and 38^{o}C). Catalases are mainly located in peroxisomes whereas GPx is distributed throughout the cytosol. GPx is primarily involved in the scavenging of the peroxides generated in the cytosol. Therefore, GPx could be the preferential route for degradation of hydrogen peroxide at

low substrate concentrations under physiological conditions (Lushchak *et al*., 2005a).

Reduced glutathione (GSH), the major nonprotein cellular thiol is a cysteine containing tripeptide with reducing and nucleophilic properties that is one of the major regulators for maintaining intracellular redox state (Nakano *et al*., 2014). GSH is the substrate for glutathione peroxidase which in turn scavenges reactive oxygen species (ROS) and lipid peroxides generated within the cells. GSH content present in cells is used as a non-enzymatic marker of oxidative stress (Sedlak and Lindsay, 1968; Halliwell and Gutteridge, 2007; Grim *et al.,* 2013). GSH activity was found to be increased with respect to control (27^{o}C) which was in accordance with the findings of Machado *et al*. (2014). Leggatt *et al.* (2007) reported similar findings in the liver and brain of killifish. Lushchak and Bagnyukova (2005) reported an increase in GSH levels in *C. auratus*. Glutathione can neutralize ROS in its reduced form (GSH) and also play an important role as a cofactor for various glutathione – dependent antioxidant enzymes (Halliwell and Gutteridge, 2007). Selman *et al.* (2000) reported that cold exposure has been up regulating the enzymatic antioxidant activities in various tissues of the short-tailed field vole *Microtus agrestis.* The increased activity of GSH in various tissues was probably the consequence of an increased need for the antioxidant defense to meet the increased peroxidative challenge due to increased pro- oxidant activity (Sahoo and Kara, 2014). GSH content was low in the liver which was in accordance with the findings of Lushchak and Bagnyukova (2006). The liver is the main source of glutathione in vertebrates and it supplies it to other organs. This may be the possible

reason for the low content of GSH in the liver. GSH plays a multifunctional role in antioxidant protection, maintaining other reductants and protein sulfhydryl groups in their reduced state, acting as a cofactor for a number of antioxidant enzymes and directly scavenging ROS and peroxides.

The disturbance of free radical process at varying temperatures affects antioxidant defenses. The long-term acclimation of zebra fish to higher and lower temperatures resulted in elevated activities in antioxidant enzymes such as GST, GPx (Malek *et al*., 2004). Abele and Puntarulo, (2004) reported that increase in ambient temperature raises antioxidant enzymes activities. This increase may occur at transcriptional, translational or post-translational levels. In the rat brain, both cold and heat stress increased the antioxidant activities like SOD, GPx, GST within 1-3 hours of exposure (Djordjevic *et al*., 2004). Fast enhancement of antioxidant defenses was found in the land snail *Helix aspersa* (Ramos- Vasconcelos et al., 2005) and during recovery from winter hibernation in frogs *Rana ridibunda* (Bagnyukova *et al*., 2003). GST activity was maximum at high temperature compared to low temperature. This result was in accordance with the findings of Bagnyukova *et al.* (2007) in which activity was maximum in summer compared to winter condition in gold fish. GST activity was maximum in gills, intermediate in liver and brain and lowest in the muscle which was similar with the findings of Lushchak and Bagnyukova (2005). The enzymes especially GPx and GST seem to play an important role in antioxidant defenses of gold fish which are subjected to other kinds of stress (Bagnyukova *et al.*, 2005a,b; Lushchak *et al.*, 2005a).

GR activity gets increased in all tissues compared to the control (27^{o}C) which are in agreement with the findings of Lushchak and Bagnyukova (2006). In the present study GR activity gets increased after 24 hours of exposure which was different from the findings of Machado *et al*. (2014) in which activity get decreased in *Notothenia rossii* after 3 and 6 days of exposure at 8^{o}C. Tatjana *et al*. (2015) reported that GR activities increased during development, but responded differently to elevated temperatures in different developmental stages in marble trout. Glutathione metabolism is continuously adjusted balancing GSH synthesis, GSSG recycling (Kidd, 1997; Griffith, 1999). GR recycles GSSG to GSH and increased GR activity was observed during the study which may be a positive measure to maintain GSH level by the organ. Overall the observed activities are the result of two processes, thermal or oxidative inactivation which occurred clearly over the heat exposure and synthesis of new molecules due to up-regulation of antioxidant defenses. Generally, the transfer from ambient condition to high and low temperature disturbed glutathione metabolism in *E. suratensis.* The quick increase in glutathione levels may serve as a signal for enhancement of overall antioxidant potential, and antioxidant enzymes activities generally showed an increase during 24 to 48 hours of exposure, a length of time that could be consistent with a longer-term enhancement of gene expression and protein synthesis. *E. suratensis* possesses high constitutive activities of antioxidant enzymes, which might be enough to cope with short term disturbance in free radical processes. Long seasonal changes in ambient temperature require an extensive reorganization of cellular metabolism including antioxidant defenses. This results in a marked increase in activities of

glutathione dependent enzymes. In nature transition from ambient condition to low to high temperature results in increase in antioxidant activities which would reflect the needs of an organism to cope with elevated oxidative stress that accompanies enhanced oxygen consumption and metabolic rate at higher environmental temperatures. But organism failed to survive due to the sustained thermal stress.

Thus the present results regarding the expression patterns of various biomarkers, such as GPx, GST, GR and reduced glutathione in response to thermal stressors suggest that severe thermal stress due to heat shock induces oxidative stress in *E. suratensis*, which may enhance oxidation of various cellular proteins in the body and cause damage to tissues. Under oxidative stress condition, the level of antioxidative substances may increase due to their *de novo* synthesis to protect tissues against oxidative damage. Results of these antioxidant parameters may be useful for improving fish fitness. An oxidative stress recently became a common theme in relation to impact of climatic change on natural ecosystems. A moderate level of oxidative stress could modulate cellular functions and have a significant impact on animal health. Manipulation of appropriate thermal treatment could be adopted to control and improve the health and production of fish.

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Chapter 4

Effect of Acute Thermal Stress on Carbohydrate, Lipid and Protein Profile in *Etroplus suratensis*

Contents

4.1 Introduction

Aquatic organisms are exposed to varying and extreme environmental conditions such as temperature. These environmental conditions cause a strong and debilitating effect on their physiology. Temperature is an important ecological factor which affects the chemical composition of tissues. There is a profound relationship between the organism and the environment which helps the organism to face the problem caused by the environment. Organisms try themselves to adapt to such environmental fluctuations. Temperature is one of the common variables in the aquatic environment that directly affects the survival of marine organisms. The ability to tolerate various temperature ranges is

different among the species (Kindle and Whitmore, 1986). Under culture conditions, fluctuations in water temperature affect the enzymatic reactions, growth efficiency, reproduction and immunity in fish and sudden fluctuations in water temperature that exceed the threshold values often cause the death of fishes (Chatterjee *et al.*, 2004; Cheng *et al*., 2013). Temperature also changes the physiological functions associated with the stress response of fish. The initial physiological stress response in fish includes the release of stress hormones, such as cortisol. An increase in plasma cortisol levels of fish is generally followed by an elevation in plasma glucose levels. Biochemical parameters, such as cortisol and glucose levels in plasma can be used as general stress indicators in fish (Santos and Pacheco, 1996). Hematological parameters such as hematocrit and hemoglobin are good indicators to assess fish health management in various conditions such as exposure to thermal stress. Water temperature dramatically changes the response to stress and the recovery dynamics.

4.2 Review of literature

Water temperature is a fundamental extrinsic factor that affects biological and metabolic processes such as survival (Brett and Groves, 1979), growth (Corey *et al*., 1983), energy level (Person-Le Ruyet *et al*., 2002), and fish vaccination (Martins *et al*., 2006) in aquatic organisms. Elevated temperature correlates with an increase in growth up to an optimum point, above which thermal stress occurs, whereas decreased temperature suppresses growth rates and metabolism (Beitinger *et al*., 2000). In animals, blood transports nutrients and metabolic waste and

carries out the body's immune functions. In fish, the blood is sensitive to endogenous and exogenous changes, such as those caused by virus infection, thermal stress, and altered nutritional status (Kenari *et al.*, 2011). Thus, a hematological examination is an essential part of evaluating the health status of fish (Rehulka, 1998). Evaluation of blood cells, blood biochemistry, and levels of the hormones can be useful for the diagnosis of diseases and to monitor the physiological status of fish, and these parameters are commonly used as indicators of the physiological stress response in fish (Lermen *et al.*, 2004). Among the various physical factors affecting the aquatic environment, the temperature is of paramount importance and is considered as the 'abiotic master factor' for fishes (Brett, 1971). Any change in the atmospheric temperature due to natural variations or pollution, may cause greenhouse effect which will influence the water temperature. Global climate change is suggested to potentially affect freshwater fisheries by lowering productivity in wild fish populations and in intensive aquaculture systems worldwide (Ficke *et al.*, 2007). As fishes are poikilotherms, drastic change in their surrounding water temperature will influence their metabolic processes, behavior, migration, growth, reproduction, and survival (Fry, 1971; Portner, 2001). Long-term changes in the environmental temperature induce ectothermic animals to display compensatory responses (which include changes in the metabolic enzymes and tissue chemistry) that are suggested to mitigate the effect of temperature on metabolism (Hazel and Prosser, 1974; Hochachka and Somero, 1971). Wedemeyer *et al.* (1999) reported that temperature beyond the optimum limits of a particular species, however, adversely affects fish health by increasing metabolic rate and subsequent

oxygen demand, invasiveness and virulence of bacteria and other pathogens which in turn may cause a variety of pathophysiological disturbances in the host. Changes in the hematological and biochemical responses to water temperature shock have been studied in various fishes like goldfish, rainbow trout, tilapia, atlantic cod, silver catfish and olive flounder (Basu *et al*., 2001; Lermen *et al*., 2004; Yang and Yeo, 2004; Gollock *et al*., 2006; Hur and Habibi, 2007; Jeong *et al.*, 2012). Hodson and Hayes (1989) reported that fish handle better when water temperature is low compared to high. Lower water temperature has several advantages such as cooler water holds more dissolved oxygen, reproduction of pathogen populations is low and the general metabolic rate of the fish is diminished. Optimum temperature for growth is expected to be different for different species and is influenced by the thermal optimum for other metabolic activities (Davis, 2004). Temperature stress influences the carbohydrate, protein and lipid profile of fishes because these molecules serve as energy sources in stress adaptation (Teoh *et al.*, 2013). A wide range of acclimatization was seen in poikilothermic animals in order to maintain physiological activities at a constant rate (Prosser and Brown, 1961). Temperature stress has been reported to produce a number of biochemical changes in fish in ion concentrations, organic constituents, enzyme activity, endocrinal activity and osmoregulation. Gerlach *et al.* (1990) reported that the adaptability of fishes and their ability to exhibit normal activity at extremes of temperature indicated that cellular processes may be maintained at appropriate levels following a period of thermal acclimation or adaptation. In ectothermic organisms, changes in temperature can be compensated by adjusting the physiological rates

(Carine *et al.,* 2004). Thermal acclimation in fish is generally determined by metabolic changes, during which an initial period of thermal stress is followed by a gradual compensation. When a stable metabolic level that is consistent between the old and new thermal state is reached, the animal is considered to be fully acclimated (Maricondi- Massari *et al.*, 1998).

Several reports are available describing the chemical constitution of fish blood (Strange, 1980; Cataldi, 1998; Barton, 2000; Lermen *et al.,* 2004). In fishes hematological parameters are used as indicators of the physiological stress in response to endogenous or exogenous changes (Adams, 1990; Santos and Pacheco, 1996; Cataldi *et al*., 1998). Plasma cortisol levels and alterations in carbohydrate, lipid and protein metabolism can be used as general stress indicators in fish (Santos and Pacheco, 1996). When compared with mammals, fishes have limited ability to utilize carbohydrates and have low metabolic rates. Walton and Cowey (1982) reported that fish utilize dietary carbohydrate poorly and thus protein is the important fuel in fish nutrition. 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). Physiological stress is a non-specific response which is composed of primary neuroendocrine component characterized by sympathetic activation and the secretion of cortisol and epinephrine (Donaldson, 1981; Schreck, 2000). A secondary phase is characterized by an increase in plasma glucose and disturbances of osmoregulatory function. In response to most stressors fish exhibits an increase in plasma cortisol levels, which is generally followed by an elevation in plasma

glucose levels. In stress, fluctuations in oxygen consumption cause an increase in energy demands of the animal, which in turn causes an increase in carbohydrate metabolism, that would meet the changing energy demand (Lacerda and Sawaya, 1986; Santos and Nay, 1987). Lipids are important sources of metabolic energy and they contain more energy per unit weight than any other biological compound. They play an important role in maintaining the structural and physiological integrity of cellular and subcellular membranes. Lipid provides a source of indispensable nutrients and they act as carriers of certain non-fat nutrients, particularly the fat soluble vitamins like A, D, E and K (New, 1986; Jauncey, 1998; Richardo *et al*., 2003). The main lipid storage site in fish varies between species, and is located either in the subcutaneous area, in the liver, in the muscle myosepta, or the mesenteric membranes. Lipid is an important fuel reserve of the fish during stress situation; it is mobilized to meet the energy needs of the organism during stress. They are essential components of all cells, making up about half the mass of biological membranes and are involved in numerous biological processes. Alterations cited in the circulating levels of total lipids and cholesterol in fish generally reflect the state of the animal as well as the integrity of the vital organs. Levels of triglycerides and cholesterol are the usual indicators of lipidmetabolism.

Proteins are important organic substances required by organisms in tissue building and they play an important role in energy metabolism (Yeragi *et al*., 2003; Remia *et al*., 2008; Yiu *et al*., 2008; Fahmy, 2012). They are important biomolecules which are involved in a wide spectrum of cellular functions (Prashanth, 2006). The structure of proteins enables them to act as the catalysts which are responsible for the control of the

rate of biological reactions. Proteins are a heterogeneous group of macromolecules which have diverse physiological functions (Lehninger, 1984). Hence, protein profiles can be considered as diagnostic tool in assessing the physiological status of a tissue or an animal as a whole (Harper, 1986; Chopra *et al*., 2001). The concentration of proteins is a balance between the rate of their synthesis (anabolism) and degradation (catabolism). In fishes temperature is known to affect the structural and functional properties of proteins. *Oreochromis mossambicus* showed a declining trend of protein, carbohydrate and lipid content in the tissues like brain, gill, kidney, liver and muscles upon exposure to stress (Ganesan *et al*., 1989) and there is an increase in chromatid break and chromosomal exchange due to stress (Rita and Milton, 2006).The catalytic properties of enzymes can be influenced by both the habitat and temperature fluctuations (Klyachko and Ozernyuk, 1998). Adequate energy reserves are required by organisms to mediate the effects of stress, which in turn serve as energy buffers during the periods of harsh environmental conditions and food shortages. Lipids, proteins and carbohydrates are important biomolecules in any model that describes the biochemical effects of stress.

4.3 Objectives of the present study

1) To investigate the effect of thermal stress on the tropical culture fish *E. suratensis* by evaluating the levels of glucose and glycogen in serum.

2) To study the pattern of variation that may occur in lipid profile during temperature stress in *E. suratensis*.

3) To find out the effect of thermal stress on protein profile in *E. suratensis*

4) To identify the difference in pattern at the selected extreme temperatures with respect to control

4.4 Materials and methods

Collection, maintenance, acclimation, determination of Critical Thermal Maximum (CT Max), Critical Thermal Minimum (CT Min), experimental design, preparation of tissue samples were the same as explained in detail in chapter 2

4.4.1 Preparation of serum samples for experimental studies

Blood was drawn from the common cardinal vein using l mL sterile plastic insulin syringe of 26 mm gauge size. The collected blood was then kept at room temperature for 30 minutes and centrifuged at 2500 x g for 30 minutes to obtain the serum. The serum was then collected and stored at -20^{o}C until use.

a) Estimation of Blood Glucose

Blood glucose was estimated by the method of Sasaki *et al*. (1972).

Principle

Ortho toluidine reacts with glucose in hot acetic acid solution to produce a blue – green colour. The absorbance is measured at 630 nm.

Reagents

1) Ortho toluidine boric acid reagent: This reagent consists of 2.5 g of thiourea and 2.4 g of boric acid in 100 mL of solvent, consisting of mixture of water, acetic acid (AR) and O – toluidine (distilled) in the ratio of 10: 75: 15.
2) Standard glucose: 100 mg of glucose was dissolved in 0.1% benzoic acid. 10 mL of this solution was diluted to 100 mL to give 100 µg of glucose per mL.

Procedure

To 0.2 mL of blood, 0.8 mL of 10% trichloroacetic acid (TCA) was added. The contents were mixed well and centrifuged at 1000 x g for 5 minutes. To 0.5 mL of supernatant added 2 mL of ortho-toluidine reagent and the tubes were then heated in a boiling water bath for 15 minutes along with a standard solution containing 20-100 µg of glucose. The absorbance due to the blue color developed was measured spectrophotometrically at 630 nm. The values were expressed as mg/ dL of serum.

b) Estimation of Glycogen.

Glycogen was estimated by the method of Montgomery, 1957.

Principle

Glycogen gets solubilized in alkali upon heating. Thereafter, glycogen is precipitated out by using alcohol.

Reagents

1) 30% potassium hydroxide
2) 95% ethanol

Procedure

The tissues (liver and muscle) were washed with water to remove adhering materials. Added 25 mL of 30% KOH to 5% tissue homogenate in an Erlenmeyer flask and kept the flask in a boiling water bath for 45 minutes. Cooled the flask and transferred the contents to another flask. Added two volumes of 95% ethanol and mixed by shaking. It was then incubated for one hour for the complete precipitation of glycogen. The contents were filtered and washed with cold water to remove adhering impurities and spread over a watch glass to air dry. Dried glycogen was weighed and the values were expressed in mg/ g wet weight of tissue.

c) Estimation of Total Lipids

Total lipid was estimated by the sulpho-vanillin reaction by Barnes and Blackstock (1973).

Principle

Lipids on heating with concentrated sulphuric acid get oxidized to ketones which form a pink colour with phosphoric acid and vanillin.

Reagents

1) Vanillin – 0.65%: 650 mg vanillin was dissolved in 100 mL of 1% benzoic acid solution.
2) A/R grade Conc. Sulphuric acid (H_2SO_4)
3) A/R grade Conc. Phosphoric acid (H_3PO_4)
4) Standard lipid solution: l g of pure groundnut oil was dissolved in chloroform-methanol and was made to 100 mL with chloroform.

Procedure

0.01 mL of serum sample was taken in a test tube. The content present in the tube was evaporated to dryness using a water bath maintained at 70°C. All the tubes were then cooled for 5 minutes. To this
1 mL of concentrated sulphuric acid was added. Simultaneously into another test tube 1 mL of vanillin followed by 4 mL of phosphoric acid was added. The tube was then mixed well, followed by 0.1 mL of serum was added. It was then shaken vigorously and was incubated at 37°C for 15 minutes. After incubation, the tubes were removed and were cooled to room temperature. The absorbance was then read against blank at 540 nm. For standard 0.1 mL of standard lipid solution and for blank 0.1 mL distilled water was used in place of the test sample in the procedure. Values were expressed in mg/ dL of serum.

d) Estimation of Triacylglycerol (TAG)

TAG was estimated by the method of Hantzsh reaction as given by Foster and Dunn (1973) and Fletcher (1968).

Principle

Phospholipids were removed using alumina isopropanol mixture. In this, alumina serves as an adsorbent for phospholipids, and TAG was extracted into isopropanol after saponification with potassium hydroxide, to yield glycerol and soap. The glycerol liberated after saponification was treated with metaperiodate which released formaldehyde, formic acid and iodide upon oxidation. The formaldehyde released reacts with acetyl acetone and ammonia forming a yellow coloured compound, the intensity of which was measured at 405 nm.

Reagents

1) lsopropanol.
2) Alumina-activity grade l: Washed with water until all fines were removed and dried in an oven overnight.
3) Saponifying reagent: Dissolved 50 g potassium hydroxide in 600 mL water and added 400 mL of isopropanol to it.
4) Sodium metaperiodate reagent: Dissolved 77 g of anhydrous ammonium acetate in about 700 mL water. To this 60 mL of glacial acetic acid was added followed by 650 mg of sodium metaperiodate. The mixture was then made up to l L with distilled water.
5) Acetyl acetone reagent: Added 7.5 mL of acetyl acetone to 200 mL of isopropanol. The mixture was mixed and to this 800 mL distilled water was added.
6) Stock triolein solution: Accurately weighed 8.85 g of triolein. It was then dissolved in l L of isopropanol.
7) Working triolein solution: l.0 mL of stock standard was diluted to 100 mL to prepare a working standard of concentration l00 µg of triolein/ mL.

Procedure

To 0.01 mL of the serum sample, added 0.4 mL of distilled water followed by 4 mL of isopropanol and the tubes were mixed well and 400 mg washed alumina was added to it. It was placed in a rotor and centrifuged for l5 minutes. To 2 mL supernatant, 0.8 mL of saponifying reagent (potassium hydroxide and isopropanol (3:2)) was added and the test tubes

were incubated at 60 - 70°C for 10 minutes. Tubes were cooled and to this l mL of metaperiodate solution was added, mixed well, and 0.5 mL of acetyl acetone reagent was added. Tubes were then incubated at 50°C for 30 minutes. For blank, 0.1 mL of water was treated in the similar manner as the test. The absorbance of the yellow coloured compound formed was read spectrophotometrically at 405 nm. Values were expressed as mg/ dL of serum.

e) Estimation of Cholesterol

Cholesterol was estimated as per Zak *et al.* (1953) using ferric chloride – sulfuric acid method.

Principle

The sample was treated with ferric chloride - acetic acid reagent to precipitate the protein. The protein free filtrate containing cholesterol and ferric chloride was treated with Conc. H_2SO_4. The reaction involved the 3-hydroxy 5- ene part of the cholesterol molecule, which was first dehydrated to form cholesta 3, 5-diene and then oxidized by sulphuric acid to link two molecules together as bischolesta-3,5diene. This material can be sulphonated by sulphuric acid to produce the highly red coloured mono- and di-sulphonic acids in the presence of ferric ion as catalyst (Solkowski's reaction). The colour developed was read at 560 nm using suitable standard and a reagent as blank.

Reagents

1) Glacial acetic acid — A/R grade.
2) Ferric chloride-acetic acid ($FeCl_3$-CH_3COOH) reagent: 0.5% solution of ferric chloride ($FeCl_3$. $6H_2O$) was prepared in purified acetic acid.

3) Conc. H_2SO_4 - A/R grade.

4) Cholesterol stock standard solution: Dissolved 100 mg of cholesterol in 100 mL of glacial acetic acid.

5) Working standard: 5 mL of stock standard was diluted to 100 mL with ferric chloride acetic acid ($FeCl_3$-CH_3COOH) reagent.

Procedure

To 0.1 mL of the tissue lipid extract sample and 0.01 mL of serum, 9.9 mL of $FeCl_3$-CH_3COOH reagent was added. The tubes were mixed well. They were allowed to stand for 15 minutes for the proteins to flocculate. The mixture was centrifuged and 5 mL of supernatant was transferred to another tube. For standards, 2 mL of working standard was taken which was then made to 5 mL with $FeCl_3$-CH_3COOH reagent. For blank 5 mL $FeCl_3$-CH_3COOH reagent was taken. Then to all the tubes, 3 mL of Conc. H_2SO_4 was added slowly. The contents present in the tube were mixed well. The standard and test were read against blank at 560 nm using a colorimeter. The values were expressed as mg/ dL of serum.

f) Estimation of Phosholipids

Phospholipids present in the serum were estimated according to the method of Rouser *et al.* (1970).

Principle

The organic phospholipid phosphorus is converted to inorganic phosphorus which reacted with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ascorbic acid formed a stable blue colour that has an absorption maximum at 660 nm.

Reagents

1) 70% Perchloric acid
2) 3% Ammonium molybdate
3) 3% Ascorbic acid
4) Standard: 35.1 mg of KH_2PO_4 was dissolved in l00 mL of water. This contains 80 µg of phosphorus/ mL.

Procedure

To 0.0l mL of serum, l.0 mL of perchloric acid was added and digested on a sand bath until it became colourless. The volume was made up to 5.0 mL with water. The blank containing 0.8 mL of perchloric acid and 4.2 mL of water was taken. Standards in the range 5-20 µg were also taken and 0.8 mL of perchloric acid was added and the contents were made up to 5.0 mL with water. To all the tubes, 0.5 mL of ammonium molybdate was added followed by 0.5 mL of ascorbic acid solution. The contents were mixed well. The tubes were heated in a boiling water bath for 6 minutes and the colour developed was read immediately at 700 nm. Phospholipid concentration was expressed as mg/ dL in serum.

g) Estimation of High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), Very Low Density Lipoprotein (VLDL)

HDL was estimated by phosphotungstate/ magnesium method adopted by Burstein *et al.* (1970).

Principle

LDL, VLDL are precipitated by polyanions in the presence of metal ions to leave HDL in solution. 3-hydroxy-5-ene part of the HDL cholesterol molecule is dehydrated and then oxidized by concentrated sulphuric acid.

This material can be sulphonated by sulphuric acid to produce the highly coloured mono -and - di-sulphonic acids.

Reagents

1) Phosphotungstate reagent: Dissolved 22.5 g of phosphotungstic acid (AR grade) in 200 mL water. To this added 80 mL of l mol/ L NaOH and was made up to 500 mL with water.
2) Magnesium chloride solution — 2 mol/ L: Dissolved 101.7 g $MgCl_2.6H_2O$ in water and was made up to 250 mL.
3) Glacial acetic acid - A/R grade.
4) Ferric chloride acetic acid reagent.
5) Conc. H_2SO_4— A/R grade.
6) Cholesterol stock standard solution: l00 mg cholesterol was dissolved in 100 mL of purified acetic acid.
7) Working standard: 1 mL of the stock solution was made up to 25 mL with $FeCl_3$-CH_3COOH reagent.

Procedure

Added 0.l mL of phosphotungstate reagent and 0.05 mL of magnesium chloride solution to 0.0l mL of the serum sample. The tubes were mixed well and centrifuged at 1300 x g for 30 minutes. To 0.1 mL of the supernatant, l0 mL of ferric chloride acetic acid reagent was added. The contents of the tube were mixed well and then kept undisturbed for 10 to l5 minutes for the proteins to flocculate and centrifuged. 5 mL of clear supernatant was taken as the test. 5 mL of $FeCl_3$-CH_3COOH reagent

was taken as blank. For standard l to 5 mL of cholesterol working standard was taken which was then made up to 5 mL using $FeCl_3$- CH_3COOH reagent. Added 5 mL of conc. sulphuric acid to all the tubes, mixed vigorously and allowed to stand for 20-30 minutes. The color developed was read spectrophotometrically at 560 nm. The values were expressed as mg/ dL of serum.

The serum levels of VLDL and LDL were calculated using Friedewald's equation (1972).

VLDL = Triglycerides / 5

LDL = Total Cholesterol – Triglycerides / 5 – HDL

h) Estimation of Total Proteins

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

Principle

The CO-NH group (peptide bond) present in the protein molecule reacted with cupric ions in alkaline medium to give a blue colour, which is measured at 660 nm.

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 reagent A and 0.1 mL of reagent 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)

5) Stock standard: 50 mg of bovine serum albumin as 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

The tissue homogenate of gills, muscle, liver and brain tissues was prepared in Tris HCl buffer of pH 7 and supernatant fractions were collected after centrifugation at 11,200 x g for 15 minutes. To the supernatant equal volume of 10% trichloroacetic acid was added to precipitate the proteins. The contents were allowed to stand for 30 minutes at room temperature and centrifuged at 1000 x g for 15 minutes. The sediment of protein was dissolved in 1 mL of 0.1N NaOH. After suitable dilution a known volume of the solution was used as samples. Different volumes of the working standard solutions ranging from 0.2 to 1.0 mL were pipetted out in to the test tubes. The volume in all the tubes was made up to 1.0 mL with distilled water. 5. 0 mL of alkaline copper reagent was added to all the test tubes. The contents in the tube 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. The absorbance of 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.

i) Estimation of Albumin

Albumin in serum was estimated by the method of Bartholomew *et al*. (l966).

Principle

Albumin present in serum bounds specifically with the dye, bromocresol green at pH 3.8 to form a green coloured complex, which brings about a change in the wave length with maximum absorption. This in turn brought about a change in the spectral profile of the dye at 637 nm. The intensity of the colour was directly proportional to the albumin concentration.

Reagents

1) 1M sodium citrate: 29.4 g of sodium citrate was dissolved in l00 mL distilled water.
2) 1M Citric acid: 21 g citric acid was dissolved in l00 mL distilled water.
3) 0.0l M Bromocresol green (BCG): 0.0698 g of BCG was dissolved in 0.98 mL of 0.1 M sodium hydroxide and was then made up to l0 mL with distilled water.
4) Buffering agent: To about 800 ml water, 17.3 mL of 1M sodium citrate, 32.7 mL of l M citric acid and 6 ml 0.01M BCG were added. The contents were then diluted to one L with distilled water. The pH was adjusted to 3.8.

5) Standard bovine serum albumin solution: 250 mg of bovine serum albumin was made up to 25 mL with 0.9% saline. 20 mL of the stock standard was made up to l00 mL with distilled water.

Procedure

To 0.02 mL of the serum sample, 0.05 mL of distilled water was added. To this 4 mL of buffering reagent was added. The contents were mixed well. Similarly standards of volume ranging from 0.1 to 0.5 mL were taken. It was then made up to 0.5 mL with distilled water. 0.5 mL of distilled water was taken as blank. 4 mL of buffering reagent was added to all the tubes and kept for 10 minutes. The absorbance of the coloured complex obtained was then read at 637 nm. The values obtained were expressed as mg/ dL of serum.

Estimation of Globulin

Globulin was estimated by subtracting albumin from total protein value.

4.4.2 Statistical analysis

The statistical analysis was carried out using the software SPSS 22.0 package. Two- way analysis of variance (ANOVA) was carried out to compare different temperature and hours of exposure. If significant differences were revealed by the ANOVA test, Tukey's test was used to further elucidate which temperature and hour of exposure were significantly different. One-way ANOVA followed by Tukey's test was carried out for the comparison between different temperatures in each hour of exposure. Significance level was set at $P< 0.05$ in all the tests.

4.5 Results

In the present investigation, the levels of blood glucose were estimated in the fish on 0th, 24th, 48th and 72th hours of exposure. The blood glucose level was elevated relative to control (27^{o}C) in fish at the 24th hour of exposure and decreased gradually towards 48th and 72th hour of exposure at 16^{o}C and 38^{o}C. The highest value of serum glucose was 62.73 ± 0.05 mg/ dL at 38^{o}C whereas at 16^{o}C it was 58.66 ± 0.05 mg/ dL (Fig. 4.1)

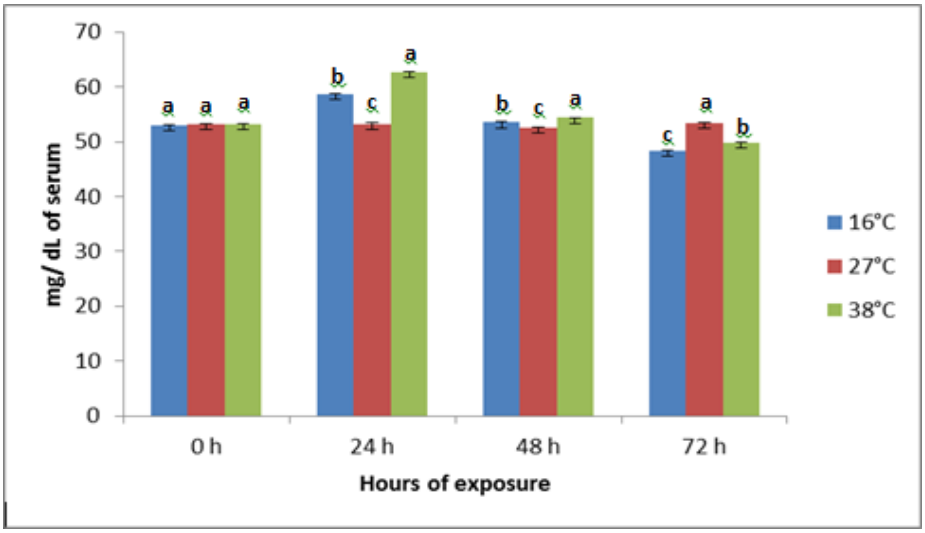


Figure 4.1 Effect of temperature stress on glucose metabolism. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature at different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.1 Effect of different temperature on glucose profile (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Glucose				
Serum	**Hours of exposure**	**Temperature**		
		16°C	27°C	38°C
	0 h	53 ± 0.161[a]	53.17± 0.2[a]	53.19± 0.18[a]
	24 h	58.66±0.05[b]	53.2 ±0.18[c]	62.73±0.05[a]
	48 h	53.5±0.11[b]	52.4±0.13[c]	54.33±0.05[a]
	72 h	48.39±0.09[c]	53.4±0.16[a]	49.8±0.1[b]

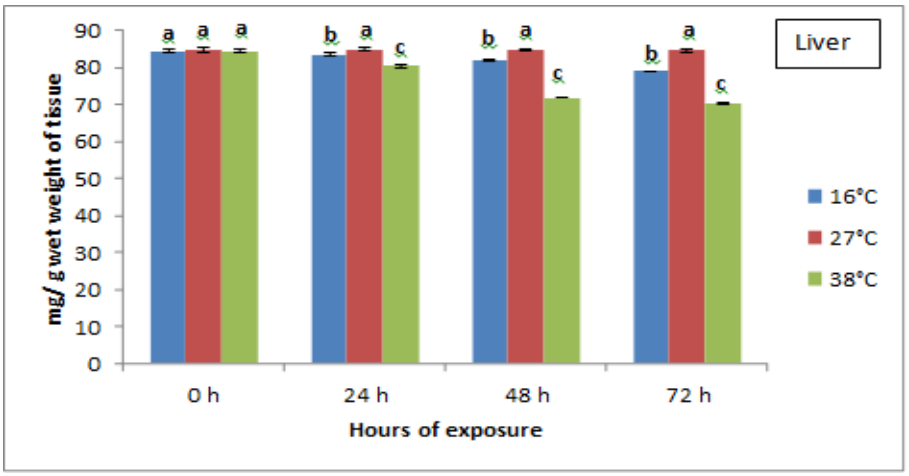
Two factor ANOVA (Table 4.2) revealed that between temperature and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests (Table 4.25) using Tukey's test revealed that between hours of exposure significant difference (P<0.05) was present. Between temperatures such as 16°C, 27°C and 38°C significant difference (P<0.05) was present (Table 4.29).

Table 4.2. Two factor ANOVA for Glucose

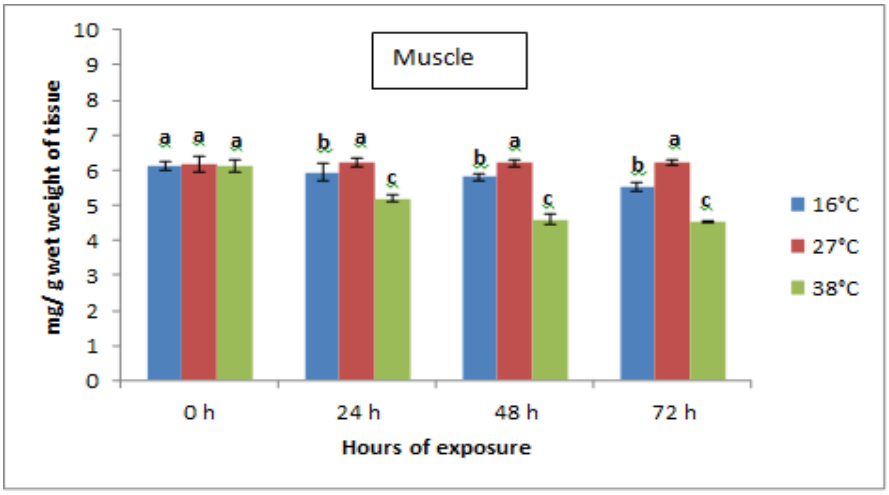
Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	275.700	3	91.900	5682.588	0.000
Temperature	24.395	2	12.197	754.224	0.000
Serum	0.000	0			
Hours * Temperature	151.795	6	25.299	1564.362	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	0.388	24	0.016		
Total	452.279	35			

df- degrees of freedom

The liver and muscle glycogen content declined during 48^{th} and 72^{th} hour of exposure. In case of liver, the value was found to be 83.59 ± 0.349 mg/ g wet weight of tissue (Fig. 4.2. A) whereas in muscle it was 5.93 ± 0.241 mg/ g wet weight of tissue at 16^{o}C (Fig 4.2. B) during 24 hours of exposure and the values declined on further exposure to temperature stress. The glycogen content was more in liver than in muscle.



(A)



(B)

Figure 4.2 A, B. Effect of temperature stress on glycogen metabolism in liver (4.2.A) and muscle (4.2.B) respectively. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly ($P<0.05$) in each temperature at different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.3. Effect of different temperature on glycogen profile (mean ± SD, n=7) present in serum of *E. suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Glycogen profile in liver and muscle				
Tissue	**Hours of exposure**	**Temperature**		
		16°C	**27°C**	**38°C**
Liver	0 h	84.46 ± 0.416[a]	84.832± 0.638[a]	84.39± 0.52[a]
	24 h	83.59±0.349[b]	85 ±0.51[a]	80.52±0.50[c]
	48 h	81.96±0.152[b]	84.8±0.43[a]	71.93±0.11[c]
	72 h	79.11±0.101[b]	84.65±0.49[a]	70.31±0.35[c]
Muscle	0 h	6.13± 0.115[a]	6.18±0.21[a]	6.15±0.187[a]
	24 h	5.93±0.241[b]	6.23±0.12[a]	5.2±0.10[c]
	48 h	5.8±0.1[b]	6.2±0.1[a]	4.6±0.14[c]
	72 h	5.53±0.115[b]	6.21±0.09[a]	4.52±0.02[c]

Values are expressed as mg/ g wet weight of tissues

Two factor ANOVA (Table 4.4) revealed that between temperature and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests (Table 4.25) using Tukey's test revealed that between hours of exposure significant difference (P<0.05) was present. Between temperatures such as 16°C, 27°C and 38°C significant difference (P<0.05) was present (Table 4.29).

Table 4.4. Two factor ANOVA for Glycogen

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	146.480	3	48.827	1896.380	0.000
Temperature	238.665	2	119.333	4634.775	0.000
Tissue	102550.147	1	102550.147	3982959.650	0.000
Hours * Temperature	133.570	6	22.262	864.622	0.000
Hours * Tissue	98.533	3	32.844	1275.645	0.000
Temperature * Tissue	141.028	2	70.514	2738.698	0.000
Hours * Temperature * Tissue	91.351	6	15.225	591.332	0.000
Error	1.236	48	0.026		
Total	103401.009	71			

df- degrees of freedom

The serum lipid profile of *E. suratensis* varied significantly when subjected to cold (16^{o}C) and warm (38^{o}C) temperature stress compared to control (27^{o}C). The fish exposed to 16^{o}C showed an increase in lipid profile. The slowly increasing trend was observed at this temperature up to 72 hours of exposure where as a slowly decreasing trend was found at 38^{o}C up to 72 hours of exposure. Total serum lipid content was maximum (Fig 4.3) with a value 398.96 ± 0.5621 mg/ dL at 38^{o}C. Cholesterol level was 111.23 ± 0.57 mg/ dL of serum at 38^{o}C whereas at 16^{o}C (Fig. 4.5) it was 100.33 ± 0.58 mg/ dL of serum. Triacylglycerol (TAG) (Fig 4.4) was 113.3 ± 0.264 mg/dL and 107.5 ± 0.5 mg/ dL during 72th and 48th hour of exposure at 16^{o}C. Serum HDL (Fig. 4.7) was highest at 16^{o}C during the 72th hour of exposure which was 14.1 ± 0.28 mg/ dL. LDL and VLDL levels (Fig 4.8 and 4.9) were maximum at 38^{o}C with values 76.5 ± 0.2 mg/ dL and 23.33 ± 0.3 mg/ dL respectively. Phospholipid content was high at 24th hour of exposure (171.2 ± 0.183 mg/ dL) at 38^{o}C (Fig 4.6).

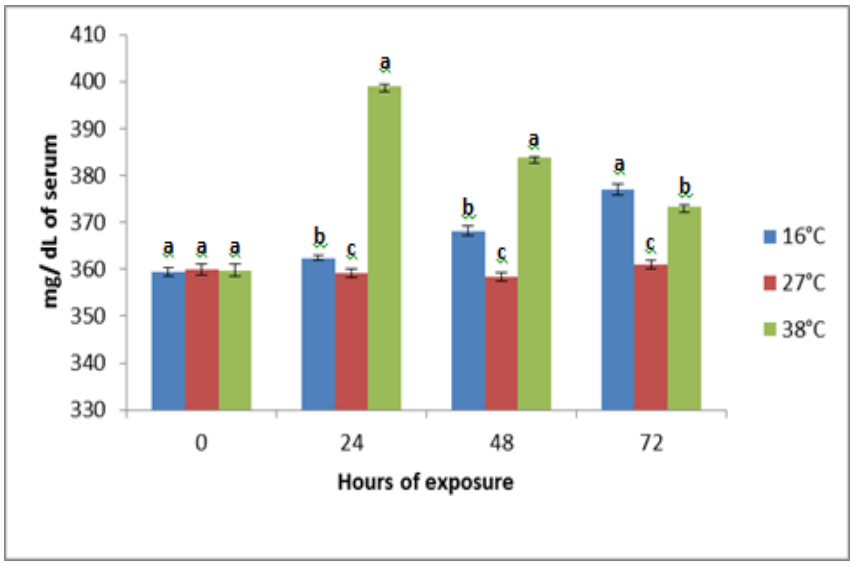


Figure 4.3 Effect of temperature stress on total lipid. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One- way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.5 Effect of different temperature on total lipid (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Total Lipid				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	359.39± 0.92^{a}	360± 1.12^{a}	359.63±1.5^{a}
	24 h	362.43±0.532^{b}	359.2 ±0.934^{c}	398.96±0.56^{a}
	48 h	368.19±1.11^{b}	358.34±0.96^{c}	383.75±0.315^{a}
	72 h	376.87±1.188^{a}	361±0.92^{c}	373.23±0.386^{b}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.6) revealed that between temperature and hours of exposure significant difference were present ($P<0.05$). Subsequent analysis by multiple comparison test (Table 4.26) using Tukey's test revealed that between 48 and 72 hours of exposure there was no significant difference. Between temperatures such as 16ºC, 27ºC and 38ºC significant difference ($P<0.05$) was present (Table 4.30).

Table 4.6 Two factor ANOVA for Total lipid

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	1028.988	3	342.996	478.139	0.000
Temperature	2313.873	2	1156.937	1612.780	0.000
Serum	0.000	0			
Hours * Temperature	2019.413	6	336.569	469.180	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	17.217	24	0.717		
Total	5379.490	35			

df-degrees of freedom

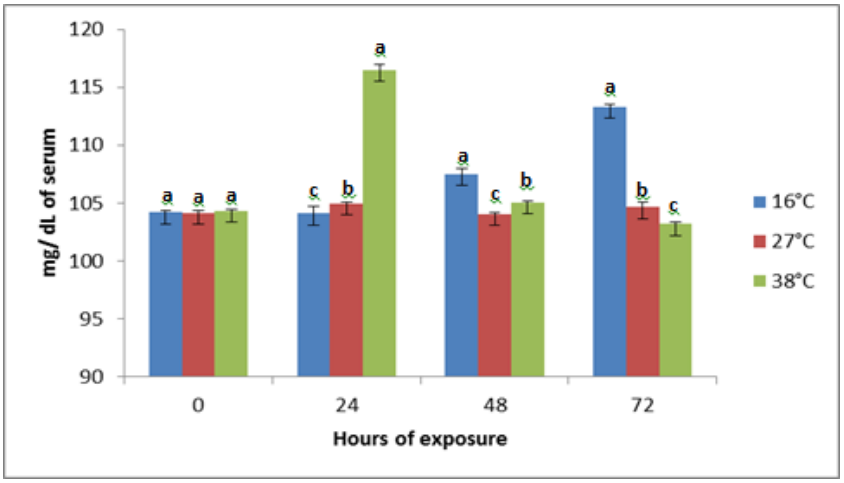


Figure 4.4. Effect of temperature stress on triacylglycerol. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.7 Effect of different temperature on triacylglycerol (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Triacylglycerol				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	104.21± 0.11^{a}	104.15± 0.21^{a}	104.32±0.163^{a}
	24 h	104.1±0.577^{c}	104.97 ±0.15^{b}	116.5±0.503^{a}
	48 h	107.5±0.5^{a}	104.06±0.1^{c}	105.06±0.115^{b}
	72 h	113.3±0.264^{a}	104.63±0.431^{b}	103.2±0.2^{c}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.8) revealed that between temperature and hours of exposure significant difference were present ($P<0.05$). Subsequent analysis by multiple comparison test (Table 4.26) using Tukey's test revealed that between hours of exposure there was significant difference present($P<0.05$) . Between temperatures such as 16ºC and 38ºC there was no significant difference (Table 4.30).

Table 4.8 Two Factor ANOVA for Triacylglycerol

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	88.806	3	29.602	341.560	0.000
Temperature	81.536	2	40.768	470.397	0.000
Serum	0.000	0			
Hours * Temperature	429.518	6	71.586	825.996	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	2.080	24	0.087		
Total	601.939	35			

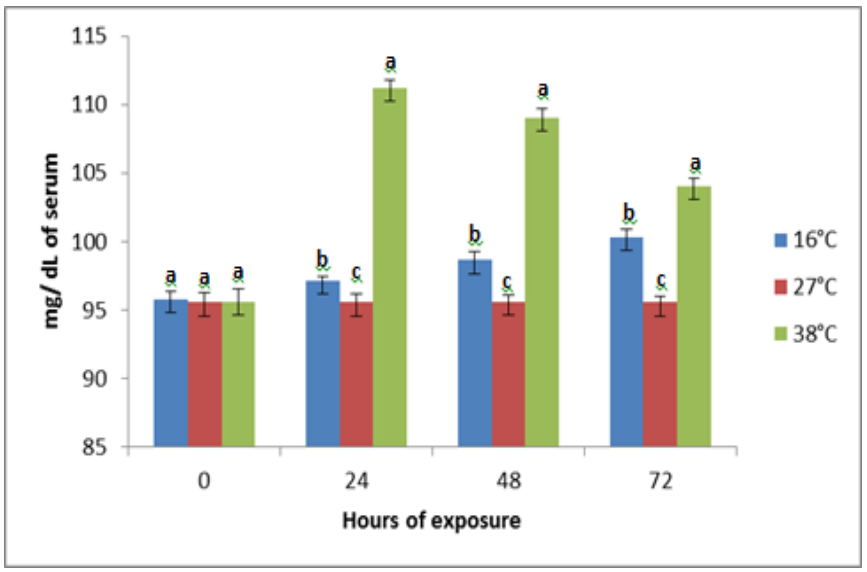


Figure 4.5 Effect of temperature stress on cholesterol. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.9 Effect of different temperature on cholesterol (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA).

Cholesterol				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	95.8± 0.513^{a}	95.56± 0.724^{a}	95.6±0.951a
	24 h	97.16±0.288^{b}	95.58 ±0.577^{c}	111.23±0.6^{a}
	48 h	98.66±0.5773^{b}	95.6±0.5^{c}	109.06±0.63^{a}
	72 h	100.33±0.59^{b}	95.56±0.43^{c}	104.06±0.54^{a}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.10) revealed that between temperature and hours of exposure significant difference was present ($P<0.05$). Subsequent analysis by multiple comparison tests (Table 4.26) using Tukey's test revealed that between 24 and 48 hours of exposure there was no significant difference. Between temperatures such as 16^{o}C, 27^{o}C and 38^{o}C significant difference ($P<0.05$) was present (Table 4.30).

Table 4.10 Two Factor ANOVA for Cholesterol

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	194.314	3	64.771	321.181	0.000
Temperature	575.909	2	287.954	1427.873	0.000
Serum	.000	0			
Hours * Temperature	278.896	6	46.483	230.492	0.000
Hours * serum	0.000	0			
Temperature * serum	0.000	0			
Hours * Temperature * serum	0.000	0			
Error	4.840	24	0.202		
Total	1053.959	35			

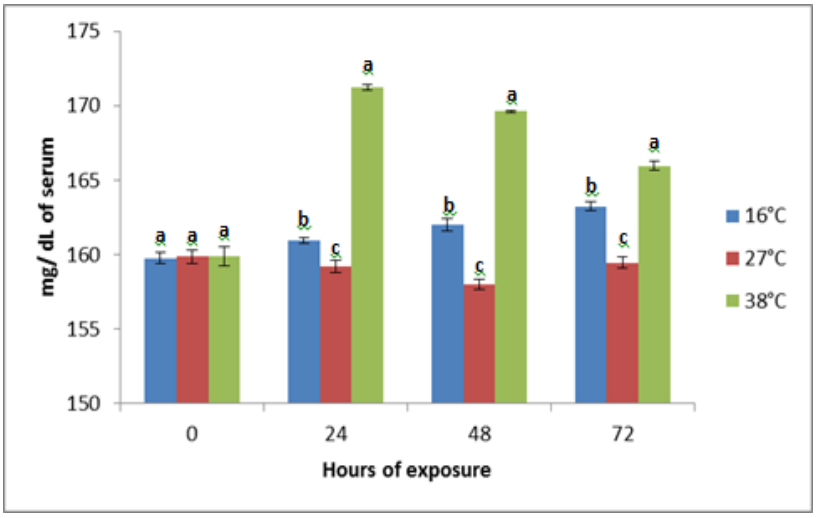


Figure 4.6 Effect of temperature stress on phospholipid. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.11 Effect of different temperature on phospholipid (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Phospholipid				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	159.76± 0.37^{a}	159.87± 0.452^{a}	159±0.9^{a}
	24 h	160.93±0.210^{b}	159.21 ±0.39^{c}	171.2±0.183^{a}
	48 h	162.02±0.421^{b}	158±0.36^{c}	169.61±0.08^{a}
	72 h	163.24±0.296^{b}	159.45±0.4^{c}	165.96±0.32^{a}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.12) revealed that between temperature and hours of exposure significant difference was present ($P<0.05$). Subsequent analysis by multiple comparison tests (Table 4.26) using Tukey's test revealed that between 24 and 48 hours of exposure there was no significant difference. Between temperatures such as 16^{o}C, 27^{o}C and 38^{o}C significant difference ($P<0.05$) was present (Table 4.30).

Table 4.12 Two Factor ANOVA for Phospholipid

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	103.611	3	34.537	320.794	0.000
Temperature	307.044	2	153.522	1425.974	' .000
Serum	0.000	0			
Hours * Temperature	148.675	6	24.779	230.158	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	2.584	24	0.108		
Total	561.914	35			

Values are expressed as mg/ dL of serum

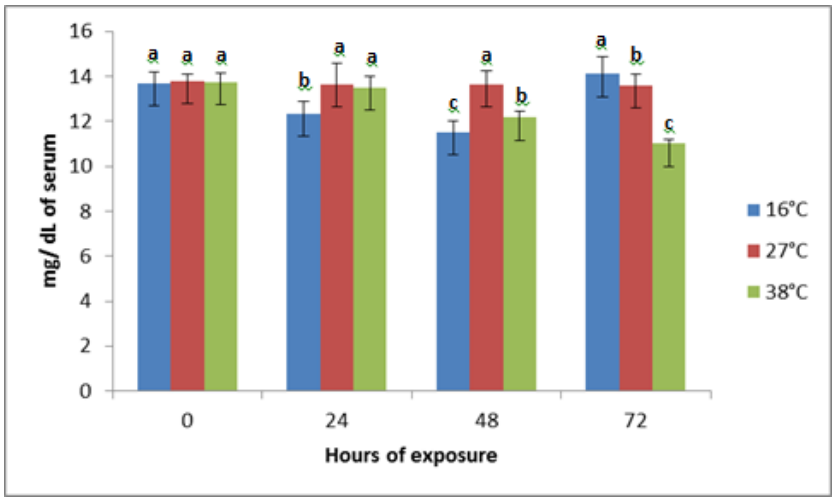


Figure 4.7 Effect of temperature stress on HDL. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One- way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.13 Effect of different temperature on HDL (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

HDL				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	13.673± 0.5^{a}	13.79± 0.288^{a}	13.73±0.428^{a}
	24 h	12.33±0.577^{a}	13.65 ±0.946^{b}	13.5±0.5^{b}
	48 h	11.5±0.5^{c}	13.62±0.621^{a}	12.16±0.288^{b}
	72 h	14.1±0.752^{a}	13.58±0.531^{b}	11±0.21^{c}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.14) revealed that between temperature and hours of exposure significant difference was present ($P<0.05$). Subsequent analysis by multiple comparison tests (Table 4.27) using Tukey's test revealed that between 24 and 48, 24 and 72, 48 and 72 hours of exposure there was no significant difference. Between temperatures such as 16^{o}C, 27^{o}C and 38^{o}C significant difference ($P<0.05$) was present (Table 4.31).

Table 4.14 Two Factor ANOVA for HDL

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	8.118	3	2.706	63.971	0.000
Temperature	16.312	2	8.156	192.803	0.000
Serum	.000	0			
Hours * Temperature	23.469	6	3.912	92.465	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	1.015	24	0.042		
Total	48.915	35			

Values are expressed as mg/ dL of serum

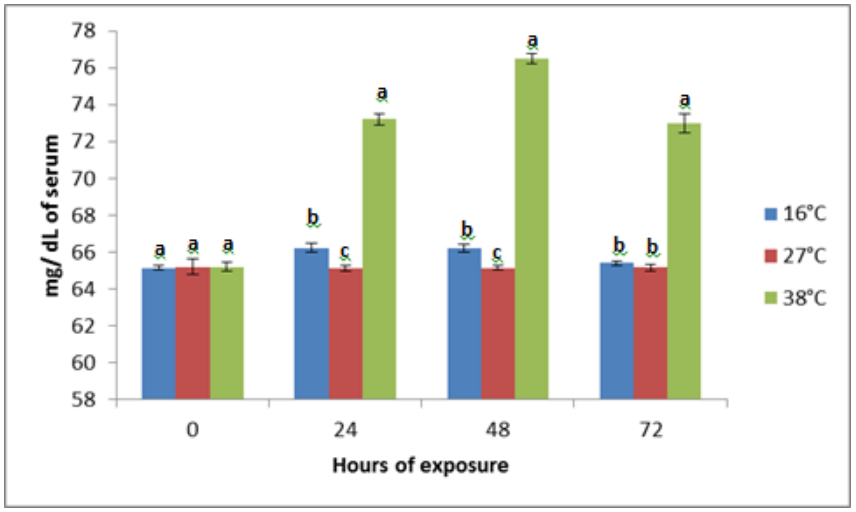


Figure 4.8 Effect of temperature stress on LDL. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.15 Effect of different temperature on LDL (mean ± SD, n=7) present in serum of *E.suratensis.* Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

LDL				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	65.13± 0.11^{a}	65.2± 0.432^{a}	65.18±0.241^{a}
	24 h	66.23±0.25^{b}	65.12±0.16^{c}	73.2±0.305^{a}
	48 h	66.2±0.2^{b}	65.13±0.12^{c}	76.5±0.288^{a}
	72 h	65.41±0.115^{b}	65.17±0.18^{b}	73±0.503^{a}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.16) revealed that between temperature and hours of exposure significant difference was present ($P<0.05$). Subsequent analysis by multiple comparison tests (Table 4.27) using Tukey's test revealed that between hours of exposure there was significant difference ($P<0.05$). Between temperatures such as 16ºC, 27ºC and 38ºC significant difference was present ($P<0.05$) (Table 4.31).

Table 4.16 Two Factor ANOVA for LDL

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	46.121	3	15.374	65.092	0.000
Temperature	100.805	2	50.403	213.404	0.000
Serum	0.000	0			
Hours * Temperature	72.086	6	12.014	50.868	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	5.668	24	0.236		
Total	224.680	35			

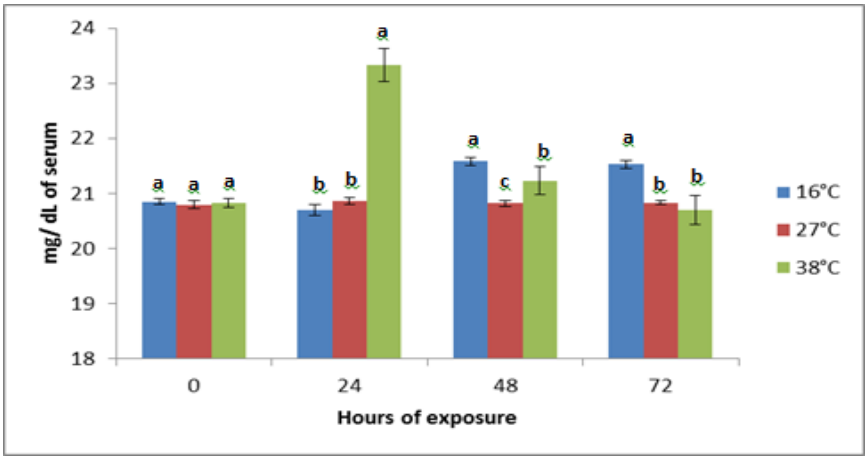


Figure 4.9 Effect of temperature stress on VLDL. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P <0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.17 Effect of different temperature on VLDL (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

VLDL				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	20.85± 0.05^{a}	20.79± 0.073^{a}	20.82±0..082^{a}
	24 h	20.7±0.1^{b}	20.864 ±0.06^{b}	23.33±0.3^{a}
	48 h	21.58±0.07^{a}	20.82±0.05^{c}	21.23±0.25^{b}
	72 h	21.53±0.07^{a}	20.832±0.04^{b}	20.7±0.26^{b}

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.18) revealed that between temperature and hours of exposure significant difference was present ($P<0.05$). Subsequent analysis by multiple comparison tests (Table 4.27) using Tukey's test revealed that between 24 and 72, 48 and 72 hours of exposure there was no significant difference. Between temperatures such as 16°C and 38°C there was no significant difference (Table 4.31).

Table 4.18 Two Factor ANOVA for VLDL

Source	Sum of Squares	df	Mean Square	F	Sig.
Hours	6.333	3	2.111	167.936	0.000
Temperature	19.613	2	9.807	780.188	0.000
Serum	0.000	0			
Hours * Temperature	33.156	6	5.526	439.636	0.000
Hours * Serum	0.000	0			
Temperature * Serum	0.000	0			
Hours * Temperature * Serum	0.000	0			
Error	.302	24	0.013		
Total	59.403	35			

Total protein profile was significantly affected by the time course of exposure and temperature investigated. Whereas, in the experimental group exposed to 16°C, protein level was 158.89 ± 0.109 mg/ dL of serum whereas at 38°C it was 163.21 ± 0.057 mg/ dL of serum during 24 h of exposure (Fig. 4.10). The maximum level of albumin was recorded during 24 h of exposure. And it was 118.28 ± 0.33 mg/dL of serum and

122.52 ± 0.511 mg/ dL of serum at 16ºC and 38ºC respectively during 24 h of exposure (Fig 4.11). Globulin level was maximum during the zeroth hour of exposure (Fig 4.12). Albumin: globulin ratio was also determined during the study (Table 4.33). During the course of study the total protein, albumin and globulin level remained steady in the case of group reared at ambient temperature and a profound change was observed in experimental groups selected for the study overtime.

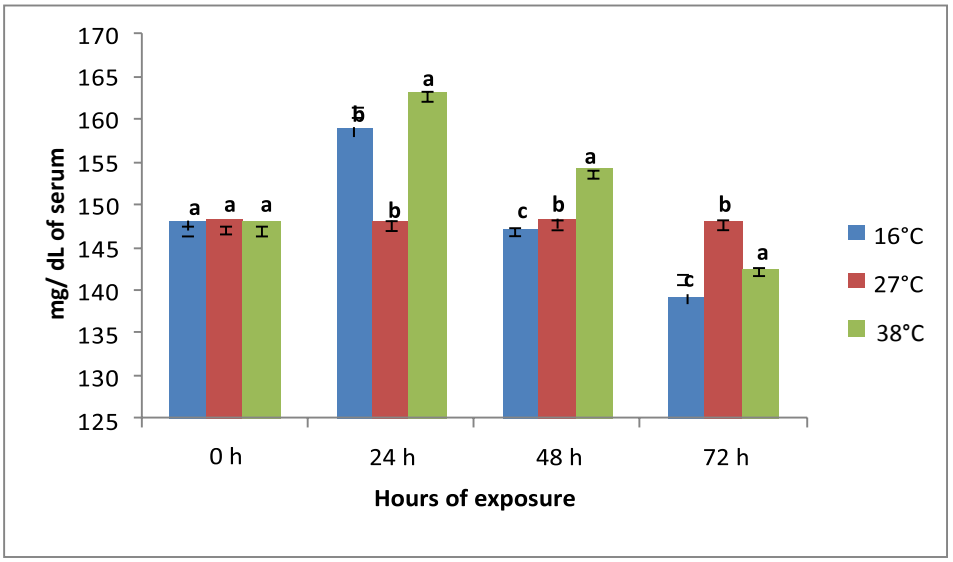


Figure 4.10 Effect of temperature stress on total protein. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.19 Effect of different temperature on total protein (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P <0.05) between treatment groups (One-way ANOVA)

Total Protein				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	148± 0.056[a]	148.17± .098[a]	148.1±0.076[a]
	24 h	158.89±0.1098[b]	148 ±0.045[c]	163.21±0.057[a]
	48 h	147.19±0.125[c]	148.23±0.062[b]	154.32±0.012[a]
	72 h	139.25±0.235[c]	148±0.056[a]	142.52±0.085[b]

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.20) revealed that between temperature and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests (Table 4.28) using Tukey's test revealed that between hours of exposure there was significant difference present (P<0.05) . Between temperatures such as 16ºC, 27ºC and 38ºC significant difference (P<0.05) was present (Table 4.32).

Table 4.20 Two Factor ANOVA for Total protein

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1395.449[a]	11	126.859	7317.617	0.000
Intercept	804330.954	1	804330.954	46396273.586	0.000
Hours	826.042	3	275.347	15882.879	0000
Temperature	116.586	2	58.293	3362.520	0.000
Serum	.000	0			
Hours * Temperature	452.821	6	75.470	4353.351	0.000
Hours * Serum	.000	0			
Temperature * Serum	.000	0			
Hours * Temperature * Serum	.000	0			
Error	.416	24	0.017		
Total	805726.819	36			

df= degrees of freedom

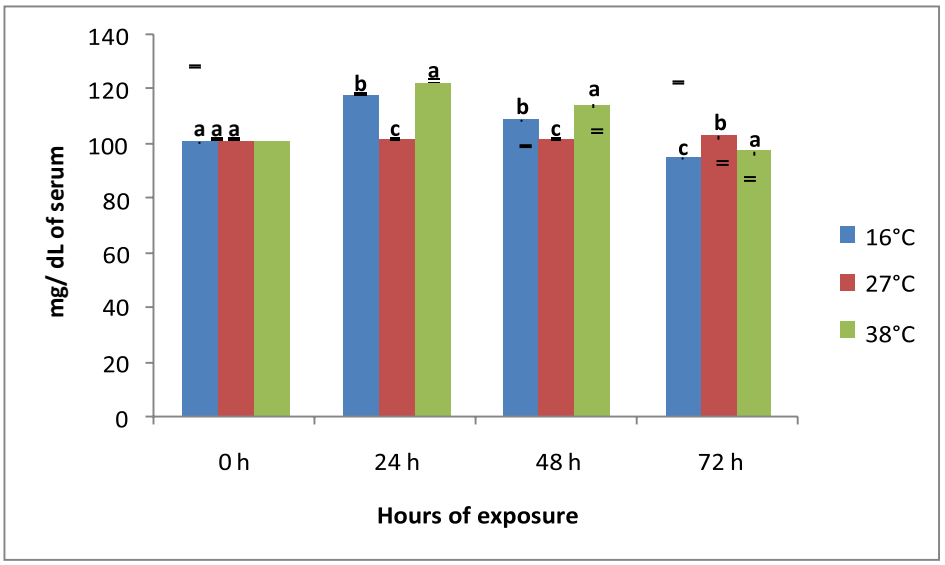


Figure 4.11 Effect of temperature stress on albumin. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different.

Table 4.21 Effect of different temperature on albumin (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Albumin				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	101.34± 0.015[a]	101.2± 0.024[a]	101±0.224[a]
	24 h	118.28±0.330[b]	101.53 ±0.068[c]	122.52±0.51[a]
	48 h	109.1±0.318[b]	102.13±0.024[c]	114.57±0.60[a]
	72 h	95.23±0.321[c]	103±0.023[a]	97.32±0.045[b]

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.22) revealed that between temperature and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests (Table 4.28) using Tukey's test revealed that between hours of exposure there was significant difference present (P<0.05). Between temperatures such as 16ºC, 27ºC and 38ºC significant difference (P<0.05) was present (Table 4.32).

Table 4.22 Two factor ANOVA for Albumin

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2417.382[a]	11	219.762	4491.559	0.000
Intercept	400826.162	1	400826.162	8192200.422	0.000
Hours	1369.671	3	456.557	9331.243	0.000
Temperature	285.347	2	142.674	2916.004	0.000
Serum	0.000	0			
Hours * Temperature	762.364	6	127.061	2596.902	0.000
Hours * serum	0.000	0			
Temperature * serum	0.000	0			
Hours * Temperature * serum	0.000	0			
Error	1.174	24	0.049		
Total	403244.718	36			

df= degrees of freedom

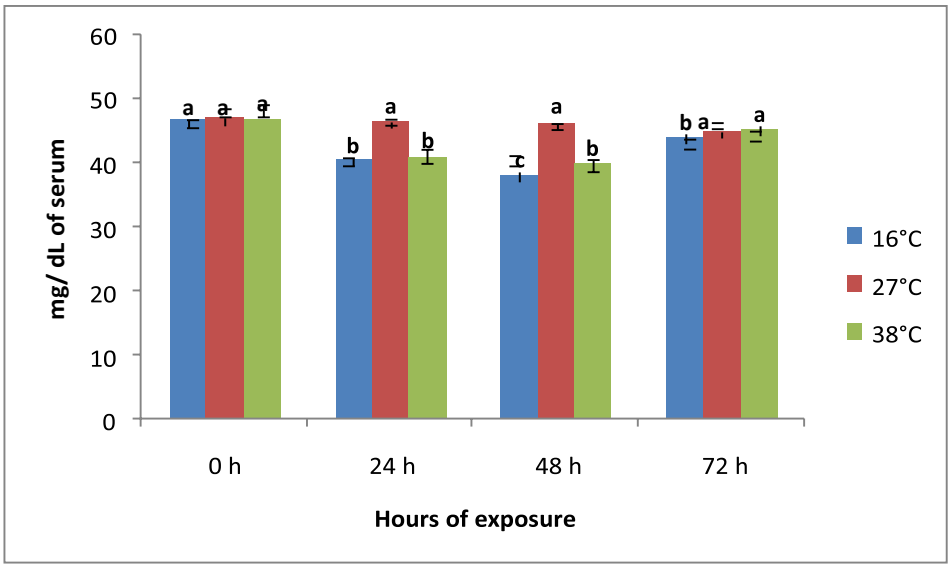


Figure 4.12 Effect of temperature stress on globulin. The vertical lines indicate means ± SD (n = 7). Bars represent different experimental temperature. On each set of bars values with different lower case letters vary significantly (P<0.05) in each temperature on different hours (One-way ANOVA). Same lower case letters on each set of bars are not significantly different

Table 4.23 Effect of different temperature on globulin (mean ± SD, n=7) present in serum of *E.suratensis*. Values of same row with different lower case letters vary significantly (P<0.05) between treatment groups (One-way ANOVA)

Globulin				
Serum	**Hours of exposure**	**Temperature**		
		16ºC	**27ºC**	**38ºC**
	0 h	46.66± 0.042[a]	46.97± 0.021[a]	46.8±0.832[a]
	24 h	40.61±0.194[b]	46.47 ±0.04[a]	40.69±0.99[b]
	48 h	38.09±0.421[c]	46.1±0.052[a]	39.75±0.73[b]
	72 h	44.02±0.631[b]	45±0.0621[a]	45.2±0.563[a]

Values are expressed as mg/ dL of serum

Two factor ANOVA (Table 4.24) revealed that between temperature and hours of exposure significant difference was present (P<0.05). Subsequent analysis by multiple comparison tests (Table 4.28) using Tukey's test revealed that between hours of exposure there was significant difference present (P<0.05). Between temperatures such as 16ºC, 27ºC and 38ºC significant difference (P<0.05) was present (Table 4.32).

Table 4.24 Two Factor ANOVA for Globulin

Source	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	343.556[a]	11	31.232	2451.200	0.000
Intercept	67666.750	1	67666.750	5310667.087	0.000
Hours	233.269	3	77.756	6102.522	0.000
Temperature	33.730	2	16.865	1323.620	0.000
Serum	.000	0			
Hours * Temperature	76.557	6	12.759	1001.399	0.000
Hours * serum	.000	0			
Temperature * serum	.000	0			
Hours * Temperature * serum	.000	0			
Error	.306	24	0.013		
Total	68010.612	36			
Corrected Total	343.862	35			

df = degrees of freedom

Table 4.25 Results of Multiple comparison using Tukey's test (Hours of exposure)

Groups	Glucose	Glycogen
0^{th} hour Vs 24^{th} hour	0.000[a]	0.000[a]
0^{th} hour Vs 48^{th} hour	0.000[a]	0.000[a]
0^{th} hour Vs 72^{th} hour	0.000[a]	0.000[a]
24^{th} hour Vs 48^{th} hour	0.000[a]	0.000[a]
24^{th} hour Vs 72^{th} hour	0.000[a]	0.000[a]
48^{th} hour Vs 72^{th} hour	0.000[a]	0.000[a]

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

Table 4.26 Results of Multiple comparison using Tukey's test (Hours of exposure)

Groups	Total lipid	TAG	Cholesterol	Phospholipid
0^{th} hour Vs 24^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 48^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
24^{th} hour Vs 48^{th} hour	0.000^{a}	0.000^{a}	0.722^{d}	0.719^{d}
24^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
48^{th} hour Vs 72^{th} hour	0.436^{d}	0.000^{a}	0.000^{a}	0.000^{a}

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

Table 4.27 Results of Multiple comparison using Tukey's test (Hours of exposure)

Groups	HDL	LDL	VLDL
0^{th} hour Vs 24^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 48^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
24^{th} hour Vs 48^{th} hour	0.024^{d}	0.000^{a}	0.000^{a}
24^{th} hour Vs 72^{th} hour	0.008^{d}	0.000^{a}	0.005^{d}
48^{th} hour Vs 72^{th} hour	0.970^{d}	0.000^{a}	0.214^{d}

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

Table 4.28 Results of Multiple comparison using Tukey's test (Hours of exposure)

Groups	Total protein	Albumin	Globulin
0^{th} hour Vs 24^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 48^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
0^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000a
24^{th} hour Vs 48^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
24^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}
48^{th} hour Vs 72^{th} hour	0.000^{a}	0.000^{a}	0.000^{a}

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

Table 4.29 Results of Multiple comparison using Tukey's test (Temperature)

Groups	Glucose	Glycogen
16°C Vs 27°C	0.000^{a}	0.000^{a}
16°C Vs 38°C	0.000^{a}	0.000^{a}
27°C Vs 38°C	0.000^{a}	0.000^{a}

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

Table 4.30 Results of Multiple comparison using Tukey's test (Temperature)

Groups	Total lipid	TAG	Cholesterol	Phospholipid
16°C Vs 27°C	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
16°C Vs 38°C	0.000^{a}	0.770^{d}	0.000^{a}	0.000^{a}
27°C Vs 38°C	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

Table 4.31 Results of Multiple comparison using Tukey's test (Temperature)

Groups	HDL	LDL	V LDL
16°C Vs 27°C	0.000[a]	0.000[a]	0.000[a]
16°C Vs 38°C	0.000[a]	0.000[a]	0.176[d]
27°C Vs 38°C	0.000[a]	0.000[a]	0.000[a]

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

Table 4.32 Results of Multiple comparison using Tukey's test (Temperature)

Groups	Total Protein	Albumin	Globulin
16°C Vs 27°C	0.113[d]	0.000[a]	0.000[a]
16°C Vs 38°C	0.000[a]	0.000[a]	0.000[a]
27°C Vs 38°C	0.000[a]	0.000[a]	0.000[a]

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

Table 4.33 Albumin: Globulin Ratio (A: G)

	Hours of exposure	Temperature		
Serum		16°C	27°C	38°C
	0 h	2.171	2.154	2.144
	24 h	2.912	2.508	3.011
	48 h	2.862	2.215	2.882
	72 h	2.163	2.288	2.153

4.6 Discussion

The changes in the environmental temperature induce certain alterations in a series of behavioral and physiological responses in an ectothermic organism so as to re-establish homeostasis (Foss *et al*., 2012). Fishes enter into metabolic depression in response to various water temperatures to alleviate the effects of temperature on metabolism (Andrea *et al.,* 2017). This has performance consequences at whole organism level (Alzaid *et al*., 2015; Costa *et al.,* 2013). In the present study fishes exposed to 16°C and 38ºC showed reduced food intake and became quiescent. The major energy source for most of the activities is glucose. Glycogen is an important energy reserve that can be rapidly mobilized during temperature stress, and in the stressed condition its catabolism and plasmatic glucose levels are closely related (Ma *et al.,* 2015). In this study with respect to controls the level of blood glucose elevated, whereas the levels of liver glycogen and muscle glycogen declined on the 24th hour of exposure. The elevation in blood glucose level and the simultaneous decrease in the levels of liver and muscle glycogen indicated the high energy demand associated with imposed temperature stress (Andrea *et al.,* 2017). Under stressed condition, the glycogenolytic pathway gets stimulated so as to meet the need for energy by mobilizing the stored carbohydrate reserve. In the current study, during the 24th hour of temperature stress increased glycemia which reflects the conversion of glycogen into glucose due to glycogenolysis for energy use. Decreasing plasma glucose levels with water temperature stress have been reported (Luis *et al*., 2008) and their results are in accordance with the present findings. Umminger (1969) observed that cold-induced

hyperglycemia may serve as a mechanism for supplying substrate for energy demands (i.e.; ATP production) or to increase the activity of metabolic pathways required for cold acclimation. Kindle and Whitmore (1986) reported that osmoregulatory mechanisms were often impaired during temperature stress; high glucose level may also help to maintain serum osmolarity in freshwater fish. Nakano and Tomlinson (1967), Larsson (1973), Dalela *et al.* (1981) and Asztalos *et al*. (1990) reported that adrenal hormones, like glucocorticoids and catecholamines may be induced during stress, which in turn elevated the blood glucose level by conversion of stored glycogen into blood glucose. The hyperglycemia observed during the 24th hour of temperature stress in *E. suratensis* indicated that glucose was used to supply energy for fish to cope with the thermal condition. All these results demonstrated that environmental temperature affected blood glucose levels. In the present study, hepatic glycogen and muscle glycogen levels were significantly declined over time at 16^{o}C and 38^{o}C. Similar results in liver glycogen were observed by Lermen *et al*. (2004) in *Ramdia quelen* exposed for 21 days at 15^{o}C and 31°C. This indicated that fish used glycogen to provide energy when they were exposed to low or high temperatures (Lermen *et al*., 2004; Chatzifotis *et al.,* 2010; Viant, 2003). The glycogen content was high in liver compared to muscle. This may be due to aerobic catabolism of glucose with the significant cooperation of liver. The declined levels of glycogen during temperature stress may also be a result of acclimatization to water temperatures (Werner *et al.,* 2006). Liver glycogen is mainly concerned with storage and export of hexose units for maintenance of blood glucose level. The function of muscle glycogen is to act as a readily

available source of hexose units for glycolysis within the muscle itself (Harper, 1985). The lower glycogen content was observed in muscle because it lacked the inherent potential to store glycogen compared to liver and is dependent on blood glucose for all its activities (Lehninger, 1983). A fall in glycogen level clearly indicated its rapid utilization to meet the enhanced energy demands during temperature stress through glycolysis or hexose monophosphate pathway (Cappon and Nicholas, 1975). The decrease in glycogen content may also be due to inhibition of the enzyme glycogen synthase or hormones which mediated glycogen synthesis (Stamp and Lesker, 1967; Edwards, 1973). Glycogen depletion is more prevalent under hypoxic conditions (Dezwaan and Zandee, 1972) and a situation similar to hypoxia may occur in temperature stress. It was found that increased phosphorylase activity may stimulate reduction in the glycogen content. Koundinya (1979) reported that stepped up glycogenolysis lead to a decrease in glycogen content. Davis (2004) reported that carbohydrate metabolism was disturbed when sunshine bass was exposed to various temperature. The decreased glycogen level was also attributed to the conversion of carbohydrates into amino acids (Gaiton *et al.,* 1965). So the organism may try to adapt with the altered situations by metabolic compensation which involved breakdown and synthesis of products.

The present investigation showed depletion in the total lipid content at 38ºC and a rapid increase was observed at 16ºC. The reduction in total lipid content may be due to increased metabolic rate which resulted in the increased energy demand. This causes the energy reservoir; lipid to be oxidized more rapidly to meet the higher energy needs. The reduction of

each lipid component may be due to reduced oxygen solubility at elevated temperatures which may retard the activity of the oxygen-dependent fatty acid desaturases (Brown and Rose, 1969). Elevation in plasma lipids can be due to alteration in their metabolism or impaired clearance. This hyperlipidemia can lead to liver dysfunction (Lu *et al.*, 2010; Javed and Usmani, 2015). Elevated triglyceride levels in plasma were observed in fish exposed to 16°C. This could indicate that fish accumulated triglycerides to cope with cold temperature. Gracey *et al*. (2004) have studied transcriptional responses in common carp subjected to a progressive cooling regime. They reported that there was an enhancement of gene expression of enzymes and proteins, involved in lipid biosynthetic processes. Carine *et al*. (2004) reported that triacyl glycerol content was high in serum at 31^{o}C compared to 15^{o}C in silver catfish which was in accordance with the present findings. Warm-acclimatized fish had significantly lower amounts of total lipid content than the cold-acclimatized animal which was different from the present findings (Norman, 1990). The correlation between the effect of environmental temperature and the utilization of fatty acids and source of metabolic energy between cold water species was demonstrated by various investigators (Oliveira *et al*., 2003; Nargis, 2006). Rao (1996) reported that in cold acclimatized rainbow trout there was an increase in the lipid content by mobilization of saturated triglycerides. He observed that cold acclimation leads to an increase in phospholipids, ketone bodies, cholesterol and lipase activity. This may be the probable reason for the elevation of lipid components during 16^{o}C up to the 72th hour of temperature stress. Triglyceride-rich lipoproteins (HDL, LDL and VLDL)

are believed to be the components of the innate, non-adaptive immune defense system, thus their decline leads to immune suppression. In lipid particularly phospholipid is the important structural component of cell membrane, subsequent alteration in lipid profile will lead to changes in the membrane system. Elevation in lipid profile is either due to a disturbance in the metabolism of lipids or may be due to impaired clearance from plasma which favors liver dysfunction. After glucose and glycogen source, it is the lipid molecules which are utilized to overcome the stress situation.

Proteins are the building blocks of the animal's body, and it is most fundamental biochemical substance to maintain the blood glucose and energy source during the stress period. Proteins play a major role in the interaction process of the cellular medium in the organisms. It is also considered as a diagnostic tool and involved in various phases of physiological events. Proteins perform a vast array of functions within living organisms including catalyzing the metabolic reaction. The level of total protein and albumin were decreased with progress of stress in the present study. Lermen *et al.* (2004) reported that protein level decreased at 31^{o}C for 21 days of exposure in silver cat fish. Low temperatures resulted in significant decreases in the levels of the different plasma protein fractions on tench and gilthead sea bream (Collazos *et al.*, 1993; Sala - Rabanal *et al.*, 2003). Similar to the present study, Xia and Li (2010) reported decrease in protein content in Salamander *Batrachupems tibetanus* when exposed to different temperatures. The protein level in the serum depends upon the equilibrium between the rate of synthesis and degradation. Anitha *et al*. (1999) reported a significant decrease in protein

content in *C.catla* when exposed to a pesticide. Reddy (1987) suggested an impairment of protein synthesis induced by stress is the reason for the quantitative reduction of protein. In support to the findings, Javed and Usmani (2015) reported a reduction in total protein, albumin levels in fish. Shingadia *et al.* (2006) reported that stress had the ability to dissociate the 80S ribosomes and cause damage to polyribosomes formation; finally it leading to inhibition of the initiation of the polypeptide synthesis. As a result altered and declined protein synthesis was observed. Decrease in protein content was not only due to impairment of protein synthesis but also due to intracellular leakage of proteins in the blood on account of cellular necrosis. The drastic reduction in the protein content suggests that the gluconeogenetic pathway has been initiated to supplement depletion of sugars by breaking down of protein to yield sugars. A decrease in the protein content may be due to the metabolic utilization of the ketoacids to gluconeogenesis pathway for the synthesis of glucose, or due to directing the free aminoacids for the synthesis of proteins, or for the maintenance of osmotic and ionic regulation (Schmidt, 1975). It could also be due to the production of heat shock proteins or destructive free radicals or could be a part of temperature stress induced apoptosis. Kumar and Gopal (2001) reported that decline in protein content might be due to degradation of proteins into free amino acids for various metabolic activities. There are other possible reasons for the reduction in protein content under stress condition such as increased proteolysis, lipoprotein formation in the repairing of cell damages and increased energy requirements in the cells. Baruah *et al*. (2004) reported that decline in protein content was due to

catabolism to meet the energy demand in the extremely stressful environment.

The present results demonstrated that in *E. suratensis* metabolic responses were distinct with respect to temperature stress. It was found that heat shock was more stressful to *E. suratensis* than cold shock. The use of different metabolic parameters as indicators of stress in *E. suratensis* was a good tool to evaluate long-term changes. The change in carbohydrate, protein and lipid metabolism seen in the animals exposed to thermal stress as compared to the control animals may be a compensatory mechanism for better survival of the fish. But the organism failed to survive under severely stressed condition because of the sustained thermal stress.

Chapter 5

Molecular Characterization of HSP 70 gene in *Etroplus suratensis* and its Expression Profile during Thermal Stress

Contents

5.1 Introduction

Heat Shock Proteins (Hsps) are a family of highly conserved cellular proteins present in every organism including fish. They are a family of proteins expressed in response to a wide range of biotic and abiotic stressors and hence also referred as 'stress proteins'. They play a pivotal role in protein homeostasis and stress response within the cell (Feder and Hofmann, 1999; Iwama *et al.*, 2004; Mao *et al*., 2005; Multhoff, 2007; Keller *et al.*, 2008). These proteins have pleiotropic effects interacting with multiple systems in diverse ways regulated by the endocrine system. Hsps are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, fidelity and translocation. Hsps trigger immune response through activities that

occur both inside the cell (intracellular) and outside the cell (extracellular). The expression of Hsp in fish has been described in cell lines, primary cultures of various cells, and in the tissues of whole organisms. The expression of Hsp is transcriptionally regulated primarily by heat shock factor (HSF) (Wu, 1995).

5.2 Review of Literature

Hsp proteins belongs to several different protein families based on their molecular weight such as Hsp90 (85-90 kDa), Hsp70 (68-73 kDa), Hsp60, Hsp47, and small Hsps (12- 43 kDa) (Park *et al.,* 2007; Hallare *et al.,* 2004). Hsps of high molecular masses (e.g. 60, 70, 90 kD) are more conserved between different taxa of organisms, compared with those with low molecular masses (e.g. 16±30 kDa).The latter may be more species-specific, and as such may offer reasonable potential for diagnostic purposes among individual species, whereas the former may be used as indicators of non-specific stressors in a wide range of organisms (Iwama *et al.,* 1998). Hsps levels remain elevated in the organism after the stressor is removed. This is an indication of long term adaptation and increased stress tolerance (Morimoto and Santoro, 1988). The heat shock response is an evolutionarily conserved mechanism for maintaining cellular homeostasis following sublethal noxious stimuli (Lindquist, 1986; Lindquist and Craig, 1988). Several Hsps act as molecular chaperones which mediate the assembly and localization of intracellular and secreted polypeptides and oligomeric proteins. The importance of Hsps in the protein folding pathway is revealed by the overexpression of a number of heat shock genes during normal cell growth. Studies with actinomycin D which is a transcription blockers

suggest that the transcription steps of protein synthesis play a major role in regulating the cellular Hsp response to various stressors (Heikkila *et al.*, 1982; Currie and Tufts, 1997).

Fish is an excellent vertebrate model to investigate the physiology, function and regulation of Hsps, as they are exposed to thermal and several other stressors in their natural environment. Hsp level may be increased in tissues of fish exposed to elevated temperatures, industrial effluents, polycyclic aromatic hydrocarbons (Vijayan *et al*.,1998), several metals such as copper, zinc and mercury (Sanders, 1993; Williams *et al*., 1996) or pesticides (Hassanein *et al.,* 1999). Oxygen radicals, toxicants, and inflammatory stress enhance the synthesis of Hsps and often give rise to an accumulation of denatured and aberrantly folded proteins within the cell. The actions of Hsps affect various aspects of fish physiology, including development and aging, stress tolerance and acclimatisation (Basu *et al.*, 2003). Hsps have been proposed as biomarkers for toxicity associated with physical and chemical stressors (Sanders, 1993; Ryan and Hightower 1994; Ovelgonne *et al*., 1995) since the expression of their genes may be activated by various stressors (Airaksinen *et al.*, 2003). The Hsp response can vary according to tissue (Smith *et al.*, 1999; Rabergh *et al.*, 2000), distinct Hsp families (Smith *et al*., 1999) and stressors (Airaksinen *et al*., 2003; Iwama *et al.*, 1998). The small heat shock protein (sHsp) family plays an important role in cell homeostasis, injury responses and disease. Zebrafish Hsp27 (zfHsp27) was expressed in mammalian fibroblasts which was phosphorylated in response to heat stress and anisomycin. It contains three conserved phosphorylated serines and a cysteine important for regulation of apoptosis.

It lacks much of a C-terminal tail domain and shows low homology in two putative actin interacting domains that are features of mammalian Hsp27. Expression of zfHsp27 and human Hsp27 in mammalian fibroblasts promotes a similar degree of tolerance to heat stress. zfHsp27 proteins enter the nucleus and associate with the cytoskeleton of heat stressed cells in vitro and in zebra fish embryos (Mao *et al.*, 2005).

The naming of Hsps is generally based on their molecular mass (kilodaltons, kDa) as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE). The commonly used categories are: 100 kDa; 90 kDa; 70 kDa; 60 kDa; and the 16 - 30 kDa group, and are usually referred to as Hsp100, Hsp90, Hsp70, Hsp60, and the low molecular weight (LMW) class of proteins, respectively (Morimoto *et al.*, 1994). Among the stress proteins, Hsp 70 which comprises both the constitutive and inducible isoforms is the most widely studied group (Ekambaram, 2010) and is highly conserved from bacteria to mammals (Schlesinger, 1990). Hsp70 family of proteins, are the most temperature sensitive and highly conserved of the Hsps. The Hsp70s are ATP binding proteins and demonstrate a 60–80% base identity among eukaryotic cells. Hsp70 is found to assist the folding of nascent polypeptide chains, acts as a molecular chaperone, and mediates the repair and degradation of altered or denatured proteins (Kiang and Tsokos, 1998). There are atleast four distinct proteins in the Hsp70 group (Hsp72, Hsp73, Hsp75, and Hsp78), and all of these proteins have several acronyms that can be redundant and confusing. Proteins in the Hsp70 group share common protein sequences but are synthesized in response to different stimuli (Kregel, 2002). Hsp70 was associated with the development of

tolerance to a variety of stresses, including hypoxia, ischemia, acidosis, energy depletion, cytokines such as tumor necrosis factor (TNF), and ultraviolet radiation. The phenomenon of acquired thermo tolerance is transient in nature and depends primarily on the severity of the initial heat stress. In general, the greater the initial heat dose, the greater the magnitude and duration of thermo tolerance (Kregel, 2002). The expression of thermo tolerance following heating will occur within several hours and last 3–5 days in duration. The variation of HSPs expression with respect to seasonal acclimatization and laboratory acclimation in fishes and invertebrates had been studied by various workers (Dietz and Somero1992; Dietz 1994; Roberts *et al*., 1997; Buckley *et al*., 2001; Tomanek and Somero 2002; Selvakumar and Geraldine 2005).

Table 5.1 Cellular locations and proposed functions of heat shock protein families (Kregel, 2002)

Heat Shock Proteins Family	**Cellular Location**	**Proposed function**
Hsp 27 (sHsp)	Mitochondria	Refolds proteins and prevent aggregation of denatured proteins, proapoptotic
Hsp 70 family:		Antiapoptotic
Hsp 72	Cytosol, Nucleus	Protein folding, cytoprotection
Hsp 73	Cytosol, Nucleus	Molecular chaperons
Hsp 75	Mitochondria	Molecular chaperons
Hsp 78	Endoplasmic reticulum	Cytoprotection, molecular chaperons
Hsp 90	Cytosol, Endoplasmic reticulum, Nucleus	Regulation of steroid hormone receptors, protein translocation
Hsp 110/104	Cytosol	Protein Folding

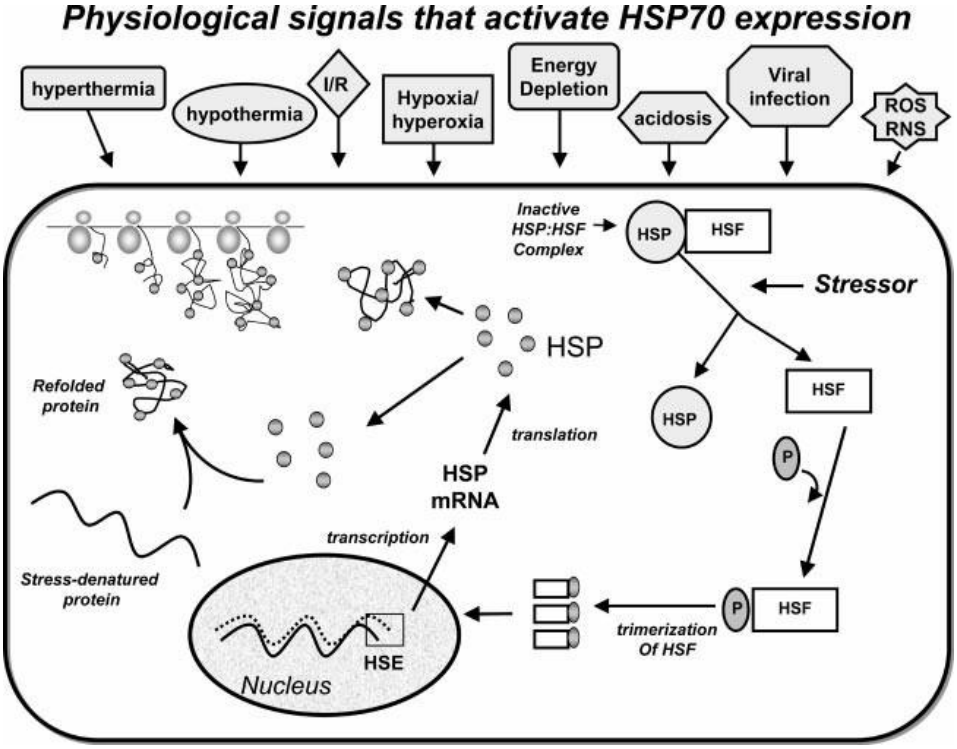


Figure 5.1. A summary of some of the major physiological signals that activate the inducible form of the 70-kDa heat shock protein (Hsp70) synthesis and a proposed mechanism for increased Hsp70 expression within a cell (Kregel, 2002).

Heat shock factors (HSFs), present in the cytosol, are bound by heat shock proteins (Hsps) and maintained in an inactive state. A broad array of physiological stimuli ("stressors") are thought to activate HSFs, causing them to separate from Hsps. HSFs are phosphorylated by protein kinases and form trimers in the cytosol. These HSF trimer complexes enter the nucleus and bind to heat shock elements (HSE) in the promoter region of the Hsp70 gene. Hsp70 mRNA is then transcribed and leaves the nucleus for the cytosol, where new Hsp70 is synthesized. Proposed mechanisms of cellular protection for Hsps include their functioning as

molecular chaperones to assist in the assembly and translocation of newly synthesized proteins within the cell and the repair and refolding of damaged proteins.

Koban *et al.* (1991) reported the induction of Hsps in the gill, liver, heart, erythrocytes, skeletal muscle and brain of mummichog (*Fundulus heteroclitus, Cyprinodontidae*) that were exposed to elevated temperatures. Dyer *et al*. (1991) studied Hsp induction in the gill, striated muscle and brain of the fathead minnow when they are subjected to heat shock. The *O. latipes* cell line expressed three Hsp 70 proteins. One of the Hsp70 proteins was barely expressed, but was highly inducible with heat, whereas the other two were expressed constitutively and showed only slight induction with heat. Luft *et al.* (1996) identified and characterized an Hsp70 member in the channel cat fish.

In fishes and other aquatic organisms, Hsps have many possible applications. The stressors cause the induction of specific proteins which offers the possibility of developing diagnostic probes for biomonitoring the condition of fish and their environment through the detection of stressed states in fish. The increased levels of Hsps induce tolerance of cells, tissues, and whole fish to subsequent stressors. This suggests that it may be possible to develop strategies to enhance tolerance to stressors by inducing the cellular stress response. In this chapter emphasize was given for the molecular characterization of Hsp 70 gene of *E. suratensis* and evaluated its expression pattern in various tissues during temperature stress.

5.3 Objectives of the Study

1) To investigate the effect of thermal stress on the tropical culture fish *E. suratensis* by molecular characterization of Hsp 70

2) An attempt is made to trace the pattern of variation in Hsp 70 in various tissue during temperature stress in *E. suratensis*.

3) To evaluate the extent of thermal stress in *E.suratensis* by expression profile of Hsp 70

4) To understand the salient features of Hsp 70 of *E. suratensis* during the thermal stress.

5.4 Materials and methods

Fish collection, maintenance, acclimatization, determination of Critical Thermal Maximum (CT MAX), Critical Thermal Minimum (CT MIN) and experimental design were same as explained in detail in chapter 2

Identification of HSP 70 by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Preparation of tissue samples

At 0^{th} and every 24^{th} hour of temperature treatment, seven fishes were randomly selected from each group and were sacrificed after placing them in a sedative over dose solution (clove oil, 1 mL/ L) for five minutes. The animal was dissected and tissues such as gills, liver, muscle and brain were collected and were wiped thoroughly using blotting paper to remove blood and other body fluids. Then they are washed in ice cold 0.33 M sucrose solution and again blotted dry. The tissues were

homogenized in a homogenizer (KEMI Model No. KHH1) in an ice cold Tris – HCl buffer (0.1 M, pH 7.5) to get 5% homogenate and supernatant fractions were collected after centrifugation at 11,200 x g for 15 minutes at 4^{o}C and is used for SDS PAGE.

20 µl of tissue homogenate was boiled in 10 µl of sample buffer (0.5 M Tris buffer of pH 6.8, glycerol, 10% SDS, 2- mercaptoethanol, 1% bromophenol blue) for 10 minutes. The samples were given a short spin and the supernatant was subjected to 10% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Electrophoresis was performed at 60 V in stacking gel and 120 V in resolving gel (BioRad, USA). After electrophoresis, gel was stained in coomasie brilliant blue stain R 250 (0.1 % coomasie brilliant blue R-250, 40% methanol and 10% acetic acid in distilled water), de-stained in de- staining solution (10% methanol and 10% acetic acid in distilled water) and photographed using Gel-DOCTM XR+ imaging system (BioRad, USA).Molecular weight of protein was determined by comparing with standards (SRL high range 1 protein marker, 20 – 220kDa).

Western Blotting

Sample preparation

Tissues were placed in ice and washed it with Tris Buffered Saline (TBS). Aspirated the TBS, then added the ice cold RIPA Buffer (Radioimmunoprecipitation assay Buffer) (1-5 mg/mL). Homogenate and gently transferred it into a pre-cooled micro centrifuge tube. Constant agitation was maintained for 30 minutes at 4^{o}C. It was sonicated three times for 10-15 seconds to lyse completely and shear DNA to reduce

sample viscosity (if, necessary). It was spun at 16000 x g for 20 minutes in 4ºC. The supernatant was transferred to a fresh tube, precooled on ice and discarded the pellet. A small volume (10-20 µl) of lysates was used to perform a protein assay. Protein concentration was determined for each cell lysate. Twenty microgram of each sample was taken and added an equal volume of 20X Laemmli sample buffer. Each cell lysate in sample buffer was boiled at 95ºC for 5 minutes. It was then centrifuged at 16000 x g in micro centrifuge for 1 minute.

Protein separation by gel electrophoresis

Equal amounts of protein (20 µg) were loaded into the wells of SDS PAGE gel, (BOLT BISTRIS 4-12%) along with molecular weight markers. The gel was run for 5 minutes at 50V.Voltage was increased to 100-150V to finish the run about in one hour

Transferring the protein from the gel into the membrane

The proteins were immunoblotted using iblot2 dry system (BOLT BISTRIS PLUS 4-12%; SKU No: NW04120BOX) as per manufacturer's instruction (Invitrogen, USA). The run was performed at 25V for 15 minutes and the nitrocellulose membrane was used for further development.

Antibody incubation

The blot was rinsed briefly in water and stained it with Ponceau solution to check the transfer quality. Ponceau solution stain was rinsed off with three washes with TBST (Tris buffered saline + tween 20). It was

then blocked in 3% BSA in TBST at room temperature for 1 hour. It was incubated overnight in the primary antibody solution (Hsp70, BioLegend) against the target protein. The blot was rinsed 3-5 times for 5 minutes with TBST. It was then incubated in the HRP (horseradish peroxide) conjugated secondary antibody (rabbit) solution (Abcam) for 1 hour at room temperature. The blot was rinsed 3-5 times for 5 minutes each with TBST. H_2O_2DAB (3,3' Diaminobenzidine) solution was applied and incubated for 30 minutes.

Imaging and data analysis

The bands formed were captured using a standard gel documentation system (E gel imager, Life technologies, USA)

RNA Extraction

The water and other required materials (including glass wares, homogenizers, gloves, scissors and forceps), used for RNA isolation were made free of RNase by treating with 0.1% Diethyl pyrocarbonate (DEPC). For the purpose the required materials and solutions were incubated with 0.1% DEPC overnight followed by autoclaving at 15 lbs pressure for 1 hour. Autoclaving for an hour ensures complete evaporation of DEPC as ethanol and CO_2 leaving behind RNase free utensils and water.

Total RNA Extraction

Total RNA was extracted from the tissues using TRI® Reagent (Sigma), following the manufacturer's instructions. Briefly, one mg of

tissue was homogenized with TRI Reagent (1 mL) in a tissue homogenizer. The homogenate was transferred to 1.5 mL micro centrifuge tubes (MCTs) and allowed to stand for 5 minutes at room temperature. To the homogenate was added 0.2 mL chloroform, shaken vigorously for 15 seconds, allowed to stand at room temperature for 15 minutes and centrifuged at 12,000 x g for 15 minutes at 4^{o}C. Centrifugation separated the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was transferred to a fresh MCT and 0.5 mL isopropanol was added. The samples were allowed to stand for 5-10 minutes at 4^{o}C and then centrifuged at 12000 x g for 10 minutes at 4^{o}C. The RNA pellet obtained was washed by adding 1 mL 75% ice cold ethanol. The samples were vortexed and centrifuged at 7500 x g for 5 min at 4^{o}C. RNA pellets were dried for 5-10 minutes and dissolved in RNase free water by repeated pipetting with a micropipette at 55-60^{o}C for 10-15 minutes.

Determining Quality and Quantity of RNA

The purity and quality of the RNA was visualized using electrophoresis on 0.8% agarose gel. RNA was quantified and qualified by measuring optical density (O.D) at 260 and 280 nm in a UV Spectrophotometer (U-2900, Hitachi). The ratio of absorbance at 260 to 280 nm is an indication of RNA quality. Only RNAs with absorbance ratio (A260: A280) $\geq$1.8 were used for further processing.

Amplification of near full length HSP 70 gene and bioinformatics analysis

Both the cDNA synthesis and PCR were carried out in a single tube in single step using PrimeScript OneStep RT-PCR Kit Ver.2 (TaKaRa) following the manufacturer's protocol. As the Hsp 70 gene ORF is approximately 1954 bp long, it was amplified as two fragments of 1124 and 1036 bp each at 5' and 3' ends of the entire gene. A combination of primers, Hsp 70NF/R1 and Hsp70F1/NR designed for *Etroplus suratensis* Hsp70 gene (Es_HSP 70) based on the conserved sequences of Hsp in teleosts was used for this purpose. PCR products were analyzed by electrophoresis in 1.5% agarose gel.

Primers for Hsp70 (based on *Oreochromis niloticus*, G9M615)

Hsp 70 NF CATGTCAAAGGGACCAGCAGTTG

Hsp 70 NR TTAGTCGACCTCCTCAATGGTTGG

Hsp 70 F1 TGCTGACCTCTTCCGTGGCAC

Hso70 R1 AGCAGCTCCATAGGCCACAGC

The PCR amplification of Hsp 70 genes with specific primers were performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 mM dNTPs, 0.4 mM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The PCR condition involved an initial denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 30 seconds for denaturation, 58°C for 30 seconds for annealing, 72°C for one minute for extension,

72^{o}C for one minute for final extension. PCR products were analyzed by electrophoresis in 1.5% agarose gel.

Following amplification, the DNA bands were excised from gel, purified and sequenced from Scigenome (India). Generated sequences were assembled, a contig was formed and was deposited in NCBI GenBank (GenBank ID: MH686211**)**. The final sequence was analysed by BLAST available at NCBI. The ORF of the sequence was predicted using ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder). The largest ORF was analysed by SmartBLAST (https://blast.ncbi.nlm.nih.gov/smartblast/ smartBlast.cgi) and the result was obtained. The result presented the closest hit and the phylogenetic associations. The secondary structure of the derived amino acid sequence was predicted by homology modelling at SwissProt website (swissmodel.expasy.org).Generated protein secondary structure was visualized and analysed using RasMol.

Semiqunatitative RT-PCR

For expression analysis an RT-PCR assay was developed. 200 ng of total RNA was used for semi-quantitative RT-PCR using PrimeScript OneStep RT-PCR Kit Ver.2 (TaKaRa) and the quality and quantity of RNA was observed and analyzed by nanodrop. Semi-quantitative primers were designed and synthesized based on the above sequences (submitted to Genbank ID: MH686211) which are supposed to amplify a 403 bp region within using the same protocol as above except reduced extension time for 30 seconds. PCR products were analyzed by electrophoresis in 1.5% agarose gel.

Primers for semi quantitative PCR

Forward EU816596_F1	TGCTGAAGCCTACCTCGGAA
Reverse EU816596_R1	CTTGGCCCTCTCACAAGCTG

Agarose Gel Electrophoresis

For analysis, 0.8% Agarose gel was prepared for RNA and 1.5% gel for PCR products. Agarose gel was prepared in 1 X TBE buffer (Tris-base -10.8 g, 0.5 M EDTA- 4 ml, Boric acid- 5.5 g, double distilled water- 100 ml, pH- 8.0) and SYBR safe (1 µl / ml of agarose gel) was added. After cooling to 45^{o}C, the agarose was poured on to gel tray and was allowed to solidify. The gel tray was submerged in 1 X TBE buffer filled in a buffer tank. Ten microlitre of PCR product was mixed with 2 µl of 6 x gel loading buffer (1% Bromophenol blue- 250 µl, 1% xylene cyanol-250 µl, glycerol– 300 µl, double distilled water–200 µl) and loaded into the well. Electrophoresis was carried out at a voltage of 3-5 volt/cm. The gel was visualized on a UV transilluminator using the Gel Doc XR system and the quantity one programme (Bio-Rad Hercules, Ca).

5.5 Results

A single band of Hsp 70 was observed in muscle (Fig. 5.2), liver (Fig. 5.3), gills (Fig. 5.4) and brain (Fig. 5.5) when the fish was exposed to 38^{o}C

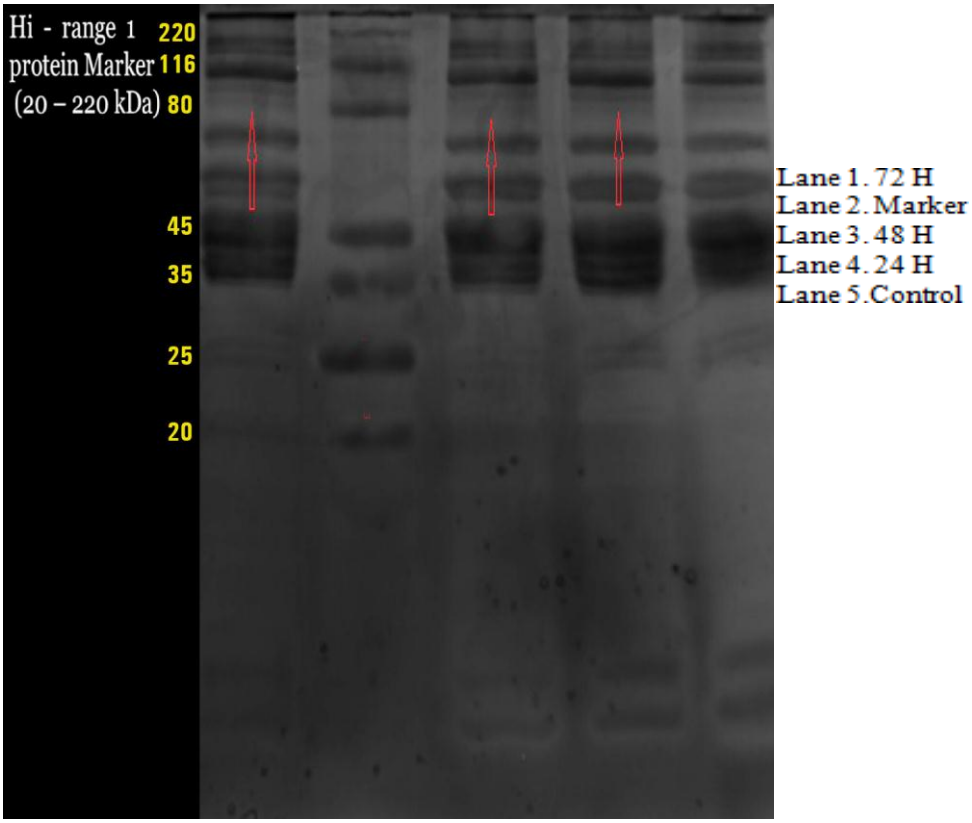


Figure 5.2 SDS - PAGE (10%) gel image of muscle exposed to 38ºC

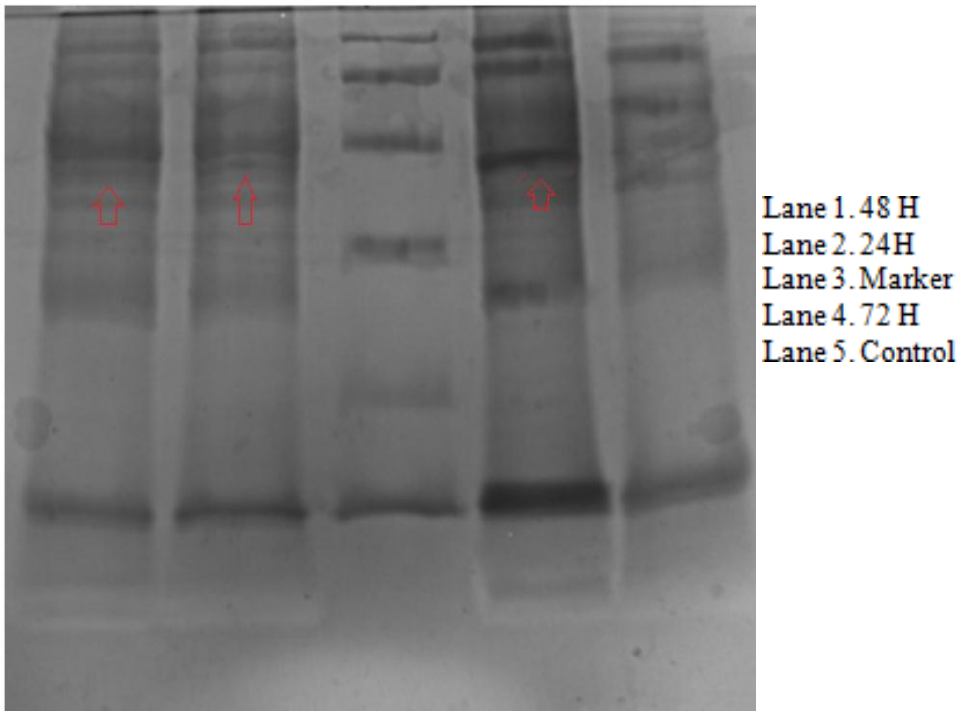


Figure 5.3 SDS - PAGE (10%) gel image of liver exposed to 38^{o}C

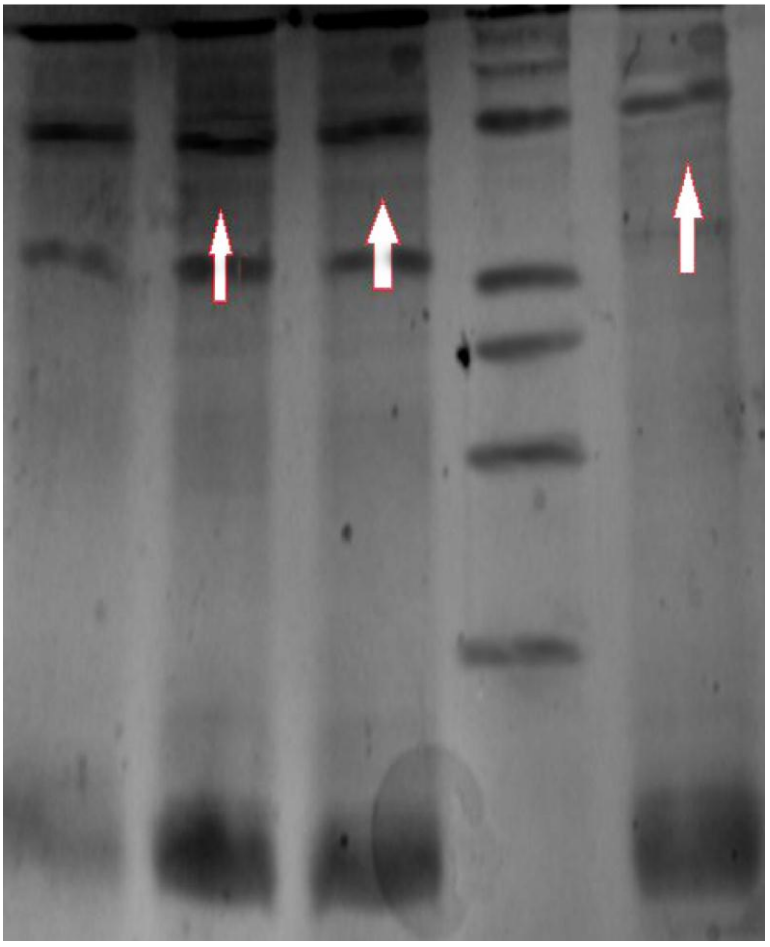
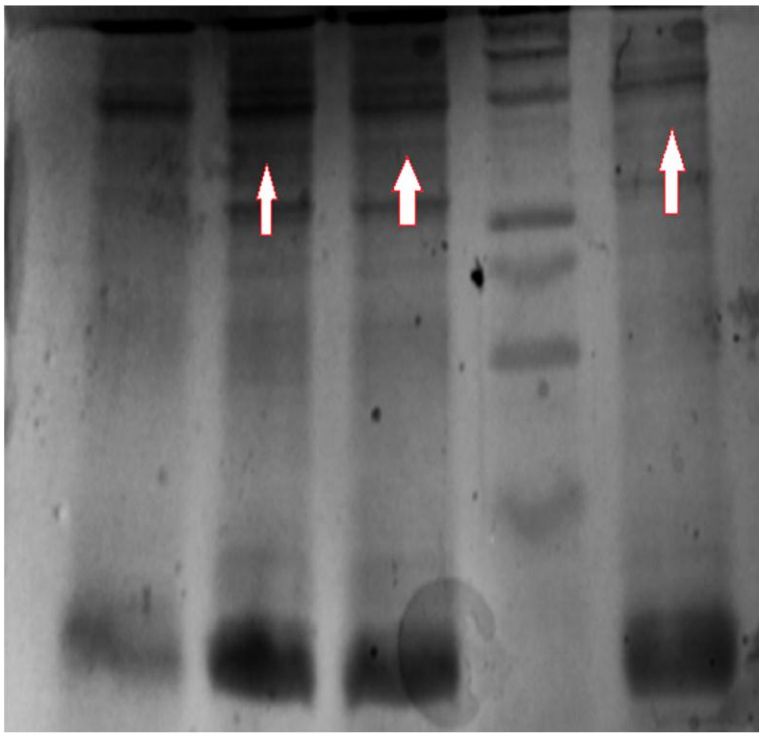


Figure 5.4 SDS - PAGE (10%) gel Image of gills exposed to 38ºC



Lane 1. Control
Lane 2. 24 H
Lane 3. 48 H
Lane 4. Marker
Lane5. 72 H

Figure 5.5 SDS - PAGE (10%) gel image of brain exposed to 38ºC

A single band of HSP 70 was observed in liver (Fig. 5.6), muscle (Fig. 5.7), gills (Fig. 5.8) and brain (Fig. 5.9) when fish was exposed to 16^{o}C

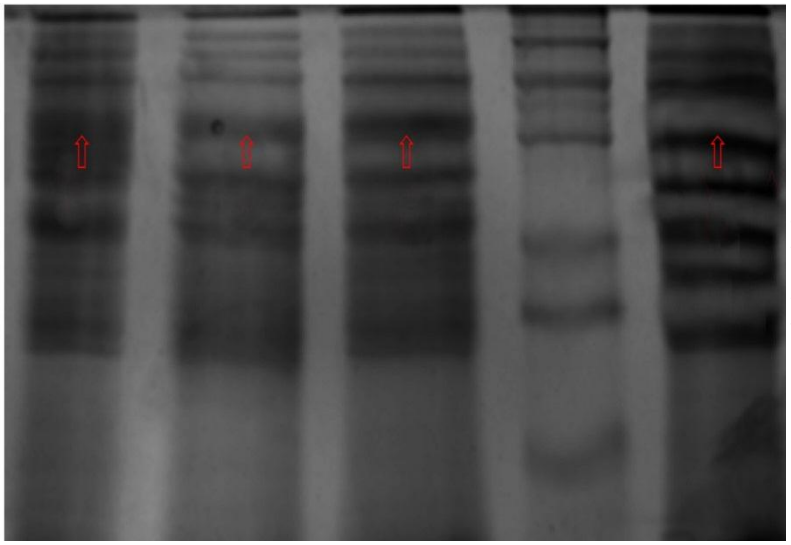


Figure 5.6. SDS- PAGE (10%) gel image of liver exposed to 16^{o}C

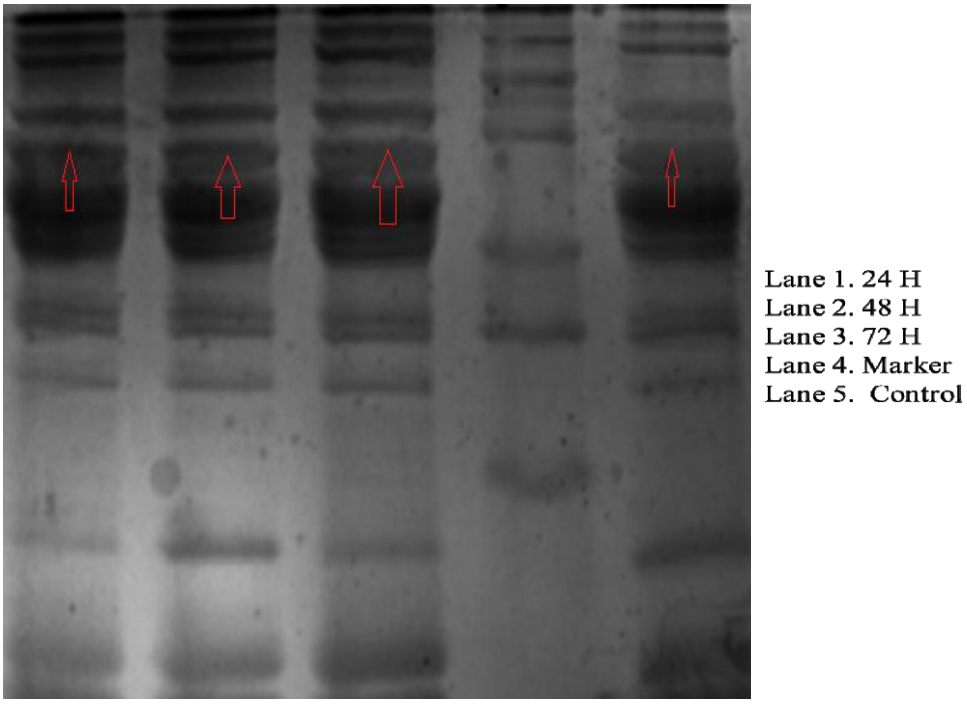


Figure 5.7 SDS- PAGE (10%) gel image of muscle exposed to 16^{o}C

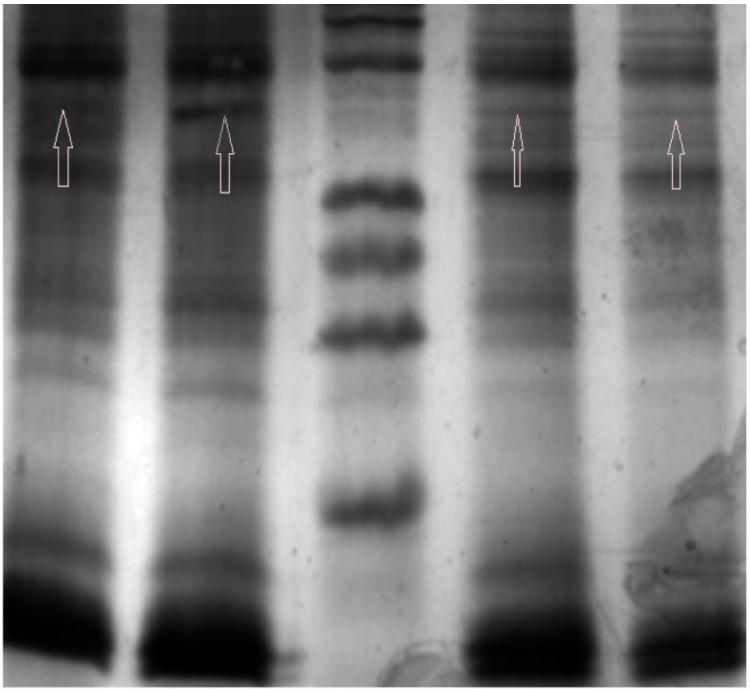


Figure 5.8 SDS- PAGE (10%) gel image of gills exposed to 16^{o}C

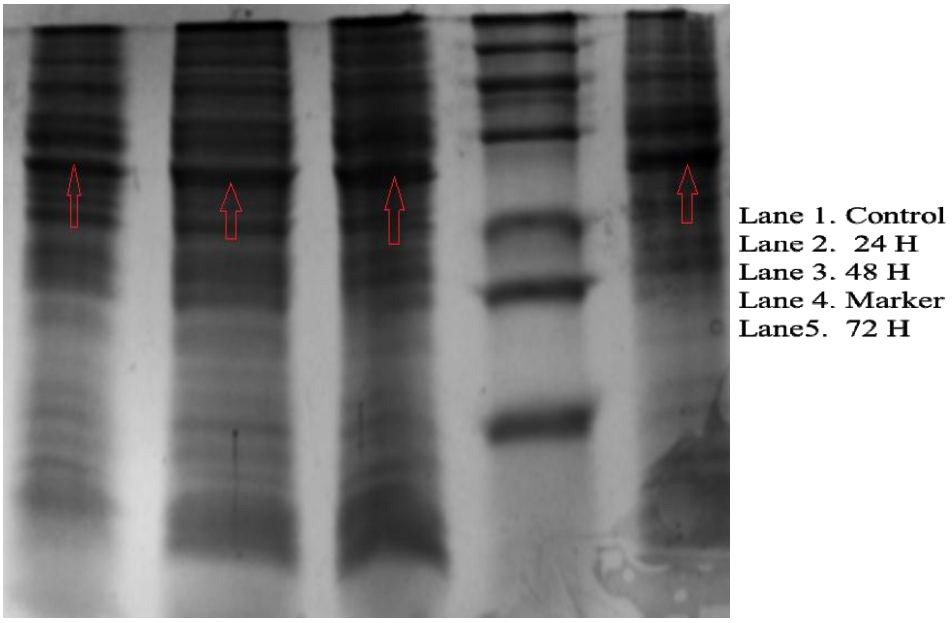


Figure 5.9 SDS- PAGE (10%) gel image of brain exposed to 16^{o}C

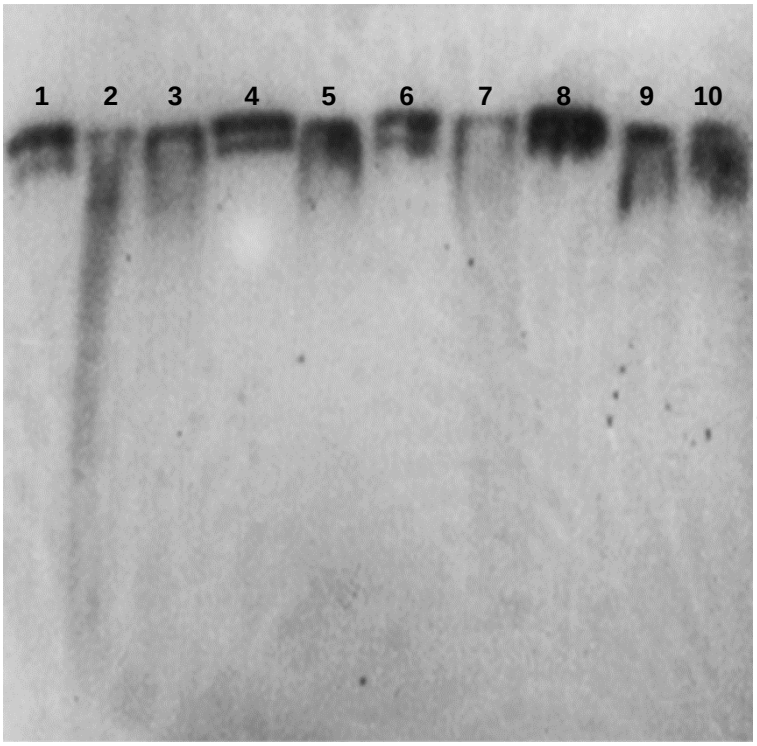


Figure 5.10 Western blots of HSP 70 expression in gills, liver, muscle and brain when fish was exposed to 16ºC and 38ºC.

Amplification of near full length HSP 70 gene and bioinformatics analysis

Near full length sequence of Es HSP 70 with a length of 1704 bp as against the 1954 bp long complete gene reported previously. The nucleotide sequence of the obtained amplicon is shown in Fig. 5.11. In the BLAST analysis, the sequence returned best hit with the sequence of *Neolamprologus brichardi* (accession no.XM_006800520.1, identity score: 96%). ORF finder predicted a number of ORFs, of which the largest spanned <2>1702. SmartBLAST analysis of the ORF returned five

closest hits and the present ORF was clustered with a zebrafish sequence (accession no. NP_001103873.1)

AGTTGAAATTATTGCCAATGACCAGGGCAACAGGACCACACCCAGCTATGT
GGCCTTCACAGATACTGAGAGGCTGATCGGCGATGCAGCCAAGAATCAGGT
TGCCATGAACCCCACCAACACAGTCTTCGATGCCAAACGACTGATTGGTCG
CAGGTTTGACGATCAAGTTGTGCAGTCAGATATGAAGCACTGGCCATTTGC
TGTCATCAATGACAACAGTCGCCCTAAGGTTCAGGTTGAGTACAAGGGTGA
AACAAAGTCCTTCTACCCAGAGGAGATCTCTTCCATGGTGCTGACAAAGAT
GAAGGAGATTGCTGAAGCCTACCTCGGAAAAACTGTCAACAATGCTGTAAT
CACAGTGCCCGCCTACTTCAATGACTCTCAGCGTCAGGCCACAAAGGATGC
TGGCACCATCTCTGGCCTCAACGTTCTGCGTATCATCAATGAGCCAACTGCT
GCTGCCATTGCTTATGGGTTGGACAAGAAGGTTGGATCAGAGAGGAACGTT
CTCATCTTTGATCTTGGTGGCGGCACCTTTGATGTGTCCATCTTGACCATTG
AAGATGGCATCTTTGAGGTAAAGTCCACTGCTGGAGACACTCATCTTGGAG
GGGAGGACTTCGACAACCGCATGGTCAACCACTTCATTGCTGAGTTCAAGC
GCAAGTACAAGAAGGACATCAGTGACAACAAGAGGGCCGTCCGTCGTCTG
CGCACCGCTTGTGAGAGGGCAAAGCGCACCCTGTCTTCCAGCACTCAGGCC
AGCATTGAAATCGATTCTCTGTACGAGGGCGTCGACTTCTACACCTCCATCA
CAAGGGCTCGCTTTGAGGAGCTCAATGCTGACCTCTTCCGTGGCACCCTGG
ACCCTGTGGAGAAGTCGCTCCGTGATGCAAAGATGGATAAGGGGCAGATTC
ATGACATCGTGCTGGTTGGTGGCTCCACCCGTATCCCCAAGATCCAGAAAC
TGCTCCAGGATTTCTTCAATGGAAAGGAGCTCAACAAGAGTATCAATCCAG
ATGAAGCTGTGGCCTATGGAGCTGCTGTCCAGGCTGCCATCCTGTCTGGAG
ACAAGTCTGAGAATGTGCAGGACCTGCTGCTTCTGGACGTCACCCCCCTGT
CCCTGGGTATTGAGACCGCTGGAGGTGTCATGACTGTCCTGATCAAACGTA
ACACCACCATTCCTACCAAGCAGACCCAGACCTTCACCACCTACTCCGACA
ACCAGCCTGGTGTGCTCATCCAGGTTTATGAGGGTGAGCGCGCCATGACAA
AGGACAACAATCTGCTGGGCAAGTTCGAGCTGACGGGCATTCCTCCTGCTC
CTCGTGGTGTGCCCCAGATTGAAGTGACATTTGATATTGATGCCAACGGCA
TCATGAATGTCTCAGCTGTTGATAAGAGCACTGGCAAGGAGAACAAGATCA
CCATCACAAATGACAAAGGTCGCCTCAGCAAAGAAGACATTGAGCGCATG
GTCCAGGAAGCTGAGAAGTACAAGGCCGAAGATGATGTGCAACGTGACAA
GGTGGCTGCTAAGAACGGCCTGGAGTCGTATGCTTTCAACATGAAGTCCAC
AGTGGAGGATGAGAAGCTTGCTGGCAAGATCAGTGATGACGACAAGCAGA
AGATCTTGGACAAGTGCAATGAGGTCATCAGCTGGCTGGACAAGAACCAG
ACTGCTGAGAAGGATGAGTATGA

Fig. 5.11 Nucleotide sequence of the obtained amplicon (1704 bp)

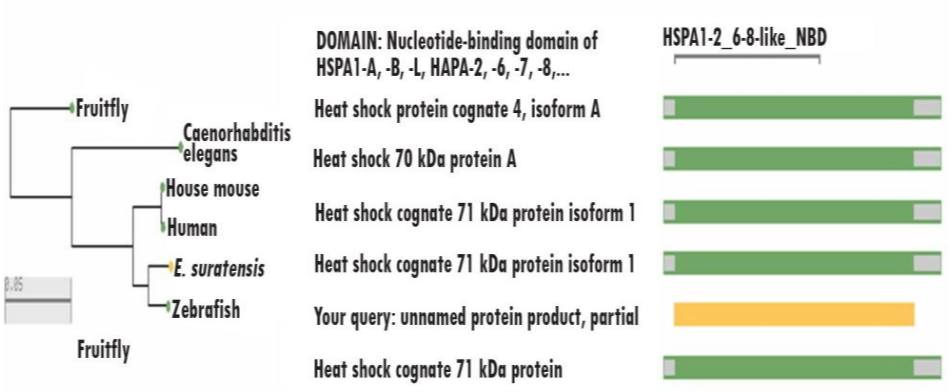


Fig. 5.12 Analysis of the largest ORF in the sequence by SmartBLAST. In the phylogenetic tree, the present sequence is grouped along with the sequence from a zebrafish.

VEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPTNTVFDAKRLIGRRF
DDTVVQSDMKHWPFNVINDNSRPKVQVEYKGETKSFYPEEISSMVLTKMKEI
AEAYLGKTVNNAVITVPAYFNDSQRQATKDAGTISGLNVLRIINEPTAAAIAY
GLDKKVGSERNVLIFDLGGGTFDVSILTIEDGIFEVKSTAGDTHLGGEDFDNR
MVNHFIAEFKRKYKKDISDNKRAVRRLRTACERAKRTLSSSTQASIEIDSLYE
GVDFYTSITRARFEELNADLFRGTLDPVEKSLRDAKMDKGQIHDIVLVGGSTR
IPKIQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDKSENVQDLLLLD
VTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERA
MTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGIMNVSAVDKSTGKENKI
TITNDKGRLSKEDIERMVQEAEKYKAEDDVQRDKVAAKNGLESYAFNMKST
VEDEKLAGKISDDDKQKILDKCNEVISWLDKNQTAEKDEY

Fig. 5.13 Deduced partial amino acid sequence of HSP70 ORF from *E. suratensis*

The predicted secondary structure of Hsp 70 of *E. suratensis* showed that the peptide contains coil, α- helix, β- sheet and turn regions as given in Table 5.2.

Table 5.2 Secondary structure analysis of Hsp 70 of *E. suratensis*

Val 1 to Ala 5, Gly 27,Phe 43 to Asp 44, His 64 to Phe 67, Tyr 90,Gly 111 to Asn 115,Asn 126, Gly 141 to Leu 142, Gly 165 to Glu 167, Gly 204, Lys 225 to Asp 227, Ser 251 to Thr 253, Asp 27,Lys 300 to Asp 302, Ile 306 to His 307,Asn 330 to Lys 334, Lys 336 to Ile 338, Ser 356 to Ser 360, Leu 366 to Asp 370, Tyr 406, Pro 411, Asn 428, Pro 447,Ile 40, Lys 472, Thr 479 to Lys 482, Ala 513 to Lys 514, Lys 525 to Glu 529	constitute15% coil region in Hsp 70.
Asp 28 to Asn 32, Pro 38 to Asn 40, Ala 45 to Arg 47, Val 57 to Lys 63, Pro 91 to Leu 110, Asp 127 to Ser 140, Glu 150 to Tyr 158, Gly 205 to Tyr 224, Lys 232 to Ser 250, Arg 274 to Ala 299, Lys 303 to Gln 305, Pro 319 to Phe 329, Glu 342 to Leu 355, Thr 425 to Asp 427, Lys 487 to Tyr 500, Glu 503 to Arg 508	constitute 33% α- helical region of Hsp 70

Val 17 to Phe 19, Arg 24 to Ile 26, The 41 to Val 42, Asn 68 to Asp 72, Arg 75 to Tyr 82, Glu 85 to Phe 89, Asn 116 to Val 121, Asn 143 to Asn 149, Arg 168 to Leu 175, Phe 180 to Glu 188, Ile 191 to Asp 200, Gln 254 to Ile 259, Phe 268 to Thr 273, Asp 308 to Leu 312, Val 371 to Thr 372, Leu 376 to Thr 380,Val 384 to Ile 389, Thr 393 to Thr 394, Thr 37 to Thr 405, Gly 412 to Glu 419, Asn 429 to Thr 437, Gln 448 to Ile 455, Met 461 to Asp 467, Glu 473 to Ile 478, Asn 515 to Gly 516	constitute 29% β- sheet region of Hsp 70.
Asn 6 to Tyr 16,Thr 20 to Glu 23,Gln 33 to Asn 37, Leu 48 to Thr 56, Asn 73 to Ser 74, Lys 83 to Gly 84,Pro 122 to Phe 125, Gly 159 to Val 164,Gly 176 to Thr 179, Asp 189 to Gly 190, Thr 201 to Leu 203, Ile 228 to Asn 231, Asp 260 to Val 266, Goly 313 to Ile 318, Asn 339 to Asp 341, Glu 361 to Asp 365, Pro 373 to Ser 375, Ala 381 to Gly 383, Lys 390 to Asn 392,Ile 395 to Pro 396, Ser 407 to Pro 410, Pro 443 to Val 446, Asp 45 to Gly 459, Lys 468 to Gly 471, Gly 483 to Ser 48, Lys 501 to Ala 502, Asp 509 to Ala 512, Leu 517 to Met 524	constitute 24% turn region of Hsp 70.

The 567 amino acids of Hsp 70 of *E. suratensis* was found to be rich in Ala (7.6%), Arg (4.9%), Asn (6%), Asp (8.5%), Cys (0.04%), Gln (3.7%), Glu (7.1%), Gly (6.5%), His (0.7%), Ile (7.2%), Leu (6.9%), Lys (8.5%), Met (1.9%), Phe (3.7%), Pro (3%), Ser (5.6%), Thr (7.6%), Trp (0.4%), Tyr (2.6%) Val (7.2%). Hsp70 was found to have a predicted molecular weight of 62.98101 kDa and a theoretical isoelectric point (p*I*) of 5.23 as predicted by PROTPARAM software. Total number of negatively charged residues (Asp + Glu) is 88 whereas total number of positively charged residues (Arg + Lys) is 76.

Fig. 5.14 Predicted secondary structure of Hsp 70 from *E. suratensis*

Semi quantitative RT-PCR

An amplicon of 403bp was produced in the semi quantitative RT-PCR assay (Fig. 5.15). It could selectively amplify the Hsp70 from different tissues of fishes experimentally treated with temperature shock (Fig. 5.16). Since equal quantity (200 ng) of RNA from different tissues were used as starting material for semiquantitative RT-PCR, the thickness of RNA bands in the agarose gel was considered as an index of expression level of Hsp 70.

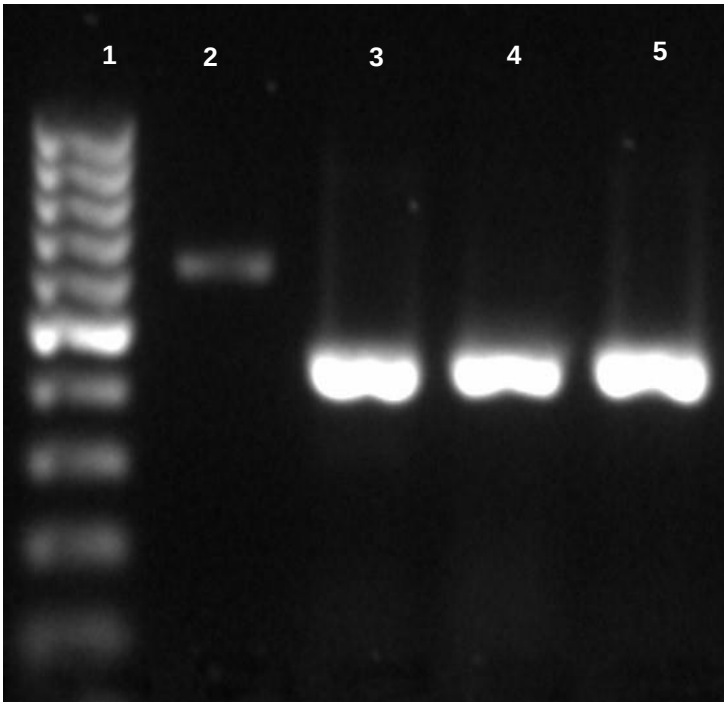


Fig. 5.15 Semi quantitative RT-PCR of Hsp 70 gene. A 100 bp ladder is loaded in well 1 for reference.

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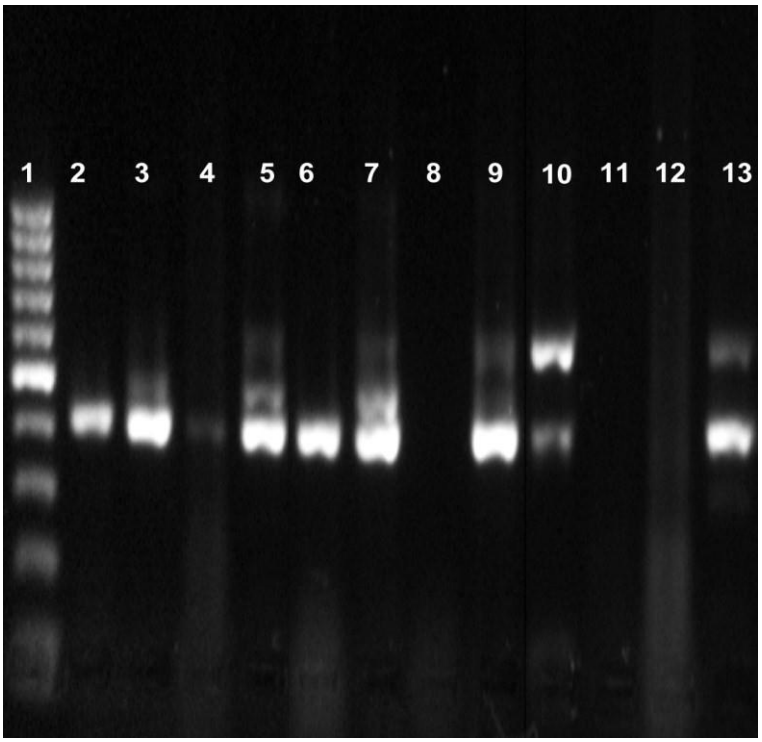

1. 100 bp ladder
2. Liver at 38°C
3. Brain at 38°C
4. Gills at 38°C
5. Muscle at 38°C
6. Liver at 16°C
7. Brain at 16°C
8. Gills at 16°C
9. Muscle at 16°C
10. Control Liver
11. Control Brain
12. Control Gills
13. Control Muscle

Fig. 5.16 Differential amplification of Hsp 70 of *E. suratensis* from different tissues at different heat shock temperatures (amplicon size – 403 bp) using the semiquantitative RT-PCR assay. A 100 bp ladder is loaded in well 1 for reference. The expression of Hsp 70 was highest in brain and lowest in gills at both 16°C and 38°C.

5.6 Discussion

Hsps have been demonstrated to increase markedly in all organisms in response to an array of stressors including temperature, exposure to heavy metals, ethanol, oxidizing and sulfhydryl reagents, chelating drugs, amino acid analogues. (Sheikh-Hamad *et al.*, 1994; Iwama *et al*., 1998, 1999; Basu *et al*., 2002). The synthesis of Hsps may assist the cells to cope with various stressors and to protect cells from subsequent stressors (Vijayan *et al*., 1997). The expression of Hsp 70 in teleosts has been studied in relation to various stressors such as heat shock (Arai *et al.,* 1994; Kultz, 1996; Iwama *et al*., 1998), aquatic toxicants (Sanders *et al.,* 1995), bacterial infection (Forsyth *et al.,* 1997), hypoxia (Gamperl *et al.,* 1998) and psychological stress (Kagawa *et al.,* 1999). The enhancement of Hsp 70 expression has therefore been regarded as an indicator of cellular stress response in fish (Thomas 1990; Iwama *et al*., 1998). Acclimation is the process by which long-term exposure to a novel environmental condition results in remodeling of cellular machinery to adapt to the new environment. Acclimation to higher temperatures results in higher basal levels of Hsp70 (Dietz and Somero, 1992). In the present investigation, Hsp70 was expressed at 16°C and 38°C in the selected tissues. However, Hsp70 was not at all expressed in control (27°C) in all the tissues. In heat shock treatments a single band of Hsp 70 was detected in gills, liver, muscle and brain by SDS- PAGE. However, there is a high possibility that the bands observed in SDS-PAGE analysis contained other proteins. Therefore, it is necessary to adopt a more precise method such as western blot analysis using specific antibodies for Hsp70 and thus it was confirmed. A single band which consist of both the constitutive

(HSC) and inducible (HSP) forms of Hsp70 has been reported in the Atlantic salmon (*Salmo salar)* (DuBeau *et al.,* 1998), eurythermal goby (*Gillichthys mirabilis*) (Lund *et al*., 2006), common carp (*Cyprinus carpio*) (Wang *et al.,* 2007), coho salmon (*Oncorhynchus kisutch*) (Arkush *et al.,* 2008), and in juvenile *Macrobrachium malcolmsonii* (Selvakumar and Geraldine 2005). By complying the agarose gel image, the Hsp 70 expression was high in brain followed by liver, muscle and gills at 16ºC and 38ºC. In this study the tissue-specific differences in Hsp70 expression are supported by similar findings in many fish species including, common carp (*Cyprinus carpio*) (Wang *et al.,* 2007), fathead minnow (*Pimephales promelas*) (Dyer *et al*., 1991) and brown trout (*Salmo trutta*) (Schmidt *et al.,* 1998). Significant differences in Hsp70 expression between tissues (brain, liver, and gill) within individual species was reported by Dietz and Somero (1993) and between four marine teleosts in response to hyperthermia (Wang *et al.,* 2007). Sanders *et al*. (1994) reported that differences in the accumulation of stress proteins might be useful in identifying the tissues that are particularly vulnerable to damage by a specific stressor and in identifying the extent of the damage. The prevailing opinion that the induction temperatures for Hsp are not genetically determined but vary depending on the environmental condition which is in accordance with the current findings (Dietz and Somero 1992; Lund *et al*., 2006). Among the various tissues the expression of Hsp 70 was lowest in the gills. Gills are directly exposed to the water; drastic changes in environmental conditions such as heat shock may have adverse effects on its vital functions leading to the death of the animal. This may be a possible reason for the lowest expression of Hsp 70 in gills. This is similar with the findings of heat-

shock protein 70 in the catfish, *Horabagrus brachysoma* (Dalvi *et al*., 2011). Gills may be rate limiting in the ability of poikilotherms to survive thermal stress, due to its limited capacity to synthesis stress proteins. The expression of Hsp 70 was highest in brain followed by liver, muscle and gills at both 16ºC and 38ºC. But in catfish (*Horabagrus brachysoma*) Hsp 70 induction was highest in liver followed by muscle, brain and gills (Dalvi *et al*., 2011). In the different organs even when the quality of the response is similar, differences of intensity can be found. Fish are thought to have only one cortisol receptor (CR) type whereas in mammals, contain distinct mineralocorticoid and glucocorticoid receptors. The structure of the CR and its association with Hsps has been established (Mommsen *et al*., 1999; Basu *et al.*, 2003). Basu *et al*. (2001) reported the possible association between cortisol and Hsps in fish and the effects of stress on this association. Glucocorticoid receptor complex in rainbow trout hepatic tissue contains Hsp 70 (Basu *et al*., 2003). As a consequence, the hepatic glucocorticoid receptors can favour the binding to the hepatic cells of the Hsp 70 proteins, thus leading to the increased expression of Hsp 70.

In natural conditions, expression of heat shock proteins in fish varies with season (less during winter than in summer). Maximum accumulation of Hsp70 was recorded in the digestive gland and gills of mussels *(Mytilus galloprovincialis*) during summer period (Mineir *et al.*, 2000). Organisms acclimated to warm environments constitutively express Hsps at higher levels and have a higher thermal threshold for Hsp induction than those inhabiting in cooler environments (Dietz and Somero1992; Barua and Heckathorn 2004; Lund *et al*., 2006). In this study higher Hsp 70 level was found in all the tissues at 38ºC compared

to 16^{o}C.Variations in the HSP levels during seasonal and laboratory acclimation studies are reported in various fish species (Currie *et al*., 2000; Nakano and Iwama 2002; Viant *et al*., 2003; Lund *et al.,* 2006). Moseley (1997), Kregel (2002) reported that mild heat-shock treatment can transiently render a biological system more resistant to a subsequent stress and have shown a positive correlation between the amount of Hsps present and the degree of thermal tolerance. The importance of Hsps in thermo tolerance and differences in their accumulation in various tissues in organisms adapted to the heat suggest a role for Hsps in acclimatization (Moseley 1997). Das *et al*. (2006) reported an increase in the inducible form of Hsp70 in the liver tissue of *L. rohita* acclimated to three temperatures (31^{o}C, 33^{o}C and 36^{o}C) and suggested that the increased level of Hsp70 was responsible for the increase in thermal tolerance (Das *et al.,* 2004).The present study suggests that the increase in the Hsp 70 renders *E. suratensis* more thermo tolerant toward thermal stress. The present results also suggest that the increase in the endogenous Hsp 70 levels in the fish at 16^{o}C and 38^{o}C compared to those at control (27^{o}C) might have resulted in an upward shift in the onset temperature of Hsp70 through a classic negative feedback mechanism. Such a model has been hypothesized for explaining the regulation of Hsp genes (Craig and Gross 1991; Morimoto 1993; Wu 1995).

In conclusion, overall results indicated that *E. suratensis* can acclimate to higher temperatures via increase in the Hsp70 level as an adaptive mechanism, which renders the fish more thermo tolerant. The present findings also showed that the expression of Hsp70 in *E. suratensis* exposed to different temperatures which supports the prevailing opinion

that Hsp induction temperatures are not genetically determined, but are dependent on the environmental conditions. Lower expression of Hsp70 in the gill suggested that gills are directly exposed to the water and vulnerable to environmental perturbations. The characteristics of the Hsp70 response to thermal shock in *E. suratensis* support the significance of these proteins in acclimatization to environmental change. Further investigations on other heat-shock proteins such as Hsp60, Hsp90, Hsp100, etc. and their gene expression may help to understand their collective role during thermal acclimatization in *E.suratensis.* One possible application is supplying exogenous Hsps, either by feeding with Hsps encapsulated in bacteria or injecting recombinant Hsp70 help the organism to overcome the stress situation. In fish, intra-coelomal injection with DnaK proteins, (equivalent to mammalian Hsp70) combined with a non-lethal heat shock, safeguards the aquatic organism from death caused by thermal stress.

Chapter 6

Summary and Conclusions

Fishes are easily affected by environmental factors that limit their abundance and distribution. Temperature is an important abiotic environmental factor that causes the body temperatures of fishes to quickly fluctuate to lethal levels, resulting in rapid metabolic variation, which can lead to disorder, affecting survival and fecundity. Because of ongoing global warming, the average temperature has increased over the past 30 years, and the frequency and extent of heat events have increased during the summer. Thus, interest in the impact of heat stress on fishes has grown. Heat stress can have rapid lethal effect on fishes, which has been widely reported. However, understanding the sub-lethal impacts of thermal stress on the development and reproduction of surviving fishes could provide important basic information for aquaculture study. When the temperature is between the lethal high and physiological limits, fishes can be affected by thermal injuries, which result in the loss of life or decline in fecundity. During evolution, fishes evolved many behavioral and physiological strategies, such as elevating antioxidant defenses and synthesizing heat shock proteins (Hsps), which are critical indicators in thermal tolerance, to overcome thermal and other types of stress. An

understanding of the impacts of environmental changes on fishes and their adaptive mechanisms is vital in studying fish-climate interactions. This would help to predict and explain the distribution and availability of fishes in different seasons and regions.

Important findings of the study can be summarized as follows

- Critical Thermal Maximum (CT Max)of *Etroplus suratensis* (Bloch, 1790) was found to be 40^{o}C
- Critical Thermal Minimum (CT Min) of *Etroplus suratensis* (Bloch, 1790) was found to be 15^{o}C

Activity of Antioxidants

Parameters Investigated	Temperature	Hour	Tissue
Superoxide Dismutase	38^{o}C	48th h	Brain
Catalase	16^{o}C	48th h	Muscle
Lipid Peroxidation	16^{o}C	48th h	Gills
Glutathione-S-Transferase	27^{o}C	48th h	Gills
Glutathione Reductase	16^{o}C	48th h	Muscle
Glutathione Peroxidase	16^{o}C	48th h	Muscle
Total Reduced Glutathione	38^{o}C	48th h	Muscle

- In all the antioxidant parameters investigated the activity was maximum on 48th hour of exposure.
- Thermal stress was maximum on 48th hour and so the organism tried to cope up by increasing the activity.

- Activity gets declined from 72th hour of exposure and the organism failed to overcome due to sustained thermal stress.
- The effect of high (38°C) and low (16°C) temperatures on antioxidants activity was almost the same.
- Among the various tissues analyzed muscle was the most affected, since it was more exposed to environment.
- In all the antioxidant parameters, between tissues, hours of exposure and temperatures significant difference was present and the significance level was set at $p< 0.05$.
- In this study with respect to controls the level of blood glucose increased, whereas the levels of liver glycogen and muscle glycogen declined when analyzed on 24th hour of exposure.
- The elevation in blood glucose level and simultaneous decrease in the levels of liver and muscle glycogen indicate the high energy demand associated with imposed temperature stress.
- When analyzed at the 24th hour of temperature stress increased glycemia was observed, possibly due to the conversion of glycogen into glucose due to glycogenolysis for energy use.
- mg/ g wet weight of glycogen content was high in liver compared to muscle.
- The possible reason for the lower glycogen content was observed in muscle because it lacks the inherent potential to store glycogen compared to liver and is dependent on blood glucose for all its activities.

- The reduction in lipid level may be due to increased metabolic rate which results in the increased energy demand.
- The reduction of each lipid component may be due to reduced oxygen solubility at elevated temperatures which may retard the activity of the oxygen-dependent fatty acid desaturases.
- During exposure to lower temperature (16^{o}C) there is an increase in all lipid components, possibly due to enhancement of gene expression of enzymes and proteins which are involved in lipid biosynthetic processes.
- Enhanced activity of lipid profile at cold condition may also due to mobilization of saturated triglycerides.
- Exposure to lower temperature (16^{o}C) leads to an increase in ketone bodies and lipase activity and this may lead to enhanced activity of lipid profile at cold condition.
- Stress cause altered and declined protein synthesis. Stress has the ability to dissociate the 80S ribosomes and cause damage to ribosomes formation; finally it leads to inhibition of the initiation of the polypeptide synthesis.
- Decrease in protein content may also be due to impairment of protein synthesis but also due to intracellular leakage of proteins in the blood on account of cellular necrosis.
- The drastic reduction in the protein content suggests that possibly the gluconeogenetic pathway has been initiated to supplement depletion of sugars by breaking down of protein to yield sugars.

- The production of Hsp 70 was observed during thermal stress which was identified by SDS-PAGE and confirmed by Western blotting.
- Among the various tissues analyzed Hsp was found to be prominent in brain at 38^{o}C
- Gills are directly exposed to water. Drastic changes in environmental conditions such as heat shock may have adverse effects on its vital function. This may be the probable reason for the lowest expression of Hsp 70 in gills.
- The 567 amino acid of Hsp 70 from *E. suratensis* was found to be rich in alanine (7.6%), arginine (4.9%), Asn (6.0%), Asp (8.5%), Cys (0.04%), Gln (3.7%), Glu (7.1%), Gly (6.5%), His (0.7%), Ile (7.2%), Leu (6.9%), Lys (8.5%), Met (1.9%), phe (3.7%), Pro (3%), Ser (5.6%), Thr (7.6%), Trp (0.4%), Tyr (2.6%), Val (7.2%)
- Hsp 70 from *E. suratensis* was found to have a predicted molecular weight of 62.98101 kDa and a theoretical isoelectric point (pI) of 5.23.
- Total number of negatively charged residues (Asp + Glu) was found to be 88 whereas total number of positively charged residues (Arg + Lys) was found to be 76.
- Val 1 to Ala 5, Gly 27, Phe 43 to Asp 44, His 64 to Phe 67, Tyr 90, Gly 111 to Asn 115, Asn 126, Gly 141 to Leu 142, Gly 165 to Glu 167, Gly 204, Lys 225 to Asp 227, Ser 251 to Thr 253, Asp 27, Lys 300 to Asp 302, Ile 306 to His 307,Asn 330 to Lys 334, Lys 336 to Ile 338, Ser 356 to Ser 360, Leu 366 to Asp 370, Tyr 406, Pro 411, Asn 428, Pro 447, Ile 40, Lys 472, Thr 479 to Lys 482, Ala 513 to

Lys 514, Lys 525 to Glu 529 constitute 15% coil region in Hsp 70 of *E. suratensis*

- Asp 28 to Asn 32, Pro 38 to Asn 40, Ala 45 to Arg 47, Val 57 to Lys 63, Pro 91 to Leu 110, Asp 127 to Ser 140, Glu 150 to Tyr 158, Gly 205 to Tyr 224, Lys 232 to Ser 250, Arg 274 to Ala 299, Lys 303 to Gln 305, Pro 319 to Phe 329, Glu 342 to Leu 355, Thr 425 to Asp 427, Lys 487 to Tyr 500, Glu 503 to Arg 508 constitute 33% α- helical region of Hsp 70
- Val 17 to Phe 19, Arg 24 to Ile 26, The 41 to Val 42, Asn 68 to Asp 72, Arg 75 to Tyr 82, Glu 85 to Phe 89, Asn 116 to Val 121, Asn 143 to Asn 149, Arg 168 to Leu 175, Phe 180 to Glu 188, Ile 191 to Asp 200, Gln 254 to Ile 259, Phe 268 to Thr 273, Asp 308 to Leu 312, Val 371 to Thr 372, Leu 376 to Thr 380,Val 384 to Ile 389, Thr 393 to Thr 394, Thr 37 to Thr 405, Gly 412 to Glu 419, Asn429 to Thr 437, Gln 448 to Ile 455, Met 461 to Asp 467, Glu 473 to Ile 478, Asn 515 to Gly 516 constitute 29% β- sheet region of Hsp 70
- Asn 6 to Tyr 16, Thr 20 to Glu 23,Gln 33 to Asn 37, Leu 48 to Thr 56, Asn 73 to Ser 74, Lys 83 to Gly 84, Pro 122 to Phe 125, Gly 159 to Val 164, Gly 176 to Thr 179, Asp 189 to Gly 190, Thr 201 to Leu 203, Ile 228 to Asn 231, Asp 260 to Val 266, Goly 313 to Ile 318, Asn 339 to Asp 341, Glu 361 to Asp 365, Pro 373 to Ser 375, Ala 381 to Gly 383 Lys 390 to Asn 392, Ile 395 to Pro 396, Ser 407 to Pro 410, Pro 443 to Val 446, Asp 45 to Gly 459, Lys 468 to Gly 471, Gly 483 to Ser 48, Lys 501 to Ala 502, Asp 509 to Ala 512, Leu 517 to Met 524 constitute 24% turn region of Hsp 70.

In the current global warming scenario, oxidative stress biomarkers have been used in the field of aquaculture to assess the health condition of fish. By supplying exogenous Hsps either by feeding with Hsps encapsulated in bacteria or injecting recombinant Hsp70 is a possible way to overcome the thermal stress. In fish, intra-coelomic injection with DnaK, a protein equivalent to mammalian Hsp70 is a possible application for the survival of thermal stress. Thus the resistance of aquatic organism to thermal stress is enhanced by endogenous Hsp70.

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References

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GenBank Submissions

Etroplus suratensis Hsp 70 mRNA, partial cds

GenBank ID: **MH686211**

List of Publications

[1] **Susan Joy**, Aneesa Painadath Alikunju, Jisha Jose, Hari Sankar Haridasanpillai Sudha, Prabhakaran Meethal Parambath, Sajeevan Thavarool Puthiyedathu, Babu Philip. Oxidative stress and antioxidant defense responses of *Etroplus suratensis* to acute temperature fluctuations. **Journal of Thermal Biology**. 2017; 70:20-16.

[2] **Susan Joy**, Aneesa Painadath Alikunju, Jisha Jose, Hari Sankar Haridasanpillai Sudha, Prabhakaran Meethal Parambath, Sajeevan Thavarool Puthiyedathu, Babu Philip. Effect of Acute Thermal Stress on Carbohydrate and Lipid Profile in *Etroplus suratensis*. **Indian Journal of GeoMarine Sciences** (accepted)

[3] Hari Sankar H.S, Jisha Jose, Remya Varadarajan, Smitha V. Bhanu, **Susan Joy**, Babu Philip. Functional Zonation of different digestive enymes in *Etroplus suratensis* and *Oreochromis mossambicus*. **International Journal of Scientific and Research Publications**. 2014; 4: 2250-3153.